ECOLOGICAL AND KINETIC ASPECTS OF AMYLOLYSIS AND PROTEOLYSIS IN ACTIVATED SLUDGE

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ECOLOGICAL AND KINETIC ASPECTS OF AMYLOLYSIS AND PROTEOLYSIS IN ACTIVATED SLUDGE

(met een samenvatting in het Nederlands)

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dr. H. C. van der Plas,
hoogleraar in de organische scheikunde,
in het openbaar te verdedigen
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van de Landbouwhogeschool te Wageningen

H. VEENMAN & ZONEN B.V. - WAGENINGEN - 1979

STELLINGEN

1\.

Een hoge extracellulaire proteolytische aktiviteit is een wezenlijk kenmerk van aktief slib.

Dit proefschrift

2\.

Extracellulaire, voor polymeren toegankelijke amylasen en proteïnasen in aktief slib zijn geïmmobiliseerd door binding aan de biomassa van het slib; hun katalytische aktiviteit is bij benadering gelijk aan die van vrije, in water opgeloste enzymen.

Dit proefschrift

3\.

Extracellulaire hydrolyse van opgeloste makromolekulaire verbindingen door levende bakteriën in oppervlaktewater geschiedt waarschijnlijk grotendeels ten eigen nutte.

4\.

Voor een goed begrip van het gebeuren in aktief slib werkt gebruik van de term 'terugvoer van gevlokte biomassa', in plaats van 'terugvoer van biomassa', verhelderend.

Dit proefschrift

5\.

Voor de blijvende aanwezigheid van een mengkultuur van mikro-organismen in kontinu met opgelost substraat belast, goed gemengd aktief slib is een scala van oecologische nissen in en op de slibvlokken, in stand gehouden door terugvoer of retentie van deze vlokken, een eerste vereiste. Externe voeding men mengsubstraat of handhaving van een hoge slibleeftijd zijn daarvoor niet noodzakelijk.

Dit proefschrift

6\.

Bij het zoeken naar mikro-organismen voor de kontinue produktie van 'single cell protein' uit polymeerbevattend afvalwater verdient het aanbeveling, onder meer uit een oogpunt van enzymkinetiek, een eerste selektie uit natuurlijk entmateriaal in een kontinue kultuur te laten plaatsvinden.

7.

Aanpassing van aktief slib aan kwalitatief veranderde voedingen geschiedt voornamelijk via populatieverschuivingen.

8.

Uitspraken over het al of niet van toepassing zijn van de kinetiek, beschreven door Monod, op mengkulturen van mikro-organismen dienen niet in eerste instantie te worden gedaan op grond van bepalingen van het totaal aan aanwezige organische verbindingen.

C. P. L. GRADY, Jr., L. J. HARLOW en R. R. RIESING. Biotechnol. Bioengng. 14 (1972) 391–410.
A. HARDER. Proefschrift, Wageningen, 1979.

9.

Het valt te verwachten dat ongeveer de helft tot nagenoeg het geheel van de opgeloste organische verbindingen in het effluent van laagbelaste aktief-slib-installaties voor de zuivering van huishoudelijk en stedelijk afvalwater niet bestaat uit rest-substraat, maar uit mikrobiële stofwisselingsprodukten.

A. KLAPWIJK. Proefschrift, Wageningen, 1978.
Dit proefschrift.
Hoogheemraadschap van Rijnland, Technische Dienst, Jaarverslag 1977, deel V.

10.

Toestaan van een relatief lange tijdsduur bij bepaalde kategorieën promotieonderzoekingen aan de Landbouwhogeschool kan de ongewenste ontwikkeling tegengaan dat risicodragend, grensverleggend en/of arbeidsintensief onderzoek minder ondernomen wordt.

11.

In verband met de belangrijke en toenemende rekreatieve funktie van de oppervlaktewateren in Nederland, is, voor een verantwoord beheer van deze wateren, meer sociaal-psychologisch inzicht vereist in de aard en de mate van de beleving van deze rekreatie.

12.

Toediening van enzympreparaten aan aktief slib, ter verbetering van het zuiveringsproces, is te vergelijken met het werpen van paarlen voor de zwijnen.

13.

In de Nederlandse politiek kan men meer duidelijkheid bereiken door de discrepantie tussen woord en daad te minimaliseren dan door verbale duidelijkheid te maximaliseren.

14.

Gebruik van de term ‘gezondheidszorg’ zou moeten inhouden dat betaling voor verleende diensten, overeenkomstig een oude Chinese gewoonte, slechts geschiedt zolang de kliënt geen patiënt is.

15.

Water wordt nooit oud.

J. M. A. JANSSEN
Ecological and kinetic aspects of amylolysis and proteolysis in activated sludge.

Wageningen, 24 oktober 1979.

VOORWOORD

De totstandkoming van dit proefschrift heeft grote inspanningen gevergd van vele betrokkenen.

Dit gold in de eerste plaats voor Prof. MULDER die mij in de gelegenheid stelde een onderzoek naar extracellulaire enzymen in aktief slib te verrichten. Grote dank ben ik hem verschuldigd voor de uiterst nauwgezette wijze waarop hij bij het samenstellen van het proefschrift heeft geholpen. Het zal voor mij wel altijd een raadsel blijven uit welke bronnen zijn nagenoeg grenzeloze energie geput werd.

Eveneens ben ik WOUT MIDDELHOVEN zeer erkentelijk voor zijn bijdrage aan dit proefschrift, speciaal voor die gedurende mijn 'Delftse periode'. Zijn specialiteit bestond uit fantasierijk duw- en trekwerk, waarbij delen van het proefschrift plotseling verdwenen, respektievelijk verschenen.

Met waardering vermeld ik de vlotte en plezierige assistentie van RENÉE DE VRIJER-ALBERS bij de uitvoering van een deel van de experimenten. ANTHEUNISSE zag kans een aantal bakteriën te identificeren, in weerwil van hun duidelijke pogingen tot obstruktie. CO TERPSTRA heeft, als doktoraal student, met mij kunnen ervaren dat eenvoudige ideeën zich niet altijd eenvoudig laten bewijzen.

Een speciaal woord van dank richt ik tot A. OTTEN (afd. Wiskunde, LH) die mij heeft kunnen weerhouden van pogingen de onhebbelijkheden van aktief slib statistisch te lijf te gaan. Naast laatstgenoemde, waren ook ZEVENHUIZEN, BULDER en JAAP VISSER (afd. Erfelijkheidsleer, LH) bereid onderdelen van het manuskript kritisch door te lezen.

VAN DE KOLK en CHRIS SCHOUTEN vertroetelden jarenlang de kontinue kulturen met steriele media, WESSELS verzorgde alle foto's, waarvan een aantal, onmiskenbaar, op naam van VAN VEEN staan, FRITS LAP glunderde bij elk technisch mankement en ANS BERNS-VAN DER SLUIS, BERTHA VAN DER SLUIS-VAN DE BRAND en Mevr. VAN HAPEREN was geen vuil glaswerk te veel.

Mevr. MÖLLER-MOL en WILLY DEN HARTOG-VAN ROOYEN, zonder jullie hulp was dit proefschrift niet in leesbare vorm verschenen (geen puzzle kan jullie nu nog te machtig zijn). VAN VELZEN besteedde grote zorg aan het tekenen van alle grafieken en deed daarenboven alles wat de anderen niet konden.

De beheerders van de zuiveringsinstallaties te Bennekom en Zeist wordt dank gezegd voor hun milde giften van aktief slib.

De Stichting voor Biologisch Onderzoek (BION) ben ik erkentelijk voor de subsidie die uitvoering van dit onderzoek financieel mogelijk maakte.

Naast al deze min of meer direkte bijdragen tot de totstandkoming van het proefschrift, zijn er indirekte bijdragen van velerlei aard, zowel van binnen als van buiten het Laboratorium voor Microbiologie; deze heb ik ten zeerste gewaardeerd ofschoon ze hier niet met name(n) vermeld worden. Allen dank ik voor hun vriendschap en belangstelling.

Tenslotte spreek ik de wens uit dat de resultaten van dit proefschrift op enigerlei wijze een positieve bijdrage zullen leveren tot de theorie en praktijk van de waterzuivering.

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1. INTRODUCTION

An investigation has been made of the occurrence and functioning in activated sludge of extracellular enzymes hydrolysing macromolecular substrates like starch and proteins, in relation to the food-to-micro-organism ratio and other growth conditions. For this purpose an ecological model study was carried out with activated sludge grown under controlled laboratory conditions with starch, maltose or glucose as the sole carbon and energy source. Systems with and without retention of flocculated biomass were applied and particular attention was paid to the performance of the sludge regarding starch removal.

1.1. ACTIVATED SLUDGE

Biological waste treatment plants are man-made microbial ecosystems in which the transformation flux is much higher than in any natural or agricultural ecosystem. This type of treatment has become the largest microbiological industry (LA RIVIÈRE, 1977). Until recently, its methods were applied and innovated empirically by civil engineers; microbiologists became interested only during the last decades and the interest of biochemists until now is meagre. This may be due to the fact that the systems applied do not lend themselves easily to rigorous and meaningful analysis. The systems are more or less continuously fed with a complex substrate (sewage) and are inhabited by a flexible, mixed microbial population selected by the chemical and physical conditions of the process (LA RIVIÈRE, 1977).

Biological treatment methods include aerobic, anaerobic or photosynthetic transformations. The most extensively used method is the aerobic activated-sludge process in which sludge flocs are supplied with waste water and are agitated for keeping the units aerobic and preventing settling of the flocs. After a period of interaction, sludge flocs and liquid (including suspended cells) are separated by gravitation. The supernatant, from which most of the organic pollutants have been eliminated by the sludge, is removed as effluent. The sludge flocs are partly returned to the aeration basin and supplied with waste water once again. Excessive amounts of sludge are discarded. Sludge production is to be minimized as in its turn it imposes a waste problem. Maintenance of a low food-to-micro-organism ratio reduces the growth rate and the yield of the sludge biomass. This reduction is achieved by recycling of the activated-sludge flocs. The physiological basis of this procedure is that relatively large amounts of substrate are consumed for maintenance functions of micro-organisms, i.e. result in mineralization without concomitant growth. Lysis of micro-organisms also reduces sludge yield.

Activated-sludge flocs are to a large extent made up of aerobic bacteria. Protozoa, rotifers and fungi can also be present. Chemoheterotrophic bacteria are responsible for the elimination of organic substrates. The undefined nature of the sludge population and the influent hampers the detailed description of the activated-sludge process.

An important fraction of the organic compounds present in domestic sewage and certainly in some industrial wastes consists of macromolecules. The same may be true of material released from decaying activated sludge as microbial cells almost completely consist of macromolecules. These polymers must be hydrolysed by extracellular enzymes to enable their permeation through the cell wall and the cytoplasmic membrane.

1.2. EXTRACELLULAR ENZYMES

Bacterial enzymes can be distinguished as follows as to their location in, on or around the cell.

1. Intracellular enzymes, occurring inside or in the cytoplasmic membrane.
2. Periplasmic enzymes (MITCHELL, 1961), located outside the cytoplasmic membrane but inside the molecular sieve constituted by the outer layer of the cell wall. This space is called the periplasmic space; it occurs only in Gram-negative bacteria. Periplasmic enzymes are inaccessible to substrate molecules with a hydrodynamic radius above a critical value.
3. Surface-bound enzymes, located on the outer layer of the cell wall or in the capsule; they are accessible to macromolecular substrates.
4. Truly extracellular enzymes, set free in the culture fluid by intact cells.

In this thesis extracellular proteins are defined as proteins that have been secreted through the cytoplasmic membrane (GLENN, 1976), i.e. belonging to classes 2, 3 or 4. Gram-positive organisms generally excrete extracellular enzymes into the culture fluid, whereas many Gram-negative organisms do not. The differences in protein secretion observed between Gram-positive and Gram-negative bacteria appear to be related to the physico-chemical nature of the cell wall and not to any fundamental ability to transport proteins across the cytoplasmic membrane (GLENN, 1976). In Gram-negative bacteria cell-bound hydrolytic enzymes are often found in the periplasmic space (COSTERTON et al., 1974).

α-Amylases are generally considered to be almost completely and truly extracellular. However, this property appears to be largely confined to Gram-positive bacteria (various *Bacillus* species, *Clostridium acetobutylicum*, *Streptococcus bovis*) and to fungi. POLLOCK (1962) mentions only one Gram-negative bacterium, viz. *Pseudomonas saccharophila*, producing only truly extracellular α-amylases. As to *Klebsiella aerogenes*, α-amylase and pullulanase are totally cell-bound (HERNANDEZ and PIRT, 1975). In many studies, however, the bacterial cells themselves were not examined for amylolytic activity.

Localization of cell-bound proteinases outside the cell membrane is hampered by the presence of intracellular proteinases involved in the endogenous metabolism of living cells and by the diversity among bacterial proteinases. The property of production of truly extracellular proteinases appears also to be largely confined to Gram-positive bacteria (various *Bacillus* species, clostridia, micrococci, streptococci, staphylococci, *Sarcina flava*). However, also some Gram-negative bacteria produce truly extracellular proteinases, viz. *Pseudomonas myxogenes*, *Ps. pyocyanea* and possibly *Proteus* X_{19} and *Serratia marcescens* (POLLOCK, 1962).

Literature data on extracellular enzymes hydrolysing polymers in activated sludge are scarce. At the onset of the present study (1973) nothing was known about the substrate affinity and other kinetic parameters of these enzymes. Several hydrolytic enzymes including amylase and proteinases were detected in activated sludge by INGOLS (1939). ALLEN (1944) enumerated several bacterial types present in sewage and activated sludge and observed a shift from a non-proteolytic to a proteolytic flora when sewage was aerated and sludge was built up for a period of 4 weeks. BANERJI et al. (1967, 1968a) studied short-term starch removal patterns by activated sludge previously acclimatized to loading with starch, glucose etc. for a period of 7 to 10 days under laboratory conditions. They found that most of the enzymes were bound to the sludge and concluded that starch degradation by sludge was initiated by (partial) adsorption of the starch to the sludge. Acclimatization of glucose sludge to starch was suggested to be brought about by increased induced enzyme production rather than by a change in the predominant micro-organisms. Like GAUDY and ENGELBRECHT (1961), they found no appreciable lag associated with the starch removal by glucose sludge. BANERJI et al. (1968b) concluded that the removal of starch in acclimatized activated sludge followed first-order kinetics during the initial aeration period; their initial starch concentration was high (1.5 g COD/l) and the effect of low substrate concentrations was not studied. SRIDHAR and PILLAI (1973) estimated proteolytic activities in sewage, sludges and effluents, investigated some procedures to extract protease from activated sludge and studied some properties of this protease.

1.3. OBJECTIVE AND OUTLINE OF THE PRESENT INVESTIGATION

The present study was intended to increase the understanding of fundamental and quantifiable aspects and characteristics of the activated-sludge process. An investigation of the enzymic degradation of externally supplied and internally released polymers was expected to allow a quantification of the removal of organic compounds from waste water and of the concomitant production (and subsequent degradation) of biological solids (biomass) from these organic compounds. Maximization and minimization of substrate removal and sludge production, respectively, are the main objectives of biological treatment of waste water.

The study of whole activated sludge was preferred to that of isolated bacterial cultures as the population composition of sludge is diverse and variable so that designation of representative organisms would be hardly possible. This is the more true as isolation and cultivation of sludge bacteria is difficult to perform. Moreover, the characteristic heterogeneous environment as occurring in activated-sludge flocs containing a large proportion of dead cells would be difficult to imitate.

The elucidation of the following problems was aimed at. Literature data are compiled in the relevant Chapters.

1. Which is the limiting factor as to polymer elimination and purification in general under conditions of continuous sludge loading or shock loadings (diffusion of substrate into sludge flocs, extracellular hydrolysis, uptake and utilization of hydrolysis products or other factors)? How does activated sludge respond to loading, under laboratory conditions, with a macromolecule as the sole carbon and energy nutrient? Is such a macromolecule, e.g. starch, immediately and adequately removed or is an acclimatization period required (Chapter 4)? Is adsorption preceding extracellular hydrolytic attack of starch by amylolytic enzymes (Chapter 6)? Several amylases have been described in the literature. Which one prevails in starch-loaded activated sludge (Chapter 3)? What is the end product of extracellular amylolysis, glucose or oligosaccharides (Chapter 6)?
2. The known amylases display their half-maximum reaction rates in the presence of 0.2 to 16 g amylose, amylopectin or starch per litre, substrate constants being too high to warrant nearly complete starch removal. Do sludge amylases show a more favourable substrate affinity or is the effect of a high substrate constant compensated by the production of large amounts of enzymes (Chapter 6)?
3. The location of amylases in activated sludge with retention of flocculated biomass imposes a problem. If amylases are excreted into the sludge-free liquid, they will be washed out soon after their production. If, on the other hand, amylase molecules are bound to the sludge mass, their reactivity and substrate accessibility may be adversely affected, also by diffusion limitation (Chapters 6 and 7).
4. What is the effect of sludge loading (food-to-micro-organism ratio) and of retention of flocculated biomass on starch removal, the yield of sludge biomass, the role of micro-organism decay, the number of viable bacteria, the activity and stability of amylases and other parameters (Chapters 5 and 6)?
5. The occurrence of dead cells in activated sludge, especially at low loadings, is well-known. Material released from these cells, e.g. proteins, might be reutilized by neighbouring cells. Is extracellular proteinase activity a measure for this turnover process; which factor is limiting this process (Chapter 5)?
6. By which mechanism acclimatizes activated sludge to starch loading (Chapter 4)? Induction of amylase synthesis in an existent population and selection of amylolytic bacteria, viz. a population shift, should be considered. Does starch loading result in the predominance of one amylolytic organism or does a mixed culture emerge (Chapter 8)? If the latter is true, would all organisms hydrolyse starch for themselves or would part of the species produce amylase in behalf of the whole population (Chapter 4)?

7. Which types of bacteria emerge (Chapter 8)? Does continuous loading of activated sludge with starch result in the selection of strains showing a particular regulation mechanism with regard to amylase synthesis, e.g. constitutive enzyme production (Chapter 8)?

2. MATERIALS AND METHODS

2.1. ACTIVATED-SLUDGE PLANTS

Activated-sludge samples withdrawn from the sewage purification plants at Zeist and Bennekom were used as experimental material and as initial sludge for laboratory scale cultivation (2.4.2).

The plant in Zeist is a usual one with grit chamber, rack, primary settling tanks, aeration tanks (with brush aerators of Kessener type), secondary settling tanks and (warmed) anaerobic sludge digesters. The total supply of about 60,000 population equivalents (PE) originates from domestic sewage (40,000 PE) and industrial waste water (20,000 PE), discharged by dairy industries, slaughter houses, galvanic industries and laundries. The installation is somewhat overloaded. The average sludge loading per g of sludge per day is about 0.5 g BOD_5 (1.5 g COD) and the mean hydraulic retention time in the activated-sludge unit is about 6 h (minimum $1\frac{3}{4}$ h). The pH in the activated-sludge tanks is usually about 7.0–7.5; nitrification does not occur.

The oxidation ditch at Bennekom, a modification of the oxidation ditch described by PASVEER (1959), is developed for the extended aeration of the domestic waste water of the combined sewerage of Bennekom (about 14,000 PE). It contains 6 brush aerators, no grit chamber and no primary and separate secondary settling tanks. Settling of sludge occurs by alternate closing of parts of the ditch. The average sludge loading per g of sludge per day approximates 0.03 g BOD_5 (0.063 g COD). The mean retention time of the sewage is about 2–3 days. The pH is usually above 7; a strong nitrification is observed.

2.2. CHEMICALS

The compounds used as constituents of cultivation media, as reagents in analytical-chemical determinations, or for enzymic determinations were obtained from different firms.

Abbreviations used: BBL: Division of Becton, Dickinson and Company, Cockeysville, Maryland, U.S.A.; Difco: Difco Laboratories, Detroit, Michigan, U.S.A.; Fluka: Fluka AG, Buch SG, Switzerland; Juste: Juste S.A., Madrid, Spain; Labaz: Labaz, Brussel-Maassluis, Belgium-The Netherlands; Lamers-Indemans: Dr. Lamers and Dr. Indemans N.V., 's-Hertogenbosch, The Netherlands; Merck: E. Merck AG, Darmstadt, W. Germany; NBC: Nutritional Biochemicals Corporation, Cleveland, Ohio, U.S.A.; Novo: Novo-industri A/S, Copenhagen, Denmark; OPG: 'de Onderlinge Pharmaceutische Groothandel' GA, Utrecht, The Netherlands; Oxoid: Oxoid Limited, London, England; Sigma: Sigma Chemical Company, St. Louis, Missouri, U.S.A.

Reagents: Folin-Ciocalteu phenol reagent (Merck); anthrone p.a. (Merck);

iodine resublimated (Ph. Helv. V, Fluka); perchloric acid 70 % (Merck); bovine serum albumine (Sigma).

Substrates etc.: glucose (Merck, Art. 8342); maltose (Lamers-Indemans, P-0547) and maltose (Merck, Art. 5912); maltotriose (containing c. 0.5% isomaltotetraose and less than 0.1% maltose) and DE-20 (preparation of dextrins, containing an average number of about 5 glucose units per molecule), donation of Th. M. van Bellegem, 'Proefstation voor aardappelverwerking, T.N.O.', Groningen, The Netherlands; soluble starch (Merck, Art. 1253); vitamin-free casein (NBC) and Hammersten casein (Merck, Art. 2242); vitamin-free, salt-free casein hydrolysate (NBC); tryptone (Oxoid, L42); yeast extract (Merck); trypticase soy broth (BBL); Bacto-agar (Difco certified, purified agar) and Opti-agar (Juste); silicone anti-foaming agent (Merck); nystatin (Labaz); chloramphenicol (OPG, Ph. Ned. VI).

Enzymes: α-amylase from *Aspergillus oryzae* (Fungamyl 1600, Novo); lysozyme chloride 3 × cryst. (NBC); proteases from *Streptomyces griseus* (Pronase E, Merck).

2.3. MEDIA

The media were prepared with demineralized water and autoclaved for 20 min at 120 °C, unless otherwise stated. Glucose, maltose and potassium phosphate solutions were sterilized separately when added to liquid media, with the exception of media D and F and the agar media.

2.3.1. *Basal medium*

The basal medium (Table 2.1) was prepared from concentrated stock solutions. The vitamin solution was stored in deep freeze.

TABLE 2.1. Composition of the basal medium.

Basal salts	mg/l	Vitamins	mg/l
$CaCl_2.2H_2O$	147	Ca-D-pantothenate	2
$MgSO_4.7H_2O$	75	D-biotin	0.02
NaCl	100	myo-inositol	10
Trace elements[1]		nicotinamide	5
		p-aminobenzoic acid	0.3
$FeCl_3.6H_2O$	3	pyridoxol hydrochloride	1
$ZnSO_4.7H_2O$	0.1	riboflavin	0.1
$CoCl_2.6H_2O$	0.05	thiamin hydrochloride	0.2
$CuSO_4.5H_2O$	0.1	vitamin B_{12}	0.005
$MnSO_4.H_2O$	5		
$Na_2MoO_4.2H_2O$	0.05		
H_3BO_3	0.1		

[1] The stock solution of trace elements was adjusted with HCl to pH 2.2.

TABLE 2.2. Composition of the media for discontinuous cultures.

Medium	The concentrations are given as g/l, unless otherwise stated										
	A_1	A_2	A_3	A_4	B_1	B_2	B_3	C	D	E	F
Basal salts[1]	1 ×	1 ×	1 ×	1 ×	1 ×	1 ×[2]	1 ×[2]				
$MgSO_4.7H_2O$[3]						0.225	0.225			1.00	
K phosphate pH 7.0 (mM)	10	15	15	3	100	100	5–20				
K_2HPO_4										1.00	
$CaCO_3$				0.15			0.50–0.75	2.00			
Soil extract (ml/l)			25	25	25	25	25	1000			
Vitamins[1]	1 ×	1 ×	1 ×	1 ×	1 ×	0.1 ×[4]	0.1 ×[4]				
$Glucose.H_2O$		0.01	0.01	0.01		0.01	0.01				
Trypticase soy broth			0.02	0.10			0.10				
Yeast extract		0.10	0.10	0.10		5.00	2.50	2.00	7.00		4.00
Nutrient broth										3.25	8.00
Tryptone											8.00
$(NH_4)_2SO_4$	0.133	0.237	0.237	0.237	0.937					1.00	
Carbon source[5] (g COD)	0.750 S or M or 0.563 T + 0.150 S + 0.038 M	1.20 S or 0.960 T + 0.240 S	1.20 S or G or 1.25 C[6]	1.20 S	5.00 G or S	5.00 G or S	5.00 G or S	2.00 S[6] + 2.00 G[6]	10.00 G[6]	5.00 S[6]	5.00 G[6]
Opti-agar								10.0	12.0[7]	12.0	12.0[7]
Bacto-agar		10.0[7]		10.0			10.0[7]				
Water	D	D	D	D	D	D	D	Tw	Tw	Tw	Tw

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[1] 1 × = equal to the concentrations recorded in Table 2.1, 0.1 × = 0.1 of that concentration; [2] no trace elements added; [3] additional to that in basal salts; [4] vitamin B_{12}: 0.005 mg/l; [5] S: starch; M: maltose; G: glucose; C: casein; T: tryptone; D: demineralized water; Tw: tap water; [6] as g/l; [7] with or without.

Table 2.3. Final concentration of medium constituents and their ratio in reactor influent streams.

	Reactor						
	with retention of flocculated biomass (I)				without retention of flocculated biomass (II)		
Sludge loading[1]	0.075	0.3	1.2	2.4	0.121	0.25–0.33	2.12
(designated as:)	(very low)	(moderate)	(high)	(very high)	(low)	(moderate)	(very high)
Hydraulic space loading[2]	1.0	1.0	1.0	3.0	0.05	0.10	1.0
Basal salts[3]	1 ×	1 ×	1 ×	0.67 ×	4 ×	1 ×	1 ×
Potassium phosphate pH 7.0 (mM)	0.3	0.6	2.4	1.6	4	2.67	2.4
Vitamins[3]	0.1 ×	0.1 ×	0.1 ×	0.067 ×	0.4 ×	0.1 ×	0.1 ×
Vitamin B_{12}[3]	1 ×	1 ×	1 ×	0.67 ×	4 ×	1 ×	1 ×
$(NH_4)_2SO_4$ (g/l)	0.066	0.266	1.06	0.708	5.31	3.54	1.06
Glucose, maltose or starch (g COD/l)	0.188	0.750	3.00	2.00	15.00	10.00	3.00
C/N ratio (g/g)	5	5	5	5	5	5	5
COD/P ratio (g/g)	20.2	40.4	40.4	40.4	121	121	40.4

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[1] SL (g COD/g sludge.day) planned for the reactor with sludge retention and averaged for the reactor without sludge retention. The sludge concentration of the first-mentioned reactor was planned to be 2.5 g (dry weight) per l; [2] dilution rate (D): l influent/day.l reactor medium; [3] related to the concentrations recorded in Table 2.1.

2.3.2. *Media for discontinous cultures*

Table 2.2 shows the various media used. The weight to weight C/N ratio was 9 in media A and B with glucose, maltose or starch as the only carbon and energy source and $(NH_4)_2SO_4$ as the nitrogen source (except for medium A_1: C/N = 10); yeast extract and trypticase soy broth were not taken into account in this calculation. The initial pH in media A and B was always 7. If no $CaCO_3$ was added the molarity of the phosphate buffer was usually 4–5 times as high as the molarity of $(NH_4)_2SO_4$ in the medium. Soil extract was prepared from garden clay soil; 1 kg was extracted with 1 l boiling tap water and the extract was passed through filter paper. The casein (NBC) used in medium A_3 was dissolved (separately) before sterilization by stirring magnetically for at least $1\frac{1}{2}$ h at pH 7.5. Separately sterilized potassium phosphate (0.75 M) and glucose. H_2O solutions (123.86 g/l) were added in quantities of 0.1 and 0.05 ml, respectively, to tubes (for counting of bacteria) with 5 ml of sterile medium by means of a repeating dispenser (Hamilton; total volume of syringe: 2.5 ml). Slants were usually supplied with $1\frac{1}{2}$ ml sterile demineralized water after sterilization and solidification of the agar. Starch yeast extract media (medium A_2) can be stored after sterilization at room temperature for at most 20 days before inoculation. The tryptone starch (4:1) yeast extract medium A_2 can be stored for at least 9 weeks without apparent loss of its growth-supporting quality.

2.3.3. *Media for continuous-flow cultures*

The composition of the media used for the continuous-flow sludge cultures is given in Table 2.3. This applies to systems with (I) and without (II) retention of flocculated biomass (cf. 2.4.2).

In order to minimize the number of sterilization operations, the medium constituents were distributed over two influent streams. One stream (the substrate solution) which was sterilized, carried $\frac{1}{4}$ or $\frac{1}{6}$ of the total flow and contained the carbon and nitrogen sources, potassium phosphate and vitamins in concentrations 4 or 6 times, respectively, the final concentrations. The other stream (the dilution water) was not sterilized (low pH) and contained the other basal salts in concentrations $\frac{4}{3}$ or $\frac{6}{5}$ times, respectively, the final concentrations.

The splitting up of the influent stream was applied to all continuous-flow sludge cultures with retention of biomass (I, activated sludge) and to the very high-loaded continuous sludge culture without retention of biomass (II, see 2.4.2). Starch was almost completely dissolved during sterilization but after this process it precipitated slowly due to retrogradation, especially in the concentrated media. Any inhomogeneity of the influent stream due to this phenomenon was prevented by continuous magnetic stirring of the substrate reservoir. The weight to weight C/N ratio of the substrate for all sludge loadings was 5. For carbohydrate substrates this value corresponds with a COD/N ratio of 13.3. The applied C/N ratio was adjusted at this low value in order to approach the C/N ratio in domestic sewage (PAINTER and VINEY, 1959; KOOT, 1974) and to ensure carbon-limited growth of the activated sludge as a whole.

The latter condition was expected to prevent bacterial production of intracellular polysaccharides and slimes which might hamper filtration and precipitation of the sludge. The COD/P ratio of the substrate for the continuous-flow sludge cultures with retention of biomass (I) was also low (40.4). At SL $= 0.075$ more phosphate was supplied than was in agreement with this ratio, in order to facilitate pH regulation (at low phosphate concentrations the pH fluctuated as the result of its adjustment by titration with NaOH).

For the other nutrients no constant ratios were used. The daily loading of sludge I with basal salts and vitamins was constant, except for the activated sludge with SL $= 2.4$, of which the loading was twice as high.

2.4. GROWTH CONDITIONS

2.4.1. *Discontinuous cultures*

All discontinuous cultures (bacterial counts and cultivation of isolated bacteria or sludges) were incubated at 25°C.

Agar plates were dried for one night at 30°C or for one h at 60°C. Pure cultures of bacteria were maintained on agar slants. Liquid batch cultures were usually grown in 100 ml B_2 or B_3 medium in 300 ml conical flasks incubated in a Gallenkamp cooled orbital incubator at 200 revolutions/min. The inoculum mostly consisted of 1 or 1.25 ml of a preculture grown during at least 3 days in starch medium B_2 or B_3 or of a small volume of a suspension of bacteria, derived from slant cultures, in sterile demineralized water.

2.4.2. *Continuous-flow cultures*

A completely mixed and continuous-flow activated-sludge reactor was constructed which was operable under controlled conditions. This laboratory scale apparatus enabled the quantification of effects of changes in operational variables on extracellular enzymes in activated sludge.

The experimental unit is shown on Plates 2.1 and 2.2 and diagrammatically in Fig. 2.1. The activated-sludge flocs were kept in the reactor by an internal polyether filter element (material purchased from Flygt, Rotterdam). The filter system was a slight modification of the system of RENSINK (1970). It had the shape of a cylinder (volume about 68 cm^3). The use of a spongy filter may ensure the maintenance of a high and constant sludge concentration in the system despite the inconstant and poor settling characteristics of activated sludge grown under laboratory conditions. Consequently, the system was tested only for its biochemical performance and not for its overall performance which includes settleability of the sludge flocs.

The partly closed continuous-flow system (I, i.e. with retention of flocculated biomass but not of culture fluid) could simply be changed into an open continuous-flow system i.e. without retention ('feedback') of biomass (II) by removing the filter. As recycling is a salient property of the activated-sludge process, the term 'activated sludge' will be used in this thesis only for the sludge

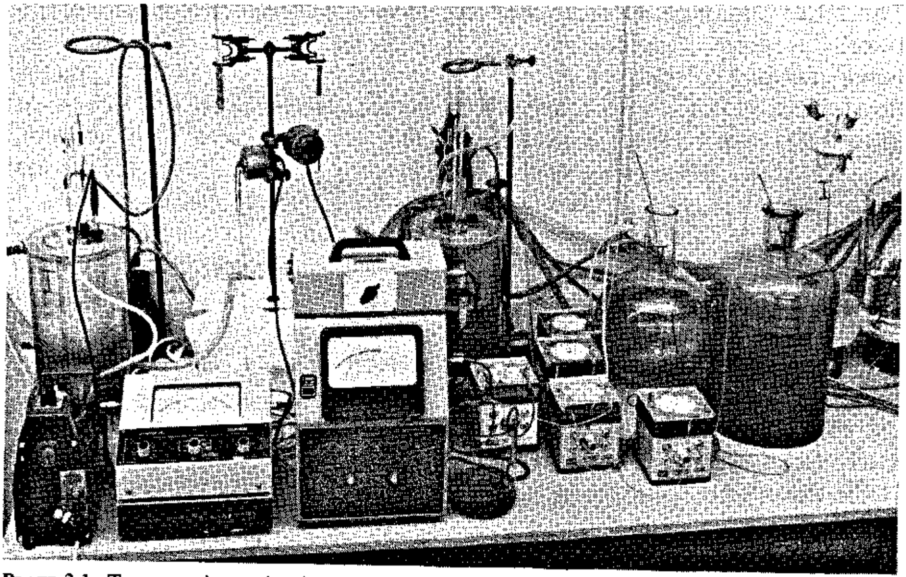

PLATE 2.1. Two experimental units operating with retention of flocculated biomass. Left-handed reactor: light-coloured sludge, SL = 2.4. Right-handed reactor: dark-coloured sludge, SL = 0.075.

in continuous-flow cultures with internal or external feedback of flocculated biomass.

Substrate solution and dilution water (see 2.3.3) were supplied separately and continuously from 10 and 15 l bottles of Pyrex glass, respectively, using sterilizable silicone rubber tubing and peristaltic pumps (Varioperpex and Multiperpex, LKB-produkter AB, Bromma, Sweden). Reactors and substrate reservoirs were stirred magnetically ('Cenco'). The outlets of the air distribution tubes were just above the teflon-coated stirring bars. The air was moistened previously by passage through gas distribution tubes contained in two flasks of demineralized water of 18.5°C. The aeration rate was controlled with Tri-Flat Variable-Area flowmeters (Fischer and Porter Co., Pennsylvania) at the beginning of the investigation.

The double-walled cylindrical reactor (inner diameter: 12.5 cm) consisted of transparent (non-sterilizable) 'Perspex' (polymethylacrylate) except for the centre of the bottom which was made of glass. The cover, containing 8 holes, was surrounded by a rubber ring (O-ring) and was provided with a handle so that the cover could easily be moved up and down and taken away for cleaning the filter at will. Effluent was removed continuously and automatically by gravitation or by suction. The cultivation temperature of 20 ± 0.2°C was maintained by circulating water of a water bath through the outer compartments of the two reactors used. The temperature of the water bath (about 18.5°C) was controlled with the aid of a cryostat and a thermostat. In all cases the pH (7.0) was controlled automatically with the aid of non-sterilized 2 N NaOH, unless otherwise stated. Infection of the substrate in the tubing and the reservoirs was

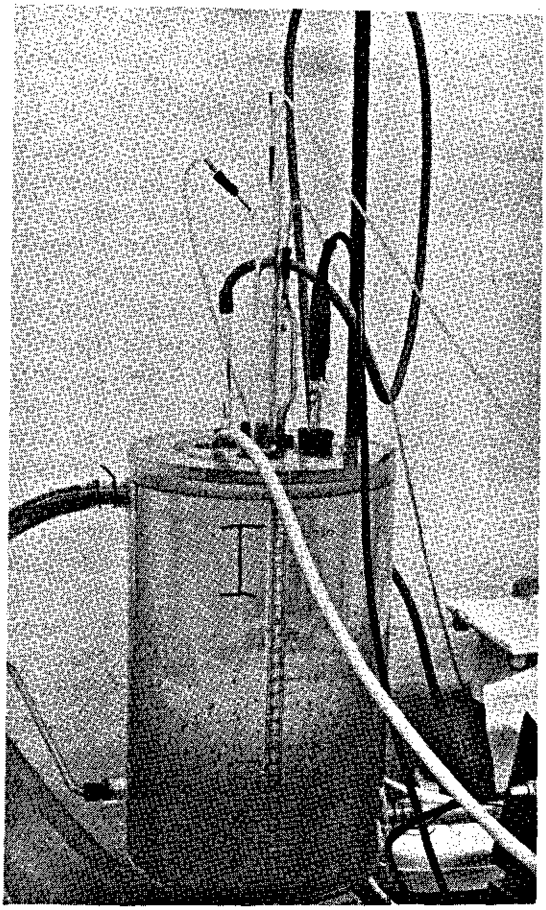

PLATE 2.2. A double-walled reactor operating with retention of flocculated biomass (SL = 2.4).

prevented by passing the NaOH solution through the substrate inlet (Fig. 2.1, detail).

Clogging of the filter, frequently occurring as a result of slimy sludge, had to be eliminated by cleaning the filter. In order to prevent loss of sludge as a result of reduced discharge of effluent, the capacity of the reactor (3.5 l) was more than twice the working volume (1.5 l). Foaming occurred rarely and only in the very high-loaded starch activated sludge. It was suppressed by the addition of a few drops of silicone anti-foam (Merck). Wall-attached sludge was resuspended, if necessary. For the rest the system appeared to be completely mixed.

The planned concentration of cell material in the reactors with retention of biomass was always 2.5 g/l. Excess of cell material was removed at intervals. The frequency of these interventions was dependent on the organic space loading (VL) which was carefully kept constant. Dry weight of starch sludge with planned SL = 0.075 was always lower than (or equal to) 2.5 g/l, resulting in

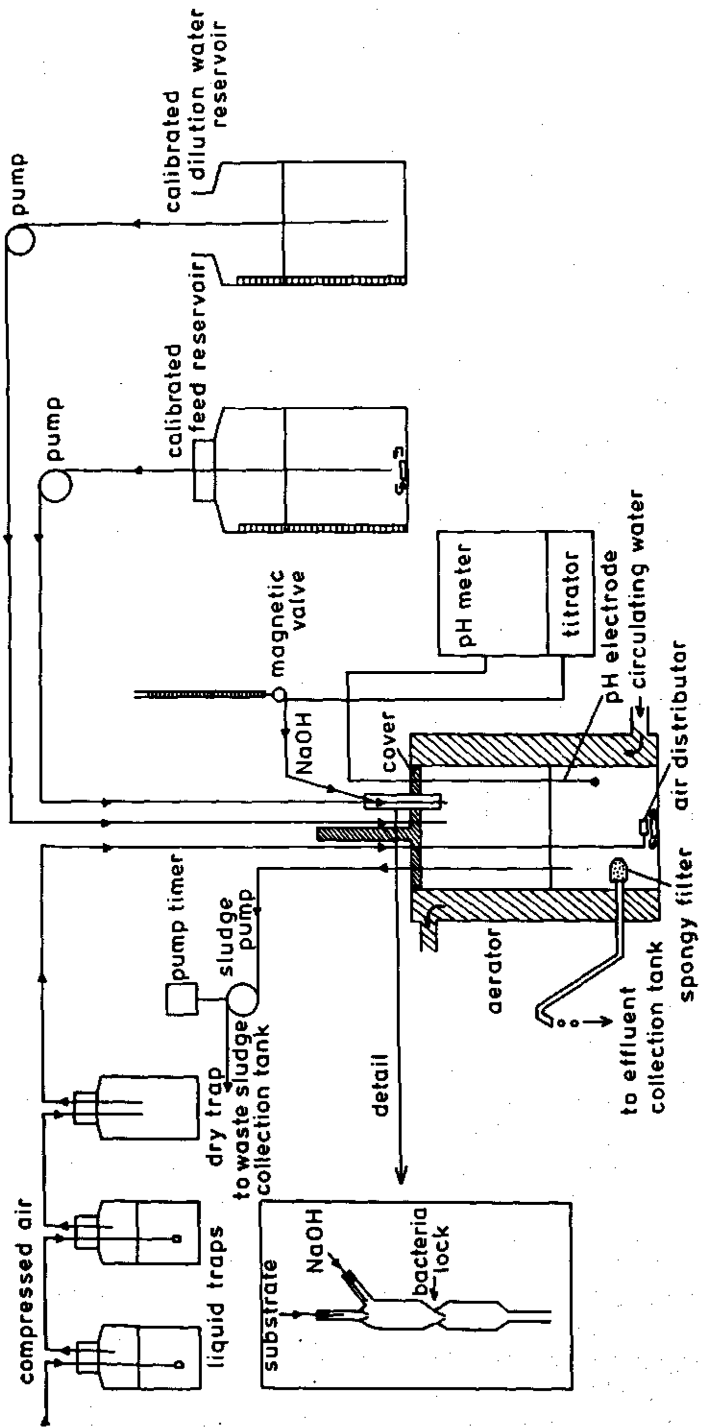

FIG. 2.1. Continuous-flow unit with retention of flocculated biomass.

sludge loadings higher than or equal to SL = 0.075. Increase of the sludge concentration to values of about 2.5 g/l always resulted in partial washing out of this sludge. The real SL values of the activated sludge with planned SL = 0.3 usually varied with a factor of about 2; once in 10–14 days half of the sludge was removed and replaced by reactor effluent or tap water. About $\frac{1}{3}$ of the starch sludge with planned SL = 1.2 was daily removed discontinuously. Excess starch sludge with planned SL = 2.4 was automatically removed every 2 h during 15–20 min by means of 6-mm diameter tubes and a waste sludge pump supplied with a timer (Watson-Marlow pump, Buckinghamshire, England). Extreme dry weights were about 2 and 3 g/l.

When this study started (July 1973) activated sludge from the Zeist plant was grown in starch medium and in maltose medium. These sludges were cultivated for $3\frac{1}{2}$ years in the laboratory; the substrate was occasionally changed from maltose or glucose to starch or vice versa. The planned loading of starch, maltose or glucose sludge, used for the different experiments, was 0.3 g COD/g of sludge.day, unless otherwise stated. Only during the third year the loading was altered.

In the experiments with very high-loaded sludge without retention of flocculated biomass (II), the system of Fig. 2.1 was used, the conditions being the same as those with sludge I. Only the waste sludge pump was replaced by a vacuum pump (Reciprotor, Copenhagen, Denmark) and a (vacuum) bottle connected to the effluent tube. The filter was replaced by a tube lengthened to the surface of the sludge liquid. The timer was maintained to remove excess sludge quickly every 4 hours. Only for the low-loaded sludges without retention of biomass a different reactor was used viz. a Soxhlet accessory with side-arm. The working volume was 0.535 l and the cultivation temperature slightly above room temperature (usually 23–24°C with extremes from about 20 to 27°C). Sludge was removed discontinuously once a day or once a few days. Magnetic stirring of the sludge was omitted and all substrate components were supplied from one bottle.

2.5. BACTERIOLOGICAL METHODS

2.5.1. *Viable counts*

Almost all viable counts of heterotrophic bacteria (expressed as most probable numbers, MPN) were performed according to the dilution frequency method, using liquid media in tubes. The same technique applied to agar media was used only once (plate dilution frequency technique: HARRIS and SOMMERS, 1968). All sludge samples were treated according to a slight modification of the procedure of PIKE et al. (1972). Homogenization of a sludge sample was performed conveniently by ultrasonic treatment of the sample, usually immediately after withdrawal and tenfold dilution in sterile demineralized water. The test tube containing the sample, was immersed in a 'Branson' ultrasonic cleaning bath (model Bransonic 32, 150 W, 50 kHz) for 1 min. This indirect ultrasonic treatment has only little lethal effect; it improves the reproducibility of counts and the period of treatment of samples is not critical (PIKE et al., 1972). However, big, stable flocs, occurring frequently in sludge loaded with 1.2 and 2.4 g COD/g of sludge.day, were pretreated, without prior dilution, for 5 min with a Vibro Mixer (40 W, Chemie-Apparatebau, Zürich). This vibrator, a circular metallic plate provided with holes was in direct contact with the sample.

Immediately after the ultrasonic vibration, serial dilutions up to 10^{-10} were made in sterile demineralized water. From the suspensions of dilutions 10^{-10} until 10^{-5}, 0.1 ml was injected by means of a repeating dispenser into 5 or 10 tubes containing 5 ml (sometimes 2 ml) growth media A_1, A_2 or A_3. The syringe had been sterilized before with 70% alcohol and rinsed successively with sterile demineralized water and part of the sample. After mixing, using a Vortex Mixer (Retsch), the tubes were incubated without shaking at 25°C.

The range of dilutions chosen was such that all tubes inoculated with the highest dilution remained sterile, whereas all those with the lowest dilution showed growth after incubation. Almost all information provided by a series of tubes is contained in the usually small range of intermediate dilutions at which part of the tubes show growth. The tables used to estimate the most probable number of viable bacteria per ml of homogenized sludge take into account the numbers of tubes showing growth in a set of only three consecutive intermediate dilutions. The tables of PORTER (1946; derived from the tables prepared by HOSKINS, 1934) were used for experiments with 5 tubes per dilution whereas the tables of HALVORSON and ZIEGLER (1933) cover the case of 10 tubes per dilution. PORTER (1946) gives various rules to select the three numbers to be used. Factors for obtaining the upper and lower approximate-97.5% confidence limits (95% confidence intervals) for the true bacterial density were obtained from COCHRAN (1950). These values were calculated using the fact that log (MPN) tends to a Normal limiting distribution when the sample size tends to infinity. The same is true of the MPN itself, but the log-transformation improves the quality of approximations for a small sample size. The above-mentioned factors fulfil the same general purpose like the standard error would have done, had it been appropriate to attach the latter to the estimated density. For a dilution ratio of 10 and a number of tubes of 5 and 10 per dilution, the factors (f) are 3.30 and 2.32, respectively. By multiplying and dividing the density estimate (MPN) with these factors, one obtaines an interval which includes the number of viable bacteria per ml with approximately 95% probability.

In order to conclude that two bacterial densities, estimated independently with 5 or 10 tubes per dilution, are different at the 5%-significance level, the estimates have to differ a factor of at least $3.30^{\sqrt{2}}$ (5.41) or $2.32^{\sqrt{2}}$ (3.29), respectively.

It is also possible to calculate an estimate and an approximate-95% confidence interval for the bacterial density if several independent counts (dilution series) are obtained from the same sludge, sampled at the same time. For example, the data might be combined into one series, but this technique cannot be used since (due to increase of sample size) MPN tables are not available. A more convenient method is to determine first the MPN separately for each of the series. Next, one combined estimate for the density is obtained by calculating their mean value (preferably on a logarithmic scale). When n estimates are combined, the factors for obtaining an approximate-95% confidence interval are $f^{1/\sqrt{n}}$ and $f^{-1/\sqrt{n}}$.

The table of COCHRAN (1950) is likely too optimistic with respect to the precision and accuracy of the MPN-based method since the incorporated statistical model does not cover some inavoidable sources of variations and of bias that appear in practice due to 'experimental errors' (e.g. resulting from pipetting and occurrence of bacteria in flocs). However, we expect the effect of experimental errors to be relatively small since the dilution method itself is of low precision. To some extent, a check on the model is possible in the situation discussed above where several MPN's are available. For example, the observed standard deviation between series of ln (MPN) can be compared with the theoretical value of COCHRAN (1950). However, in our experiments different series from the same sludge were taken at different times. In this case the theoretical value will be systematically exceeded due to possible density fluctuations in the sludge system during time of loading.

With the MPN counts in the media A_1, A_2 and A_3, ('total') numbers of viable bacteria and numbers of bacteria growing on starch, glucose, maltose or casein were estimated; this was done in sets of tubes used for separate counts (every dilution series gives one count) as well as in sets of tubes used for combined counts (every series gives 2 or 3 counts) (4.2). The tubes were inspected for growth after 10 days of incubation and after 15 or 20 days. Only the latter were used for determination of the MPN.

The spread-plate count was used for counting the numbers of the different types of bacteria present in activated sludge. The count was made after a prolonged incubation of the low-concentrated agar media e.g. A_2 or A_4 (about 3 weeks).

2.5.2. *Isolation of bacteria*

In the beginning of the investigation, bacteria were isolated from the tubes of highest dilution showing growth, by streaking on agar media and isolating the most frequently occurring colonies. Because this is not a representative isolation method, it was replaced by direct spreading of 0.1 ml (in duplicate or triplicate) of sludge dilutions $10^{-5}-10^{-6}-10^{-7}$ on low-concentrated agar media A. After 12 or more days of incubation the different types of bacteria were inspected under the phase contrast microscope and types and numbers of colonies observed with the aid of a stereomicroscope and counted. Cultures of various types were grown pure by streaking on agar media A and were usually maintained on agar slants containing media A, B_3 or C.

2.6. ANALYTICAL METHODS

2.6.1. *Dry weight of sludge and bacterial cultures*

Well mixed, representative samples of usually 10 ml were passed through a previously dried and weighed membrane filter (Oxoid Nuflow N°. 100; pure cellulose acetate; diameter 47 mm; nominal pore size 0.45 μm; maximum pore size 0.95 μm). After rinsing once with demineralized water, the filters were dried overnight at 105°C. Results are reported as dry weight, (dry) biomass or (mixed liquor) suspended solids, (ML)SS. The repeatability was within 5%.

Filtration was often hampered by slimes produced by and extracellularly bound to bacteria. Sometimes sludge or cells were harvested by centrifugation at 38,000 × g during 30 min, washed once with demineralized water, transferred to a weigh glass and weighed after drying overnight at 105°C.

2.6.2. *Ash content of sludge*

Samples of 50 or 100 ml of sludge were harvested by centrifugation, washed once and transferred to a crucible, previously ignited at 600°C and weighed. The crucible with contents was dried overnight at 105°C for the determination of the dry weight of the sludge sample. In some instances dry weight was determined simultaneously with the aid of a membrane filter (2.6.1).

The ash content was determined after incineration of the sample in an electric muffle furnace at 600°C for 1 h. Results are reported as percentages of ash or of (mixed liquor) volatile suspended solids, (ML)VSS (i.e. weight loss of MLSS by incineration).

2.6.3. *Chemical oxygen demand*

The total amount of soluble organic matter in sludge-free liquids (centrifuged for 30 min at 38,000 × g) was estimated as chemical oxygen demand, COD, and expressed as the amount of oxygen (mg) needed for the total oxidation of most of the organic matter present per litre of sludge-free liquid. The COD was determined in the usual way, i.e. by chemical oxidation of the organic matter in the sample with potassium dichromate and subsequent determination of the excess of oxidant with ferrous ammonium sulphate. The determination was carried out according to the test methods for waste water of the 'Nederlands Normalisatie-instituut' (NEN 3235 5.3). The standard solutions used were less concentrated, however, as the samples were only slightly polluted. The solution of potassium dichromate was 0.05 N, that of ferrous ammonium sulphate about 0.03 N.

2.6.4. *Oxygen concentration and oxygen uptake rate*

Oxygen concentration and oxygen uptake rate were determined polarographically with a Biological Oxygen Monitor model 53 (Yellow Springs Instrument Co., Inc., Ohio, U.S.A.) coupled to a 100 mV potentiometer. The respiration rate was measured at 20°C in 5 or 10 ml air-saturated 0.02 M potassium phosphate, pH 7.0. Substrates were injected with a syringe. The decrease of the oxygen concentration was recorded as a function of time. For calibration deoxygenated sludge and air-saturated demineralized water were used.

Sludge was centrifuged, washed 2 to 3 times with potassium phosphate (0.02 M, pH 7.0), resuspended in such a buffer solution and kept at 20°C for about 3-8 h before the oxygen uptake rate was determined.

2.6.5. *Total carbohydrates*

Total carbohydrates were determined with the anthrone method as described by TREVELYAN and HARRISON (1952) and ZEVENHUIZEN (1966). Glucose was used as the standard (0.1 mg per ml) and the absorbance was measured in a Vitatron colorimeter against demineralized water.

The method was used for the determination of carbohydrates, especially starch and its degradation products, in cell-free sludge liquid. The sludge was filtered by using a 0.45 μm membrane filter (Oxoid), immediately after sampling, or centrifuged at 1-4°C for at least 20 min at 38,000 × g. The method was also used for the determination of the total carbohydrate content of activated sludge after washing the sludge once with demineralized water.

2.6.6. *Reducing sugars*

Reducing sugars were determined with the method of Somogyi-Nelson (SOMOGYI, 1952) as described by ZEVENHUIZEN (1966). Glucose was used as the standard (0.1 mg per tube) and the absorbance was measured in a Vitatron colorimeter against demineralized water. The method was used for the determination of reducing sugars liberated from starch by amylases.

2.6.7. *Starch and dextrins*

Samples of 10 ml of sludge were, immediately after withdrawal, membrane-filtered or ice-cooled and centrifuged or they were immediately mixed with 2 drops of 70% $HClO_4$ and centrifuged at 38,000 × g for a least 20 min. Starch was determined as its reddish brown-coloured iodine complex; a blue colour is obtained with less concentrated reagent. The iodine reagent was prepared according to ZEVENHUIZEN (1966): 200 ml of 0.3 M citrate buffer, pH 6.0, 50 ml of demineralized water and 40 ml of I_2-KI solution (6.0 and 12.0 g/l, respectively; cf. LARNER, 1955) were mixed and stored at 3.5°C in the dark in a closed bottle. A high concentration of iodine reagent was used in order to prevent interference of unidentified components with the iodine starch reaction. GILBERT and SPRAGG (1964) suggest that chemical reactions of samples or extracts from natural sources with iodine may occur and WILDING (1963) found that high protein concentrations have a decolourizing effect on the iodine starch colour; this effect is dependent on time and temperature.

The samples, usually 5 ml for those from the reactor and 0.1 ml in the case of section 2.6.8, contained at most 0.5 mg starch. They were diluted with water to a volume of 5 or 10 ml, to which 1.0 or 2.0 ml, respectively, of iodine reagent was added. The absorbance at 623 nm was measured within a few min (maximum 10 min) in a Vitatron colorimeter against demineralized water; it was corrected for the absorbance of the reagent blank and compared with that of a freshly prepared starch standard. An extinction of about 0.085 was observed with 5 ml starch solution of 10 mg/l. The absorbance slowly fades away after 10 min owing to the volatility of the iodine. The wavelength of 623 nm is near the maximum produced by the starch component amylose (660 nm) and at some distance from the maximum of amylopectin (530–550 nm). Although amylose accounts for only 15–27% of the soluble starch, it is responsible for the greater part (about 70%) of the absorbance at 623 nm. The colour, developed after the procedure indicated, was found to obey the Lambert-Beer law up to at least 0.5 mg starch per sample which was the maximum amount present. This method provides only a rough indication of the amount of starch-like polysaccharides present, as the wavelengths of maximum light absorption and the molar extinction coefficients vary considerably with the degree of branching and with the molecular weight. Their concentration is expressed arbitrarily as mg 'starch(-extinction) equivalents' (SE) per litre.

2.6.8. *Amylolytic activity*

The rate of enzymic starch digestion by sludge or bacterial cultures was measured as the standard dextrinizing and as the saccharifying activity. The former activity, used for routine purposes, was deduced from the decrease of iodine-stainable compounds (determination 2.6.7), the latter from the increase of reducing compounds as determined with the Somogyi-Nelson reagent (2.6.6). The sludges were not pretreated, since it was aimed to determine the amylolytic activity under conditions prevailing in the reactor.

Enzymic starch hydrolysis was followed with time. The incubation mixtures of (at least) 10 ml were reciprocated in rubber-stoppered conical flasks of 25 or 100 ml in a thermostat-controlled water bath. The mixture consisted of 1.0 ml of 1.0 M potassium phosphate, pH 7.0, 4 ml of starch solution (containing 12.5 g starch and 1.25 g chloramphenicol per litre), sample, and demineralized water. The blank reaction mixture contained all ingredients except the enzyme sample.

The final standard conditions were, unless otherwise stated:

starch	5 g/l	pH	7.0
potassium phosphate	0.1 M	temperature	30 ± 0.2°C
chloramphenicol	500 mg/l	rate of shaking	c. 100 strokes/min

Chloramphenicol was added in order to prevent enzyme synthesis and bacterial growth during the enzyme incubation. The starch chloramphenicol solution was heated in boiling water for 5 min and cooled down in tap water. It was always prepared freshly in order to avoid hazardous effects of amylose retrogradation.

The reaction was usually initiated by the addition of the starch solution, after about 10 min of pre-incubation, by means of a blow pipette supplied with a cotton-wool plug in order to avoid contamination of the starch solution and the incubation mixtures with salivary α-amylase.

After 30 sec and subsequently after different periods of incubation, samples of at least 1½ ml were freed from protein or cell material by the addition of one drop of 70% $HClO_4$ (giving a final pH of about 1.4). The samples were centrifuged at 2500 × g for 7 min or at 38,000 × g for 20 min if this was necessary for obtaining a clear supernatant required for the determination of the saccharifying activity. The first sample (30 sec of incubation) served as the control. Supernatants were analysed for starch (2.6.7), reducing sugars (2.6.6) and sometimes total carbohydrates (2.6.5). Samples of reaction mixtures with cell-free ultrasonicate of starch sludge had sometimes to be centrifuged again (7 min; 2500 × g) after the addition of the Somogyi-Nelson reagent.

The dextrinizing activity (DA) was defined as the initial decrease of starch-extinction equivalents (measured as iodine-stainable compounds), expressed as mg starch (equivalents) (SE) hydrolysed per hour per ml of enzyme sample. The saccharifying activity (SA) was defined as the initial increase of reducing sugars, expressed as mg glucose per hour per ml of enzyme sample. Specific enzyme activities were defined as above, but expressed per mg of dry biomass per hour (DA_{spec} and SA_{spec}).

Ultrasonication of sludge

Enzyme activities were also measured in cell-free extracts and resuspended precipitates of ultrasonically disintegrated sludges. Sludge aliquots of 10 ml were disintegrated in glass tubes of about 15 ml (diameter 20 mm) with the aid of an 'Ultrasonic Power Unit' (M.S.E., 0.9–1.0 ampere, diameter of probe 9.5 mm). After sonication, samples were centrifuged at 38,000 × g and 4°C for 30 min. The supernatants were usually slightly opalescent. The pellets were resuspended in demineralized water and brought to the original volumes. Samples were kept in ice during the whole procedure.

2.6.9. *Estimation of $K_{1/2}$ value of sludge amylases*

The amylolytic reaction rate was measured as DA (2.6.8) in samples of untreated starch sludge or of cell-free ultrasonicate (sonication time 10 min; 2.6.8); these samples were diluted previously with demineralized water with a factor 2.5–15 and kept in ice.

Final conditions in the assay mixtures, unless otherwise stated:

starch	5–5000 mg/l	pH	7
potassium phosphate	0.01 M	temperature	30 ± 0.2°C
chloramphenicol	omitted	rate of shaking	c. 100 strokes/min
preferred DA	0.25–0.75		

The reaction was initiated by rapidly adding 10 ml of a previously diluted enzyme sample (with a blow pipette plugged with cotton-wool at the top) to 30 ml of a solution containing the other components of the assay mixture. Initial starch concentrations were usually: 5, 7.5, 10, 15, 25, 50, 200, 500, 1000 and 2000 mg/l. Samples of 7.5, 5.0 or 2.5 ml (of initial starch concentrations 5–50, 100–200 and 500–5000 mg/l, respectively) were withdrawn from the assay mixtures; after different periods (intervals of at least 30 sec) they were blown out into centrifuge tubes containing 3, 2, or 1 drop(s) of 70% perchloric acid, respectively, and centrifuged at 38,000 × g for 20 min. Starch equivalents were determined with iodine reagent (2.6.7) in samples of 5–0.1 ml of the supernatant. The increased sample size up to 5 ml at low starch concentrations ensured a much increased sensitivity and precision of the DA method (3.5.2). The SA method was not sensitive enough and was adversely affected by reducing compounds present in the ultrasonicate and by possible adsorption of starch to sludge.

Dextrinizing reaction rates of sludge and ultrasonicate, at their original concentration, were calculated from the initial decrease of starch equivalents (starting at $t = \frac{1}{2}$ min). The given sample correlation coefficients (r) reflect the measure of the degree of closeness of the linear relationship between S^{-1} and v^{-1} (Lineweaver-Burk plots).

2.6.10. *Protein in cell material*

In some experiments total protein was determined in sludges and bacterial cultures by the biuret method.

Samples were centrifuged at 38,000 × g for 30 min, washed once with demineralized water and resuspended in demineralized water. Protein was determined according to a modification of the method of ROBINSON and HOGDEN (1940), as described by HERBERT et al. (1971). It was quantitatively extracted from whole cells upon treatment with 1 N NaOH at 100 °C for 5 min.

The biuret colour is extremely reproducible and strictly proportional to the amount of protein added. Its absorbance was read in a Vitatron colorimeter against demineralized water. Bovine serum albumine served as the standard. Its absorbance (corrected for that of the reagent blank) read off at 570 nm is 0.141, when 1 g is present per litre. The absorbance of vitamin-free casein (NBC) at the same concentration is 0.123.

2.6.11. *Protein degradation products*

The determination was carried out according to a modification (DEMOSS and BARD, 1957) of the method of Lowry c.s. (1951) with the 'phenol reagent' of Folin and Ciocalteu (1927). At alkaline reaction a blue colour ('molybdenum blue') is formed after interaction of phosphotungstic and phosphomolybdic acids ('phenol reagent') with tyrosine and, to a less extent, with tryptophan in the protein or in the free form. Other amino acids have little effect, but it was found (HERRIOTT, 1941) that the pretreatment of proteins with alkali and a trace of copper greatly increases (3 to 15 ×) the absorbance developed with the Folin reagent, presumably due to the formation of Cu^{2+} complexes with the other amino acids. This modification of the original Folin method not only increases its sensitivity but also reduces somewhat the very unequal chromogenicities of different proteins.

Reagents: 1) 216.0 g $Na_2CO_3.10H_2O$/1 demineralized water; 2) 0.6 g $CuSO_4.5H_2O$ and 1.2 g potassium sodium tartrate tetrahydrate per 1 demineralized water (after dissolving separately); 3) 1 part of Folin-Ciocalteu phenol reagent and 2 parts of demineralized water (kept at 4 °C).

Procedure: samples were made up (if necessary) with demineralized water to a total volume of 0.5 ml; 5.0 ml of copper carbonate reagent (equal volumes of reagents 1 and 2 mixed just before use) was added and the test tubes were left at room temperature for 10 min. Diluted Folin-Ciocalteu reagent (0.5 ml) was added and the contents of the tubes immediately mixed thoroughly. After exactly 30 min the absorbance was read at 665 nm in a Vitatron colorimeter against demineralized water. The absorbance of a reagent blank was subtracted. A standard curve was prepared with vitamin-free casein (NBC; 0–2 mg/ml in the original solution). The curve is not linear, but well reproducible. This method was usually used for the determination of perchloric acid-soluble split products of casein (2.6.12).

Another modification of the method of Lowry is described by HERBERT et al. (1971), being about 2.4 times as sensitive as that described by DEMOSS and BARD (1957). It was sometimes used, omitting the boiling in 0.5 N NaOH during 5 min and reading the absorbances at 665 nm.

2.6.12. *Proteolytic activity*

The determination of proteolytic enzyme activity can be based upon determinations of either the reaction products or of the remaining substrate. Assays based on determinations of low-molecular protein degradation products are more sensitive. For this reason the release of perchloric acid-soluble peptides from casein was chosen as a measure of proteolytic enzyme activity. Casein preparations of high purity are commercially available in large amounts; casein contains a relatively high proportion of aromatic amino acids (BLOCK and BOLLING, 1947) and it was found (SRIDHAR and PILLAI, 1973) that activated-sludge proteases hydrolyse casein more extensively than haemoglobin and egg albumin.

The use as an enzyme substrate of small natural peptides of known sequence or of (whether or not chromogenic) synthetic substrates was not considered, as proteolytic enzyme activity of activated sludge results from a mixture of many enzymes with unknown reaction mechanisms and substrate specificities.

Since it was the purpose to determine the enzyme activity under conditions prevailing in the reactor, no pretreatment was applied to the samples of sludge. For the determination of standard proteolytic enzyme activities, incubation mixtures of (at least) 10 ml were reciprocated in rubber-stoppered conical flasks of 25 or 100 ml capacity in a thermostat-controlled water bath. The mixture contained 1.0 ml of 1.0 M potassium phosphate, pH 7.5, 4 ml of casein solution (containing 12.5 g casein and 1.25 g chloramphenicol per litre) and 5 ml of sample. Sometimes, part of the sample was replaced with demineralized water. The blank reaction mixture, incubated if very low proteolytic activities had to be determined, contained all ingredients for the enzyme assay with the exception of the enzyme.

The final standard conditions were, unless otherwise stated:

casein	5 g/l	pH	7.5
potassium phosphate	0.1 M	temperature	30 ± 0.2°C
chloramphenicol	500 mg/l	rate of shaking	c. 100 strokes/min

Chloramphenicol was added in order to avoid bacterial growth and proteinase synthesis and to prevent assimilation of liberated amino acids and peptides. Vitamin-free casein (NBC) was dissolved in a chloramphenicol solution by magnetic stirring for at least 1.5 h at room temperature; NaOH was added occasionally in order to raise the pH value to about 7.5. This stock solution was stored at 3.5°C for at most one month. The reaction was initiated by the addition of the substrate solution by means of a blow pipette, after about 10 min of pre-incubation.

Samples of 2 ml were withdrawn after 30 sec and subsequently after different periods of incubation. The first sample (30 sec of incubation) served as the control. The reaction was stopped by the addition of 0.1 ml 70 % $HClO_4$ (final pH about 1). Cell material or cell protein and undigested casein precipitated due to this treatment and were subsequently separated by centrifugation for 20 min at 38,000 × g. Peptides and amino acids released by proteolytic enzymes from casein were determined in the decanted supernatant by the modified Lowry method (2.6.11). This method was preferred to other well-known methods (biuret reaction, ninhydrin reaction, UV absorbance at 280 nm) because of its sensitivity, specificity and reproducibility. UV-absorbing substances released from activated sludge interfered with the simple spectrophotometric method (280 nm) of KUNITZ (1947), described by LASKOWSKI (1955).

The proteolytic activity (PA) was defined as the initial increase of perchloric acid-soluble casein constituents, expressed as mg casein hydrolysed per h per ml. Specific enzyme activities were defined as above, but expressed per mg of dry biomass per h (PA_{spec}).

2.6.13. *Enzyme inactivation rate during sludge starvation and in adsorption experiments*

A sample of sludge was centrifuged (usually for 25 min at 23,000 × g), resuspended in the supernatant or in demineralized water at 2–2.5 times the original concentration and pre-incubated for 10 min at 30°C. In some experiments cell-free ultrasonicate was prepared according to the method described in 2.6.8.

A sample of sludge, cell-free ultrasonicate, or, if applied, a solution of an enzyme preparation like Fungamyl 1600 was added at t = 0 to the other components of the inactivation mixture contained in rubber-stoppered conical flasks of 100 ml.

The final (standard) conditions in the inactivation mixture were, if not otherwise stated, as follows:

potassium phosphate	0.1 M	rate of shaking	c. 100 strokes/min
chloramphenicol	500 mg/l	sludge or ultrasonicate	40–100% of original concentration
pH	7.0		
temperature	30 ± 0.2°C	enzyme preparation (if added)	20 mg/l

After $\frac{1}{2}$ min and subsequently after different periods of inactivation a sample of this mixture was added to a pre-incubated enzyme assay mixture for the immediate determination of DA (2.6.8) or PA (2.6.12). The final molarity of potassium phosphate in the enzyme assay mixture was 0.1 and the pH for both

DA and PA was 7.0; no additional chloramphenicol was added. The final sludge or ultrasonicate concentration was $\frac{1}{2}$ or $\frac{1}{4}$ of that of the original sample (or less if Fungamyl 1600 was added). Enzyme activities were computed at the original concentrations.

2.6.14. *Bacteriolytic activity*

Sarcina lutea was used as substrate for bacteriolytic enzymes since the organism is extremely sensitive to the peptidoglycan-splitting muramidase (lysozym) and glucosaminidase and to a lesser extent to endopeptidases.

One ml of a cell suspension obtained from a young culture of *S. lutea* grown on an agar slant (medium D or F) was inoculated in a 100 ml – conical flask containing 50 ml of medium D. After about 1 day of cultivation at 30°C the bacteria were centrifuged for 15 min at 5100 rpm in a Rotosuper centrifuge (swing out), washed once with demineralized water and diluted appropriately. A typical reaction mixture consisted of: 4 ml of *Sarcina* suspension, 1 ml 0.03 M potassium phosphate, pH 7.0, and 5 ml of enzyme solution. The nephelometer used (EEL, Evans, Halstead, Essex, England) was adjusted to 50 units with the aid of a 100 units standard tube; the initial value of the whole incubation mixture was planned to be 70–100 units. The experiments were carried out at room temperature. When whole sludge was tested for extracellular bacteriolytic activity by adding a suspension of *S. lutea*, clearing up of the *Sarcina* cells was followed microscopically and that of the *Sarcina* suspension with the naked eye (turbidity of supernatant of settled sludge).

3. ENZYMIC HYDROLYSIS OF STARCH AND CASEIN BY ACTIVATED SLUDGE

3.1. INTRODUCTION

A successful study of microbial, ecological and sanitary-engineering aspects of amylolytic and proteolytic enzymes in activated sludge requires knowledge of the properties of these extracellular enzymes and reliable assay conditions for the determination of their activities. However, little if anything is known about extracellular enzymes in activated sludge. For this reason some properties and assay conditions of these two enzymes were studied in whole sludge.

Starch-degrading enzymes. Starch is a mixture of the homopolysaccharides amylose (straight chains of 1,4-α-linked D-glucose residues), and amylopectin (branched polymer of straight chain fragments of 1,4-α-linked D-glucose residues joined by 1,6-α-linkages). Amylolytic (i.e. starch-degrading) enzymes may be classified as being endo-acting, exo-acting or debranching (literature: BANKS and GREENWOOD, 1975; Enzyme Nomenclature, 1972; WHELAN, 1960). The term amylases is used in the present publication for enzymes degrading starch hydrolytically.

α-Amylase (1,4-α-D-glucan glucanohydrolase, EC 3.2.1.1), by far the most common endo-amylase, hydrolyses exclusively 1,4-α-glucosidic linkages in outer and inner chains of starch, glycogen and related polysaccharides in a rather random manner. It readily converts amylose and amylopectin into dextrins, maltotriose and/or maltose, resulting in a rapid decrease of the ability to stain with iodine but only a comparatively small increase in reducing power. Amylose-derived dextrins and maltotriose are ultimately converted into maltose and glucose at a much slower rate and in a non-random manner. Maltose itself is completely resistant to α-amylolysis. The 1,6-α-glucosidic bonds of amylopectin are not attacked by α-amylase. Therefore, the hydrolysis of amylopectin ultimately leads to the accumulation of resistant, branched α-limit dextrins in addition to maltose and glucose. The smallest of these α-limit dextrins contain 3, 4 or 5 glucose residues, depending on the sources of α-amylase, joined by one 1,6-α-bond in addition to the 1,4-α-linkage(s).

Exo-acting starch-degrading enzymes hydrolyse starch, glycogen and related polysaccharides by the successive removal of low-molecular weight products from the non-reducing ends of the (outer) chains. With amylose as substrate, large amounts of small sugars are formed without any marked change of the iodine stain of the polysaccharide.

This group of enzymes includes β-amylase (1,4-α-D-glucan maltohydrolase, EC 3.2.1.2), phosphorylase (1,4-α-D-glucan: orthophosphate α-glucosyltransferase EC 2.4.1.1) and glucoamylase (syn. exo-1,4-α-glucosidase; 1,4-α-D-glucan glucohydrolase, EC 3.2.1.3). They produce maltose, glucose-1-phosphate and glucose, respectively. β-Amylase and phosphorylase cannot hydrolyse or bypass the branch linkages of amylopectin and glycogen. Therefore they form high-molecular weight β-limit (maltosyl, maltotriosyl) dextrins and phosphorylase (maltotetraosyl)-limit dextrins in addition to maltose and glucose-1-phosphate, respectively. The weight of both dextrins amounts to about half of that of the starting material.

In contrast to β-amylase and phosphorylase, most forms of glucoamylase have the ability to hydrolyse 1,6-α-glucosidic bonds in addition to 1,4-α-glucosidic linkages, be it that the reaction proceeds only when the next bond in sequence is 1,4.

Debranching enzymes hydrolyse the 1,6-α-glucosidic inter-chain linkages in amylopectin and/or glycogen and therefore enable the above-mentioned starch-degrading enzymes to convert these polymers ultimately into small unbranched sugars. Only glucoamylase quantitatively converts both amylopectin and glycogen to glucose in the absence of debranching enzymes.

For the complete hydrolysis of starch following the degradation by α- or β-amylase and debranching enzyme, the presence of α-glucosidase (α-D-glucoside glucohydrolase, EC 3.2.1.20) is required for the conversion of maltose into glucose. This enzyme is also able to split off terminal, non-reducing, 1,4-linked α-

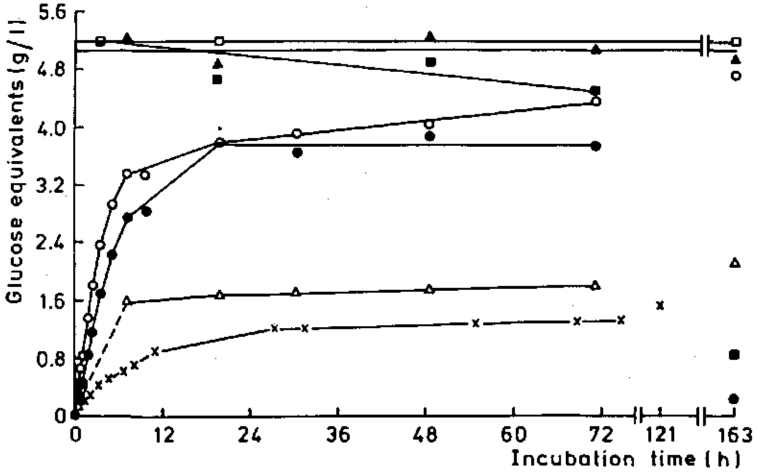

FIG. 3.1. Exhaustive starch degradation by the amylolytic enzymes of starch activated sludge, by those of the cell-free ultrasonicate of this sludge and by Fungamyl 1600. The initial starch concentration of 5 g per litre corresponds with 5.16 g glucose equivalents per litre as the moisture content of the starch was 7.2%. The activated sludge had been loaded with starch for 393 days (SL = 0.3); it was washed once with demineralized water and incubated at the original sludge concentration. Ultrasonic treatment was performed for 10 min. Standard amylase assay mixtures (2.6.8) additionally received 50 mg of nystatin per litre, 1% (v/v) ethanol and some drops of toluene and chloroform per 50 ml of incubation mixture, except for the mixture with 1 mg Fungamyl 1600 per litre. ●, ○, △, Reducing sugars produced by starch sludge, cell-free ultrasonicate and Fungamyl 1600 (25 mg/l), respectively; ■, □, ▲, total carbohydrate concentration in the presence of sludge, of ultrasonicate and of Fungamyl 1600 (25 mg/l), respectively; x, reducing sugars produced by Fungamyl 1600 (1 mg/l) under standard conditions (2.6.8).

D-glucose residues from higher oligosaccharides, be it at a lower rate than from maltose.

Finally it is remarked that in addition to the well-known exo-enzymes mentioned above, some other exo-amylases have been reported recently. One of them (from *Pseudomonas stutzeri*) specifically hydrolyses the fourth bond from the non-reducing ends (liberating maltotetraose), another (from *Aerobacter aerogenes*) specifically hydrolyses the sixth bond from the non-reducing ends (liberating maltohexaose). Moreover, the distinction between α- and β-amylases is becoming less pronounced as more information is forthcoming and amylases with quite diverse action patterns have been isolated. For instance, some amylases liberate mainly β-maltose, have a high degree of repetitive attack but have also the ability to bypass the 1,6-α-branch points of amylopectin and glycogen, indicating that they are endo-enzymes.

3.2. CHARACTERIZATION OF THE AMYLOLYTIC ENZYME SYSTEM OF ACTIVATED SLUDGE

3.2.1. *Introduction*

To study the mechanism of starch degradation in sludge I, the final degree of starch hydrolysis and the ratio of dextrinizing (DA) to saccharifying (SA) activities were estimated whilst the substrate specificity of the saccharifying enzymes was investigated. The DA and SA assay methods were compared as to their suitability for routine measurements of amylolytic activity.

As the amylolytic enzymes are completely bound to the activated sludge (3.5.1), all experiments were carried out with whole activated sludge and/or with the (cell-free) ultrasonicate of the sludge. During experiments which were continued for more than 12 h, microbial growth was prevented by adding nystatin and some drops of toluene and chloroform in addition to the chloramphenicol present under standard conditions (2.6.8). Nystatin is a polyene antibiotic inhibiting growth of yeasts and fungi. It was administered as the ethanolic solution at final concentrations of 50 mg per litre and 1 % (v/v) of ethanol.

3.2.2. *Final degree of starch hydrolysis*

The exhaustive hydrolysis of starch by starch-acclimatized activated sludge (starch activated sludge, starch sludge) and by the cell-free ultrasonicate of this sludge was compared with that of a commercial fungal α-amylase (Fungamyl 1600) (Fig. 3.1). Ultrasonic treatment of sludge results in a considerable increase of DA and SA (see also Figs. 3.2 and 3.3). This increase is presumably caused by the release of periplasmic enzymes (7.2).

From the high degree of hydrolysis, 75 and about 90 %, brought about by starch sludge and cell-free ultrasonicate, respectively, it is concluded that under the conditions described, glucose is the main end product of sludge amylolysis. The 41 % hydrolysis finally achieved by Fungamyl 1600 (25 mg/l) suggests that maltose is the main end product of Fungamyl action. The experiment with 1 mg Fungamyl per litre suggests that intermediately a semi-stable point of hydrolysis is reached when the average degree of polymerization ($\overline{\mathrm{DP}}$) of products formed is 4. The data concerning the fungal α-amylase agree with those reported in NOVO enzyme information (Sept. 1971) and with those of WHELAN (1960 and 1964).

Comparison of the above-mentioned high degree of hydrolysis with the different hydrolysis limits of known α-amylases (HOPKINS, 1946; ADAMS, 1953; WHELAN, 1964) suggests that the amylolytic enzymes of starch activated sludge and of the cell-free ultrasonicate of this sludge under the described conditions include not only α-amylases, but in addition debranching enzymes and α-glucosidases. The presence of glucoamylases either contributing to the activity of these enzymes or even replacing them, is improbable in view of the fact that glucoamylases have not been demonstrated convincingly in bacteria. Many fungi are known to produce glucoamylase, generally in addition to α-amylase. β-Amylases and amylases with action patterns intermediate between those of α- and β-amylases were assumed not to be present in activated sludge.

The high degree of hydrolysis found justifies the expression of the SA of activated sludges in glucose equivalents, although it is usual to express this activity in maltose equivalents. The decrease of total carbohydrates and reducing compounds in the assay mixture with starch sludge (Fig. 3.1) was particularly clear after prolonged incubation; it was due to the pronounced development of yeasts.

The observation that the SA of cell-free ultrasonicate during the first 20 h of incubation decreased more readily than the SA of starch sludge, may be explained by the greater instability of dextrinizing enzymes in ultrasonicate than in sludge (Fig. 5.10).

3.2.3. *Ratios of dextrinogenic to saccharogenic activities*

Starch hydrolysis was followed with time using starch activated sludge, cell-free ultrasonicate and Fungamyl 1600 as the enzyme systems (Fig. 3.2).

Neglecting some rate changes in the very beginning of the starch hydrolysis, it is evident that the production of reducing compounds by Fungamyl 1600 was proportional to time only until 8 % of the glucosidic linkages of the starch had been hydrolysed ($\overline{\mathrm{DP}} = 12.5$). At this degree of hydrolysis the achromic point of the iodine stain (i.e. the final disappearance of iodine colouring as nearly as it can be judged) had not yet been reached. This decrease of the rate of hydrolysis cannot be attributed to instability as the enzyme had maintained 100 % activity even after 100 hours of incubation.

In contrast, the production of reducing sugars by amylolytic enzymes of starch sludge and cell-free ultrasonicate was proportional to time roughly until about 50 % hydrolysis (approximately corresponding with the achromic point) and strictly until about 33 % hydrolysis (Figs. 3.1 and 3.2). The rate of decrease of the iodine-stainable material was constant in all cases until about 55 % of the initial standard amount was left. In most of the experiments with amylolytic

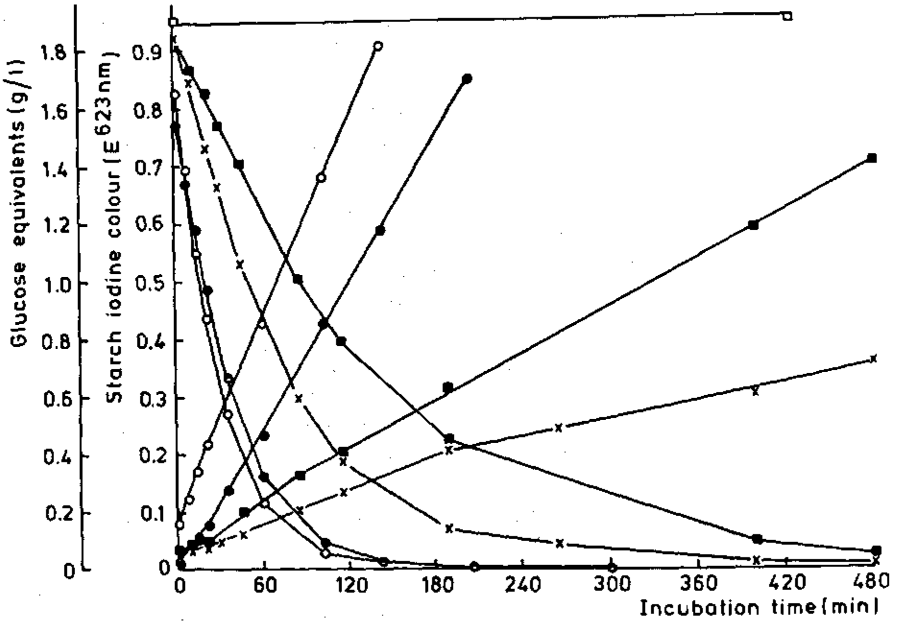

FIG. 3.2. Starch degradation by starch activated sludge (SL = 0.3), cell-free ultrasonicate, and Fungamyl 1600. Curves with negative slope show the decline of the extinction (at 623 nm) of iodine-stainable material (DA), curves with positive slope show the increase of reducing sugars (SA). The horizontal line (□) gives the E^{623nm} of a standard incubation mixture without enzyme sample after iodine reaction. For experimental conditions of sludge (●) and ultrasonicate (○): see Fig. 3.1. Standard conditions were used for Fungamyl (x) and ultrasonicate (■). ●, Starch activated sludge (after 393 days of loading), incubated at the original sludge concentration; ○, cell-free ultrasonicate (sonication time 10 min) of this sludge; incubated at original concentration; x, Fungamyl 1600 (1 mg/l); ■, cell-free ultrasonicate (sonication time 5 min) of starch activated sludge (after 388 days of loading); incubated at half of the original concentration.

enzymes of activated sludge no prolonged constant rate of production of reducing sugars was found. Deviation from strict proportionality was found after e.g. 10–20 % hydrolysis. Instability of the amylolytic enzymes, especially in the ultrasonicate and in the presence of e.g. 1–2 % of toluene, was probably the most important cause of this deviation (Fig. 5.10).

DA/SA ratios were calculated from the linear parts of the starch degradation and sugar formation curves. The DA and SA curves were linear for relatively short and prolonged periods, respectively, as shown in Fig. 3.2. The ratios are based on the expressions of the DA and SA activities (2.6.8): g starch equivalents. $l^{-1}.h^{-1}$/g glucose. $l^{-1}.h^{-1}$. Table 3.1 shows that the DA/SA ratio of the amylolytic enzyme system of starch sludge was about 10, independent of the loading of the sludge. The same ratio was found with ultrasonicate and with activated sludge from the Zeist sewage treatment plant. These values are lower than the ratio of the α-amylase of *Asp. oryzae* (Fungamyl), but higher than that of glucoamylase. SCHELLART et al. (1976) reported that the DA/SA ratio of the

TABLE 3.1. DA/SA ratios and hydrolysis percentages at the achromic points (a.p.) of the starch-iodine reaction of various amylolytic systems. In parentheses: extinctions, corrected for that of the reagent blank, at 623 nm at the approximately achromic points. Conditions during incubation: N^{0}'s 1, 2 and 5: standard; 4 and 6: with 2 and 0.5 % toluene, respectively; 3: see Fig. 3.1.

N^{0}	Source	Loading with starch (days)	Sludge loading (SL) or sampling date	Amylolytic system	DA/SA	Hydrolysis % at a.p.
1.				Fungamyl 1600 (1 mg/l)	24.6	11.7 (0.007)
						17.4 (0.004)
2.	Lab. sludge	388	0.3	cell-free ultrasonicate	9.6	⩾32.4 (0.010)
3.	Lab. sludge	393	0.3	whole sludge	9.1	32.8 (0.004)
						43.5 (0.002)
				cell-free ultrasonicate	9.4	43.8 (0.004)
						54.6 (0.002)
4.	Lab. sludge	243	2.4	whole sludge	11.4	29.0 (0.002)
				cell-free ultrasonicate	9.1	
5.	Zeist sludge		(20/11/'75)	whole sludge	11.2	
				cell-free ultrasonicate	13.6	
6.	Zeist sludge		(15/12/'76)	whole sludge	13.1	
				whole ultrasonicate	13.5	

culture filtrate of starch-grown *Trichoderma viride* was 10.7 times as low as that of the same Fungamyl preparation used in the present study. This mould degrades starch mainly by extracellular glucoamylase. α-Glucosidases, if present, are not expected to influence the ratio found with activated sludge, since they probably cannot hydrolyse polysaccharides at a significant rate (Enzyme Nomenclature, 1972). That the SA, with respect to starch, of starch sludge or ultrasonicate did not depend on the activity of α-glucosidases (at least during the initial stage of this reaction), was demonstrated by testing a cell-free ultrasonicate (sonication during 5 min) of maltose sludge which had a low DA. Only a very low DA was shown to be present in the ultrasonicate and the SA with starch as the substrate was negligible.

The DA/SA ratios of 5 pure crystalline α-amylases were shown by BERNFELD (1951) to be equal. ADAMS (1953) reported considerable differences between DA/SA ratios of 4 α-amylases, but these preparations had not been purified. KUNG et al. (1953) reported large differences between the curves relating iodine

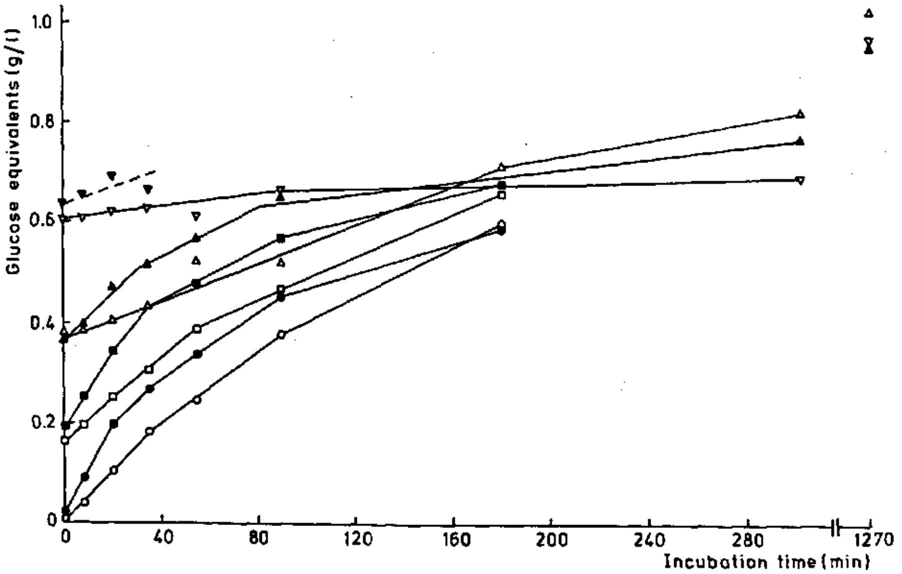

FIG. 3.3. Degradation of starch, a mixture of dextrins (DE-20), maltotriose and maltose (biochemical grade) by starch activated sludge and the whole ultrasonicate of the sludge. The activated sludge had been loaded (SL = 0.3) with starch for 237 days and was incubated at half of the original concentration. Ultrasonic treatment was performed during 20 min. The (standard) incubation mixtures (2.6.8) were supplied with toluene (0.5% v/v), chloroform (0.5% v/v), nystatin (50 mg per litre) and ethanol (1% v/v); substrate concentrations were 1.5 g/l instead of 5 g/l. The initial starch solution (after boiling for 5 min) contained 0.6% reducing sugars (related to total carbohydrate concentration); DE-20 was found to contain 9.2 instead of 20% reducing sugars. The total carbohydrate concentrations of about 1.5 g glucose equivalents per litre remained constant in all incubation mixtures for at least 5 hours. ○, □, △, ▽, Reducing sugars produced by sludge from starch, DE-20, maltotriose and maltose, respectively; ●, ■, ▲, ▼, reducing sugars produced by whole ultrasonicate, prepared from this starch sludge, from starch, DE-20, maltotriose and maltose, respectively.

stain to reducing power in the relatively early stage of α-amylolysis of amylose by different α-amylase preparations. Like ADAMS, they found recrystallized, maltase-free taka-amylase (from *Asp. oryzae*) and maltase-free, crystalline *B. subtilis* α-amylase to have the highest DA/SA ratios. Recrystallized, maltase-free hog pancreatic α-amylase had the lowest DA/SA ratio, viz. about 3 times lower than the highest ratio.

The discrepancy between the DA/SA ratio of the α-amylase Fungamyl 1600 and that of the sludge amylolytic enzymes may be also explained by the action of debranching enzymes in sludge. These enzymes cause a marked increase in the iodine-staining potency of amylopectin (GUNJA et al., 1961; ZEVENHUIZEN, 1966), which is the main component of starch. This effect of debranching enzymes may partly compensate for the decolourizing action of α-amylases on starch. However, this explanation is not plausible as amylose is responsible for the greater part (about 70%) of the absorbance at 623 nm (2.6.7).

Another difference between the amylolytic enzymes of activated sludge and Fungamyl 1600 is the higher percentage of hydrolysis of the glucosidic linkages of starch with sludge enzymes, as was shown in Table 3.1 for the achromic point. However, according to HOPKINS (1946) and KUNG et al. (1953) this percentage varies also among the α-amylases.

Comparing the results obtained in the present study with those of the literature survey (BANKS and GREENWOOD, 1975), it is concluded that the difference between the DA/SA ratio of starch sludge and that of α-amylase (Fungamyl 1600) as to the degradation of starch probably is mainly due to the different action pattern of α-amylases of sludge compared to that of *Asp. oryzae*. The predominance of β-amylase and glucoamylase in starch sludge is thought to be improbable in view of the much lower DA/SA ratios found with these enzymes (BERNFELD, 1951; SCHELLART et al., 1976). Summarizing it is stated that the amylases of activated sludge mainly, if not completely, consist of α-amylases and debranching enzymes.

3.2.4. *Substrate specificity of the saccharifying enzymes*

To investigate if and to what extent α-glucosidases are responsible for the degradation of oligosaccharides by the amylolytic enzyme system of activated sludge, an experiment was carried out concerning the substrate specificity of these enzymes. For that purpose the saccharifying activities of sludge and ultrasonicate were estimated using carbohydrates with decreasing degree of polymerization viz. starch, dextrins, maltotriose and maltose. The results obtained (Fig. 3.3) for the following reasons indicate that α-glucosidases play no important role in the reactions shown.

1) The initial saccharifying rates declined with decreasing degree of polymerization of the substrate. If the rate of saccharification of starch by e.g. starch sludge is 100 (arbitrary units), the activities with DE-20, maltotriose and maltose as the substrates would have amounted to about 89, 37 and 12, respectively. Such a decrease of saccharifying activity would not have been expected if high activities of α-glucosidases had been present. The higher SA on maltotriose

compared to that on maltose, resulting in crossing of the SA curves (Fig. 3.3), is attributed to the action of amylases and not to that of α-glucosidases. The latter enzymes degrade maltotriose more slowly than maltose (TH. M. VAN BELLEGEM, personal communication, 1977) and degrade starch insignificantly or not at all (Enzyme Nomenclature, 1972). In the case of ultrasonicate the decrease of SA with decreasing DP is somewhat less pronounced, suggesting the occurrence of a slight α-glucosidase activity. The equal increase of SA and DA, with respect to starch, upon ultrasonic treatment (Fig. 3.3, Table 3.1), was not due to the release of α-glucosidase (3.2.3) but was probably the result of the release of polysaccharide-hydrolysing enzymes from the periplasmic space (Chapter 7).

2) The decrease of reaction rate during prolonged starch hydrolysis is probably the result of the lower activity of the starch-degrading enzymes on oligomer dextrins and/or enzyme inactivation. Inactivation of dextrinizing enzymes proceeds considerably faster in ultrasonicate than in whole sludge (see also Fig. 5.10). Although the initial SA's on starch and DE-20 in ultrasonicate are about 75% higher than those in whole sludge, the reaction rates after about one h are about equal (Fig. 3.3). This suggests lability of starch-degrading enzymes in ultrasonicate which becomes still more apparent during prolonged incubation. The fact that the maltotriose and DE-20-hydrolysing enzymes show the same pattern of inactivation as the starch-hydrolysing enzymes suggests that with the three substrates the same polysaccharide-degrading enzymes are involved. From these results it is concluded that the saccharifying activity of α-glucosidases on low-molecular oligosaccharides is small (or nil) compared with the saccharifying activity which the amylases have on starch and dextrins. However, it cannot be completely excluded that intracellular α-glucosidases are inactivated during ultrasonic treatment of sludge.

The data of 3.2.2, 3.2.3 and 3.2.4 suggest that amylolysis by sludge is achieved primarily by the concerted action of α-amylases and debranching enzymes. This concerns not only the initial phase of polymer degradation but also for an important part the degradation of maltotriose and possibly that of maltose. Degradation of maltose by α-amylases would be in contrast with data of the literature.

3.2.5. *Routine determinations of amylolytic activity*

For routine determinations of amylolytic activity of whole sludge or of sludge ultrasonicate and of pure cultures of bacteria, the determination of DA was preferred to that of SA or to both DA and SA because of the following reasons.

a) The standard DA method is rapid, simple, and based on linear parts of curves.

b) Especially at very low initial starch concentrations the DA method is very sensitive and precise.

c) The ratio of DA to SA appears to be fairly constant as determined in different activated sludges and their ultrasonicates (Table 3.1).

d) Starch and its iodine-stainable degradation products cannot be taken up by bacterial cells in contrast with lower oligosaccharides.

e) Cell-free sludge liquids were shown to intervene with the Somogyi-Nelson assay used in the SA determination.

3.3. DETERMINATION OF DEXTRINIZING ACTIVITY OF SLUDGE

3.3.1. *Effect of sludge and of culture media on the starch iodine reaction*

In spite of the use of a high concentration of iodine reagent (2.6.7), some interference of the samples to be tested with the starch iodine reaction was occasionally observed. The decreased starch iodine colour (compared with that of the starch standard), sometimes observed at the beginning of the amylolytic reaction, had very likely to be attributed to (instantaneous) adsorption of starch to sludge (Fig. 3.4A and section 6.2). Sometimes other factors were responsible (e.g. Fig. 3.2: immediate loss of colour in cell-free ultrasonicate). The decrease of the extinction due to interference with whole sludge or its ultrasonicate was relatively small and is not likely to have affected the calculation of the reaction rates.

No effect of yeast extract and tryptone, in concentrations up to at least 5 g/l, on the iodine starch colour (4 g starch/l) was found. The extinctions were measured within a few minutes after mixing, as usual.

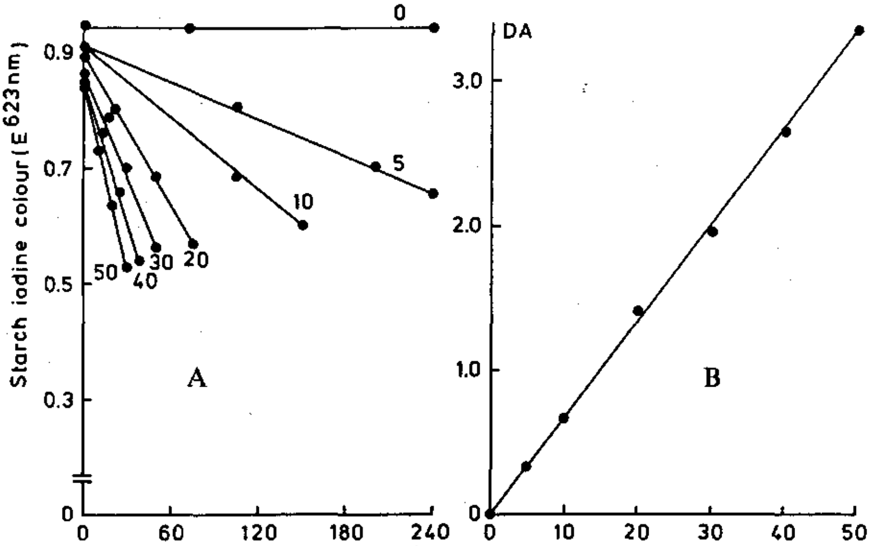

FIG. 3.4. Degradation of starch under standard conditions (2.6.8) by 2–20 times diluted very slimy starch activated sludge (after 384 days of loading; SL = 0.3; dry weight of biomass: 6.46 g/l). A. The numbers in the graphs indicate the percentages of the final (reaction mixture) volume occupied by the original sludge volume. B. Relation between DA and quantity of sludge sample. The data plotted are those of Fig. 3.4A, calculated as dextrinizing activities.

3.3.2. *Proportionality of the dextrinizing reaction with time and sludge concentration, and precision of the enzyme assay method*

The decrease of the iodine-stainable material was found to be constant until about 55% of the initial standard amount was left (Fig. 3.2). During prolonged incubation the slope of the curve decreases continuously. The linearity of the reaction for several hours (Fig. 3.4A) indicates that the amylolytic enzymes are not inactivated, at least during the first stage of the reaction.

Highly active sludges were diluted so that the iodine-stainable material did not decrease with more than about 45% in 7 minutes. The initial decrease of iodine-stainable material was only seldom not completely constant (e.g. with sludge samples from the system without cell retention, II), but such a small divergence from linearity hardly influenced a routine estimate.

Fig. 3.4B shows that the amylolytic reaction rate, if calculated from linear parts, is exactly proportional to the volume of the sludge sample. This proves that a reliable determination of DA in activated sludge is possible, in spite of the inhomogeneity of the system.

The precision of the enzyme assay method was satisfactory (repeatability within 10–15%) when the extinction value at the end of the enzyme incubation was less than 90% but more than about 60% of the initial value. When DA values were small (ΔE less than 0.10) more samples were assayed.

3.3.3. *Effect of nature of buffer and of concentration of potassium phosphate on the dextrinizing activity*

Table 3.2 shows that different buffer substances did not affect DA of different

TABLE 3.2. Dextrinizing activities of starch sludges I (SL = 0.3), incubated in various buffers and at various potassium phosphate concentrations.

Number of days of starch loading	pH during loading	pH during dextrinizing reaction	Molarity of buffer	Buffer	DA
79	7.0	5.0	0.1	citric acid-sodium citrate	2.97
			0.1	sodium cacodylate-HCl	3.96
10	7.0	6.0	0.1	citric acid-sodium citrate	11.61
			0.1	potassium phosphate	14.64
16	7.0	7.2	0.05	sodium cacodylate-HCl	15.06
		(7.2)[1]	0.10	potassium phosphate	14.66
		(7.2)[1]	0.05	potassium phosphate	15.56
		(7.0)[1]	0.01	potassium phosphate	15.62
			0.05	tris-HCl	13.73
65	8.8	7.2	0.1	sodium cacodylate-HCl	20.17
			0.1	potassium phosphate	20.25
			0.1	tris-HCl	20.59
70	8.8	9.0	0.1	tris-HCl	17.07
			0.1	glycine-NaOH	16.78

[1] pH measured after reaction.

sludges at different pH values, except 0.1 M citrate buffer that appeared to be significantly (20–25%) inhibitory (cf. inhibition of serum, salivary and urinary amylases, STREET, 1960). Potassium phosphate buffer of pH 7.0–7.2 in a concentration range of 0.01–0.1 M did not affect DA; the reaction was initiated, according to the standard procedure, by the addition of the starch chloramphenicol solution (cf. 3.4.3). In the standard assay mixture 0.1 M potassium phosphate, pH 7.0, was chosen as the buffer. This buffer ensured a constant pH value without prior adjustment of the pH of the sludge samples also when the sludge was grown at a different pH value; normally the sludge was grown at pH 7.0.

3.3.4. *Effect of chloramphenicol and nitrogen deficiency on growth and on production of amylases in batch cultures of activated sludge*

For the determination of dextrinizing enzyme activities of (sludge) samples with a low DA, incubation of such samples with a starch solution for periods up to 7 h (exceptionally 1–2 days) was necessary. Realizing that sludge samples generally contain considerable amounts of assimilable nitrogen, the question arose whether synthesis of amylolytic enzymes and assimilation of low-molecular starch degradation products by the sludge organisms would occur

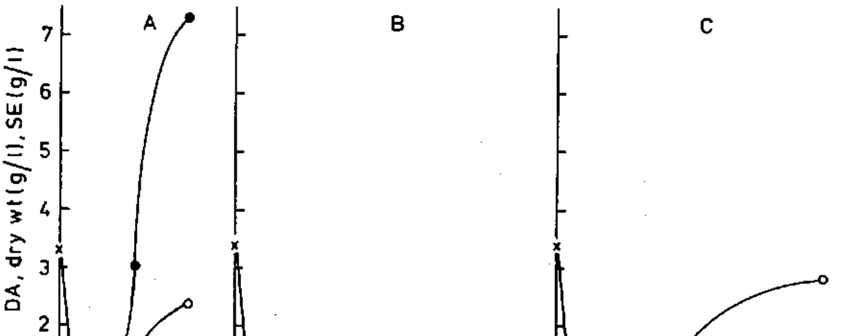

FIG. 3.5. Effect of nitrogen deficiency and added chloramphenicol on the production of biomass and amylases by starch activated sludge. The sludge had been loaded during 141 days with SL = 0.3, contained 2.45 g dry wt/l and $\mathrm{DA}_{\mathrm{spec}}$ was 2.25. The sludge was centrifuged at 2500 × g, washed once with potassium phosphate buffer (0.02 M; pH 7.0) and resuspended in such a buffer. It was inoculated at a final concentration of 0.5 g/l in batch cultures (25°C; 200 rev./min). Iodine-stainable compounds were determined in membrane filtrates of the cultures without adding $HClO_4$ (2.6.7). A, Complete starch medium B_1; B, starch medium without $(NH_4)_2SO_4$; C, complete starch medium B_1 with chloramphenicol (500 mg/l); ●, DA; ○, biomass (g dry wt/l); x, starch-extinction equivalents (g/l).

during such periods of prolonged incubation. For this reason an experiment was carried out in which the response of sludge I to added starch was investigated under various conditions (Fig. 3.5). This and other experiments showed that batch cultures of washed starch activated sludge (SL = 0.3) did not increase substantially in biomass and in DA in periods up to 10 or sometimes 18 h of incubation in complete starch ammonium medium. In this connection it is remarked that the initial amylolytic activity of the starch sludge was so high that the starch had been degraded to non-iodine-stainable compounds (i.e. cell-available compounds; see Chapter 6 or Fig. 4.3E) before DA increased substantially.

Sludge freed from nitrogen by washing and incubated without added $(NH_4)_2SO_4$ remained rather constant as to biomass and DA for at least 50 h (Fig. 3.5B). This suggests that in this starch sludge amylases are not synthesized without growth.

Addition of 500 mg chloramphenicol/l to the complete starch ammonium medium (Fig. 3.5C) prevented the increase of DA for at least 2 days. However, within this period an increase of white-coloured biomass likely due to growth of yeasts and/or fungi was observed.

From the results of this experiment it can be concluded that estimation of sludge amylolytic enzyme activity under standard assay conditions, i.e. with unwashed (nitrogen-containing) sludge, 500 mg chloramphenicol/l added, at 0.1 M buffer and 30°C, synthesis of amylolytic enzymes is excluded during incubation periods up to 2 days.

3.3.5. *Effect of the conditions of storage of activated sludge on the dextrinizing activity*

Freezing and thawing of various activated-sludge samples from outdoor plants resulted in an increase of DA of 40 to about 70% (Table 3.3). Continued storage of the samples in the frozen condition during periods up to at least 20

TABLE 3.3. Dextrinizing activities of different types of activated sludge before and after freezing of separate samples.

Activated sludge	Treatment	DA
Bennekom (20/11/'75)	control	2.40
	7 days at −10°C	3.53
Bennekom (11/12/'75)	control	2.96
	4 days at −23°C	⩾4.38
	20 days at −23°C	⩾4.64
Zeist (11/12/'75)	control	3.86
	4 days at −23°C	⩾5.42
	20 days at −23°C	⩾5.58
Starch sludge (SL = 0.075)	control	0.51
	2 hours at −10°C	0.72
	4 days at −10°C	0.70

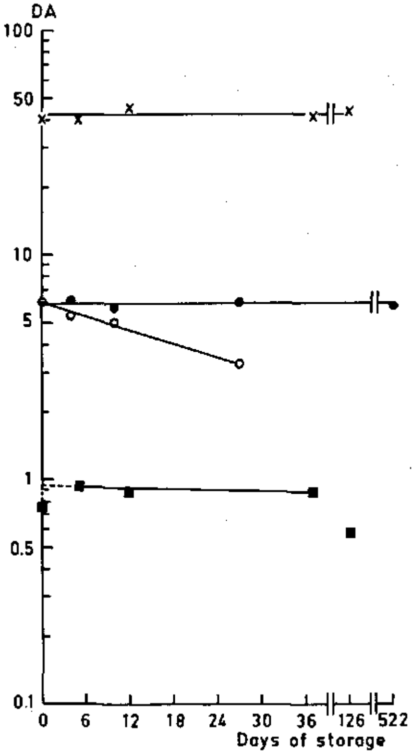

FIG. 3.6. Stability of amylases of starch activated sludges during storage. x, Starch sludge (SL = 2.4) samples stored separately for different periods at −10 °C; •, starch sludge (SL = 0.3), stored at −10 °C, and periodically thawed, sampled and refrozen; O, same starch sludge (SL = 0.3), stored at 3½ °C; ■, starch sludge (SL = 0.075) samples stored separately for different periods at −10 °C.

days did not bring about any further change of DA. Storage at −10 °C of starch activated sludge grown in the laboratory at SL = 0.3–2.4 was not accompanied with any significant change in DA up to at least 1½ year. Even when the same sludge sample (SL = 0.3) was frozen and thawed several times, no change was observed (Fig. 3.6). This was in contrast with very low-loaded starch sludge (SL = 0.075) and with activated sludge from outdoor plants (Table 3.3 and Fig. 3.6).

Storage of starch activated sludge (SL = 0.3) at 3½ °C resulted in a slow decay of DA. As a consequence of these results, sludge samples were allowed to stand at 3½ °C for at most a few days, if necessary for routine determinations. For more precise determinations of DA, samples were assayed immediately after withdrawal. Freezing of samples prior to DA determinations was allowed only for laboratory starch sludge loaded with SL = 0.3 or more.

3.3.6. *Effect of some other conditions on the dextrinizing activity*

Limited starch retrogradation was shown to have no significant effect on DA of starch activated sludge. The same value was observed no matter whether a freshly prepared starch solution was used as the substrate or a slightly turbid solution kept for one day at room temperature. Nevertheless starch solutions were always prepared freshly (2.6.8).

For the determination of certain amylolytic activities, toluene was added to incubation mixtures in order to prevent uptake of oligosaccharides by bacteria and moreover to prevent production of amylases. However, toluene may affect the DA of activated sludge in at least two ways. a) It can increase the DA of activated sludge with about 25–30 %. Between 1 and 10 % toluene the influence of the concentration did not change clearly. The increase might be explained by the contact between substrate molecules and periplasmic amylolytic enzymes (7.2). Starch activated sludge (SL = 1.2) and sludge from the plant at Bennekom were used to test the toluene effect on DA. These sludges were pre-incubated for about 10 min in the presence of toluene. b) Toluene may accelerate the inactivation of dextrinizing enzymes during long periods of incubation, at least in the absence of starch (Fig. 5.10A).

The effects of pH, temperature and starch concentration on the amylolytic activity of activated sludge are dealt with elsewhere (3.5.3, 3.5.2 and 6.3, respectively). A temperature of 30°C instead of 20°C was chosen for routine purposes to accelerate the reaction. At a starch concentration of 5 g/l the maximum amylolytic activity was measured under the conditions mentioned (6.3).

3.4. DETERMINATION OF PROTEOLYTIC ACTIVITY OF SLUDGE

3.4.1. *Unit of proteolytic activity*

Proteolytic enzyme activity (PA) is expressed as mg casein hydrolysed per ml per h under standard conditions (2.6.12). The assay method is based on the determination of perchloric acid-soluble products of casein hydrolysis with Folin-Ciocalteu reagent (2.6.11). Undigested casein is used as the standard. Expression of the data in the units mentioned above is valid only if the extinction coefficient of enzymically hydrolysed casein is the same as that of whole casein. The experiment of Fig. 3.7 was carried out to check this. Casein was hydrolysed by Pronase E, a mixture of several proteolytic enzymes, including endo- and

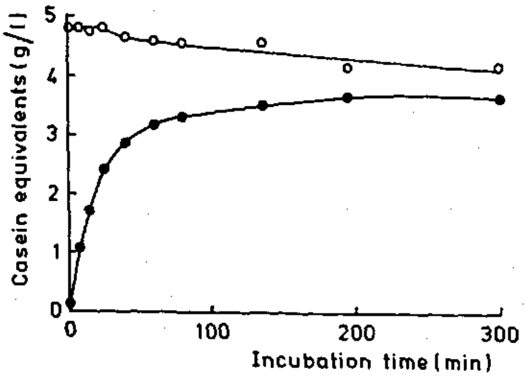

FIG. 3.7. The effect of (exhaustive) casein degradation by Pronase E on the content of Lowry-positive material. The final concentration of Pronase E in the standard incubation mixture was 20 mg/l. Samples of 2.0 and 0.1 ml, withdrawn from the incubation mixture, were added to 0.1 ml 70 % v/v $HClO_4$ and to 2.5 ml Na_2CO_3 solution (part of the Lowry reagent), respectively. ●, g $HClO_4$-soluble Lowry-positive material per litre; ○, total amount of Lowry-positive material (g/l).

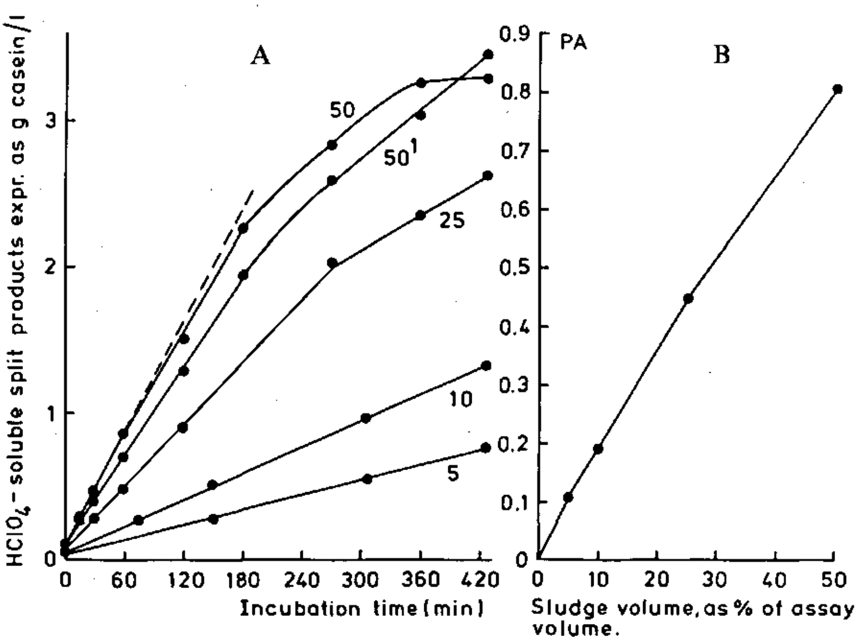

FIG. 3.8. Degradation of casein under standard conditions (2.6.12) by 2–20 times diluted Zeist sludge (July 1975). A. The numbers in the graphs indicate the percentages of the final assay volume occupied by the original sludge volume. 50^1 : as 50, but with 1 % toluene in the reaction mixture. B. Relation between PA and volume of sludge sample. The proteolytic activities were calculated from the linear parts of the curves of Fig. 3.8A.

exopeptidases, obtained from *Streptomyces griseus* K-1. It hydrolyses 70–90 % of the peptide linkages, compared to complete hydrolysis with acid. Fig. 3.7 shows that the total amount of casein equivalents during proteolysis remained constant, except for a slight decrease observed after approximately 50 % digestion of casein. The experiment clearly shows that the primary degradation products possessed the same extinction coefficient as whole casein. Hence, expression of proteolytic enzyme activity as mg casein hydrolysed per ml per h is justified.

3.4.2. *Proportionality of the proteinase reaction with respect to time and sludge concentration, and precision of the enzyme assay method*

Casein was digested by Zeist activated sludge under standard conditions (2.6.12). Perchloric acid-soluble degradation products increased proportionally to time until about 2 of the initially present 5 g casein per litre had been hydrolysed (Fig. 3.8A). The linearity of the reaction during many hours indicates that the proteolytic enzymes are stable, at least during the first stage of the reaction. Addition of toluene as a growth inhibitor may slightly decrease the proteolytic activity (see also 3.4.5), but it apparently prevents uptake of casein degradation products by micro-organisms of the sludge at a rather advanced stage of the reaction.

Plotting the initial reaction rates calculated from Fig. 3.8A versus the sludge concentration (Fig. 3.8B) shows a relationship slightly deviating from proportionality. Deviations have repeatedly been shown to occur with proteinases acting on proteins (DIXON and WEBB, 1964; Methods in Enzymology Vol. II and XIX, Eds. COLOWICK, S. P. and KAPLAN, N. O.).

The results shown in Fig. 3.8A and B show that a reliable determination of proteolytic activity in activated sludge is possible, in spite of the inhomogeneity of the system, the many different peptide bonds in casein and the mixture of enzymes present. The linear relationship shown in Fig. 3.8A suggests a high repeatability of the assay method (far within 10%).

If not otherwise stated, proteolytic activities were estimated in twice diluted sludges (50% sludge volume); the routine reaction period was 30 min or longer, if necessary, but 15 min when the PA was exceptionally high and decreased slightly even before 30 min.

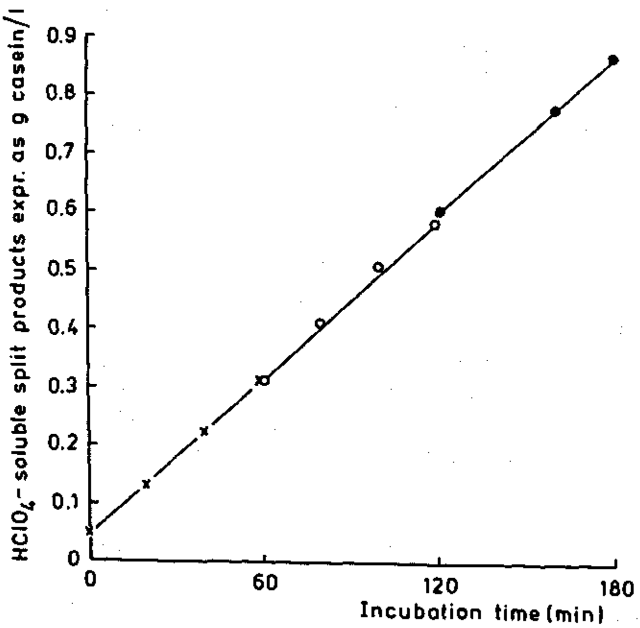

FIG. 3.9. Effect of added potassium phosphate on the reaction between casein and proteolytic enzymes of starch activated sludge (SL = 0.3). The sludge, at 25% of its original concentration, was incubated at pH 7.5, with 5 g casein and 500 mg chloramphenicol per litre. NaOH (0.1 N) was added occasionally to readjust the pH to 7.5. Potassium phosphate was added to final concentrations of 5 and 100 mM after 60 and 120 min, respectively. The data of the third hour were corrected for the dilution of the incubated components. x, PA in the absence of potassium phosphate; O, PA at 5 mM of potassium phosphate; ●, PA at 100 mM of potassium phosphate.

TABLE 3.4. Proteolytic activities of starch activated sludge (SL = 0.3) at various potassium phosphate concentrations. The sludge, at 30% of its original concentration, was incubated for 80 min in beakers with 5 g casein and 500 mg chloramphenicol per litre and with different amounts of potassium phosphate. The reaction was initiated, according to the standard procedure, by the addition of the casein chloramphenicol solution. The pH of the incubation mixture was maintained at 7.5 by periodically adding NaOH solution.

Potassium phosphate (mM)	Relative PA[1]
0	110
5	127 (117)[2]
20	114
100	100
200	80

[1] As % of PA at 100 mM potassium phosphate; [2] PA without pH correction. The pH dropped to 7.3 in 80 min and to 7.0 in 2–3 h.

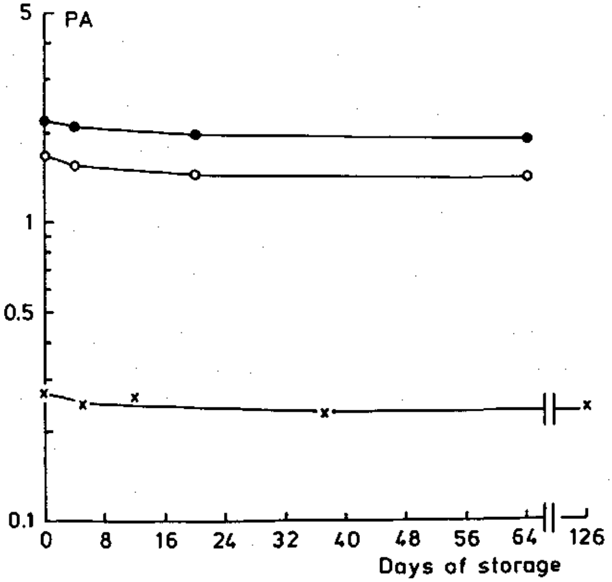

FIG. 3.10. Stability of proteinases of activated-sludge samples stored separately for different periods in deep-freeze. ●, Bennekom sludge (11/12/'75; PA_{spec} = 0.66) stored at −23 °C; ○, Zeist sludge (11/12/'75; PA_{spec} = 0.61) stored at −23 °C; x, starch sludge (SL = 0.075; PA_{spec} = 0.32) stored at −10 °C.

TABLE 3.5. Proteolytic activities of activated sludges before and after freezing of separate samples.

Activated sludge	Treatment	PA
Bennekom (20/11/'75)	control	2.15
	7 days at −10 °C	2.16
Zeist (20/11/'75)	control	1.60
	7 days at −10 °C	1.55
Starch sludge (SL = 0.075)	control	0.305
	2 hours at −10 °C	0.295
	4 days at −10 °C	0.265

3.4.3. *Effect of potassium phosphate concentration and of the assay procedure on the proteolytic activity*

Adjustment of the pH of sludge samples prior to assaying PA, as well as the decrease of the pH during enzymic casein digestion, can be avoided by using a concentrated buffer. Therefore 0.1 M potassium phosphate was included in the standard incubation mixture for estimating PA of activated sludge.

The effect of the potassium phosphate concentration on PA of activated sludge was investigated in some detail because in preliminary experiments, in which the pH was not kept constant, inhibition by this buffer had been observed. According to REJ and RICHARDS (1974) a sodium phosphate buffer does not interfere with the estimation of protein by the Lowry procedure. In the experiment recorded in Table 3.4 the pH was maintained at 7.5 by titration with NaOH solution. A significant inhibition by increased potassium phosphate concentration was observed, but at low concentrations (about 5 mM) the reaction was slightly stimulated. The nature of the action of potassium phosphate is obscure; the stimulation by 5 mM and the inhibition by 100 mM were hardly observed when phosphate was added after starting the proteolytic reaction (Fig. 3.9). This might mean that proteolytic enzymes bound to the substrate are insensitive to potassium phosphate.

In the standard assay procedure (0.1 M phosphate buffer) the PA is inhibited only to a small extent compared to that in the absence of buffer. The latter situation resembles that in the sludge reactor.

3.4.4. *Effect of the conditions of storage of activated sludge on the proteolytic activity*

The effect of the conditions of storage of activated sludge on PA is different from that on DA. Freezing and thawing of the activated sludges investigated, including also those from Bennekom and Zeist and the very low-loaded

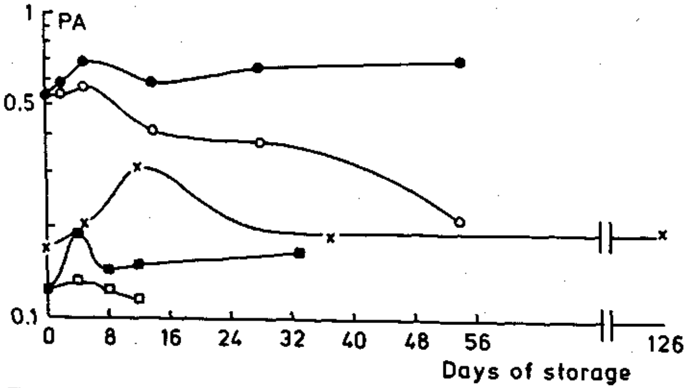

FIG. 3.11. Stability of proteinases of samples of starch activated sludge (SL = 2.4) stored separately for different periods at different temperatures. x, Sludge with $PA_{spec} = 0.056$; storage temperature: -10°C; ●, $PA_{spec} = 0.20$; -10°C; ○, same sludge, $PA_{spec} = 0.20$; 3½°C; ■ $PA_{spec} = 0.050$; -23°C; □, same sludge, $PA_{spec} = 0.050$; 3½°C.

laboratory units, did not result in an immediate increase of PA as it was found with DA (Tables 3.5 and 3.3). Storage, however, resulted in a slow decrease of PA of activated-sludge samples of outdoor plants and of very low-loaded starch sludge (Fig. 3.10). However, very high-loaded starch sludge showed a clear increase of PA during storage in frozen condition. Prolonged storage of such sludge in deep freeze resulted in a decrease of proteolytic activity (Fig. 3.11). Samples of this sludge kept at $3\frac{1}{2}$°C showed a slight rise, followed by a pronounced decrease of PA.

If necessary for routine determinations, sludge samples were allowed to stand for at most a few days at $3\frac{1}{2}$°C (or in deep freeze). However, samples of higher-loaded starch sludges were assayed immediately after withdrawal.

3.4.5. *Effect of some other conditions on the proteolytic activity*

No clear effect of the casein preparation on the proteolytic activity of starch activated sludge (SL = 2.4) was observed. About the same activities were found with vitamin-free casein (NBC; used under standard conditions) and vitamin-free Hammersten casein (Merck). Neither did chloramphenicol affect the proteolytic activity of moderate-loaded starch sludge during short periods of incubation. Chloramphenicol was always added as a growth inhibitor under standard conditions. Storage of the casein chloramphenicol stock solution (12.5 and 1.25 g/l, respectively) at 3.5°C for one month did not affect the measured proteolytic activity of different activated sludges.

Addition of toluene to the reaction mixtures (1 to 10% of the volume) as a growth inhibitor generally slightly decreased the proteolytic activity of activated sludge, at most with about 20% (see also Fig. 3.8A). Therefore this compound was not added under standard conditions. Starch activated sludge (SL = 1.2 and 2.4) and activated sludges from Bennekom and Zeist were used to test the toluene effect on PA. The sludges were pre-incubated in the presence of toluene for about 10 min before the addition of casein.

$HClO_4$-treated samples from a PA assay with sludge could be stored at room temperature for at least 20 h without affecting the Lowry absorbance. The same was true of stored supernatants of the samples.

The effects of pH, temperature and casein concentration on the proteolytic activity of activated sludge will be dealt with in 3.6.3, 3.6.2 and 6.5, respectively.

3.5. PROPERTIES OF ACTIVATED SLUDGE-BOUND AMYLOLYTIC ENZYMES

3.5.1. *Location of the amylolytic enzyme system of sludge*

Macromolecular substrates like starch are utilized by bacteria only after primary attack by extracellular enzymes. Such enzymes are defined in the present paper as being located outside the semi-permeable membrane (GLENN, 1976). They may be bound to the cell wall or may be excreted into the culture fluid (Chapter 1, Introduction). Centrifugation is probably the least destructive technique for separating cells from the surrounding medium. Activated sludges from the plants in Zeist and Bennekom and from the laboratory units were centrifuged for 30 min at 4°C and at 38,000 × g so that no cells were left in the supernatant. No dextrinizing activity was detected in the supernatant; the entire activity was recovered in the precipitated sludge after the sludge was resuspended in demineralized water (7.2).

Bacteria isolated from starch activated sludge (SL = 0.3) similarly excreted no or only small amounts of amylolytic enzymes into the medium when grown in batch culture (Chapter 8). We conclude therefore that the amylolytic enzymes in activated sludge, as determined under standard conditions, are bound to the surface of bacteria and are directly accessible to the substrate in the external cell environment. However, upon ultrasonication of the sludge, amylolytic enzymes were detected which in whole sludge were not directly accessible to starch (7.2). Since no essential difference was detected between the amylolytic action pattern and the final degree of hydrolysis of starch in whole starch sludge and in its ultrasonicate (Figs. 3.1, 3.2 and 3.3), it is suggested that the accessible amylolytic enzymes have the same abilities as the non-accessible enzymes. It is tempting to conclude that the bulk of amylolytic enzymes active in ultrasonicate is located outside the semi-permeable membrane of the bacteria. This is at least true of the enzymes which are responsible for a substantial degree of hydrolysis of starch in starch sludge as well as in its ultrasonicate (i.e. the amylases).

Rather low percentages of the dextrinizing activities of whole sludge were extracted by magnetic stirring in solutions of potassium phosphate, NaCl, $MgSO_4$ or pentasodium tripolyphosphate ($Na_5P_3O_{10}$). However, rather high percentages of amylolytic activity could be set free by ultrasonication during short periods (7.2).

The properties of the amylolytic enzymes which will be dealt with in the following sections may be affected by the binding of the enzymes to the bacterial cell wall.

3.5.2. *Effect of temperature on the dextrinizing activity of activated sludge*

The DA of laboratory-grown and of Zeist activated sludges was determined under standard conditions at different temperatures. The accuracy of the

TABLE 3.6. Q_{10} values of dextrinizing activities of activated sludges under standard conditions.

Activated sludge	Number of days of starch loading	Q_{10} (20–30°C)	Q_{10} (1–30°C)
Zeist (7/8/'74)		2.00	2.26
Starch sludge, SL = 0.075	237	1.93	
Starch sludge, SL = 0.3	26	1.95	
Starch sludge, SL = 0.3	176	2.58	2.66
Starch sludge, SL = 2.4	142	1.67	
Starch sludge, SL = 2.4	143	2.15	

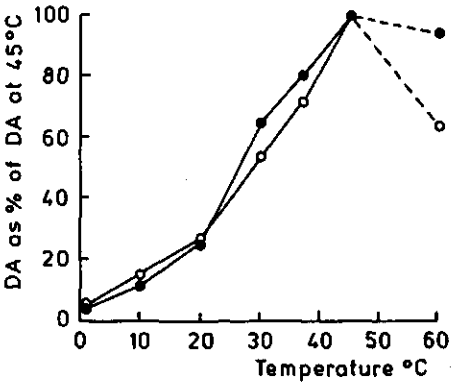

FIG. 3.12. Effect of temperature on DA of activated sludges under standard conditions. The values at 60°C represent approximate initial reaction rates; these rates decreased readily with time of incubation owing to heat instability of the enzymes. ●, Activated sludge after 176 days of starch loading (SL = 0.3); ○, activated sludge of Zeist (7/8/'74).

temperature was ± 0.2°C, except for 60°C where it was less.

The data shown in Fig. 3.12 were calculated from initial reaction rates. Both sludges behaved rather similarly at temperatures up to 45°C, showing a Q_{10} of somewhat more than 2 (Table 3.6). The enzymes of both sludges remained stable during incubation (20–42 min) at 37 and 45°C. However, at exposure to 60°C the Zeist sludge was less active than the starch-grown sludge. This was due to the greater thermolability of the former sludge; pre-incubation for 4 min and subsequent incubation in the presence of starch for 15 min at 60°C resulted in complete inactivation.

The Q_{10} of DA of other starch-grown activated sludges was also about 2 (Table 3.6). These values were calculated from experiments similar to that shown in Fig. 3.12.

3.5.3. *Effect of pH on DA of starch activated sludges grown at different pH values and effect of pH on the stability of amylases*

Activated sludge was loaded with starch (SL = 0.3) at controlled pH values. The DA of these sludges was measured as a function of pH (Fig. 3.13). When grown at pH 7.0 (Fig. 3.13B) DA showed a rather broad pH optimum which was not necessarily constant; it ranged e.g. from pH 6 to about 7.5 and from pH 5.0–5.5 to 7.5–8.0. The low activity at pH 4.0 is attributed to lability of the enzymes (Table 3.7).

The DA of starch sludge grown at a low pH (Fig. 3.13A) behaved differently. Especially after 11 days of growth at pH 5.0 (upper curve) a narrow pH optimum around pH 5.5 was observed. This was not due to fungal or yeast amylases, as these organisms were not detected microscopically even after two weeks at low pH. Obviously, loading of activated sludge with starch at pH 5 results in a bacterial population producing amylolytic enzymes with deviating pH optima.

The DA of starch sludge grown at a high pH (Fig. 3.13C) behaved hardly differently from the DA of sludge grown at pH 7.0. The optimum pH was 6–8, even after 10 weeks of cultivation. This suggests that bacteria producing

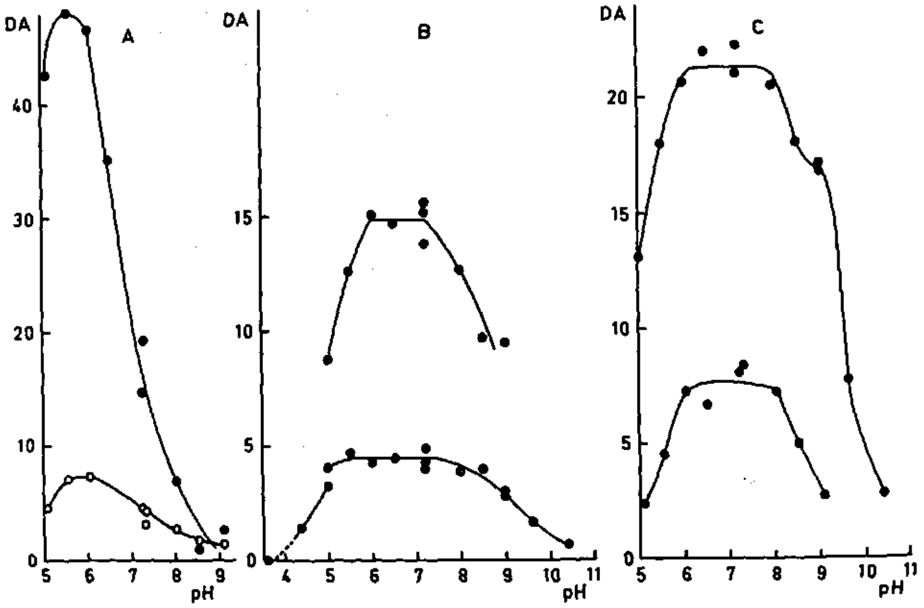

FIG. 3.13. Effect of pH on DA of starch activated sludges (SL = 0.3), grown at different pH values. The buffers used in the DA assay included: citric acid-sodium citrate (pH 3.6–5.0), sodium cacodylate-HCl (pH 5.0–7.2), potassium phosphate (pH 7.2), tris-HCl (pH 7.2–9.0) and glycine-NaOH (pH 9.0–11.4). The molarity of the buffers used was 0.1, except for one experiment (B, upper curve) where 0.05 M was used. The pH during the cultivation of the sludge was (A) 5.5–5.0: lower curve: 4 days pH 5.5 and subsequently 4 days pH 5.0; upper curve: 4 days pH 5.5 and subsequently 11 days pH 5.0; (B) 7.0: lower curve: 72 days pH 7.0; upper curve: 16 days pH 7.0; (C) 8.5–9.0: lower curve: 4 days pH 8.5 and subsequently 11 days pH 9.0; upper curve: 70 days pH 8.8.

amylases with a high optimum pH are not developing in activated sludge. Until recently all of the amylases studied had pH optima in the acid or neutral range (FENIKSOVA and ERMOSHINA, 1969; ADAMS, 1953; HOPKINS, 1946; BERNFELD,

TABLE 3.7. The residual dextrinizing and proteolytic activities (determined at 30°C) and endogenous respiration rates (determined at 20°C) after keeping activated sludge samples at room temperature at extreme pH values. The sludge had been loaded with starch (SL = 0.3) during 104 days (treatment at low pH values) or during 108 days (treatment at high pH values).

Treatment	Activity as % of that of untreated sludge samples		
	Endogenous respiration rate	DA	PA
5 min, pH 3.0	36	20	44
5 min, pH 3.5	54	19	59
5 min, pH 4.0	90	23	72
5 min, pH 11.0	87	81	87
5 min, pH 11.75	55	60	120
5 min, pH 12.5	51	5	129
5 min, pH 4.0 followed by 5 min, pH 11.0	72	15	71

1951). Recently amylases with high pH optima (and with unusual properties) were isolated from species of *Bacillus* and *Pseudomonas* (FOGARTY and KELLY, 1975, reported by FOGARTY and GRIFFIN, 1975; BOYER and INGLE, 1972; ROBYT and ACKERMAN, 1971). However, such species were not isolated from starch activated sludge, cultivated at pH 7.0 (Chapter 8).

The effect of extreme pH values on the stability of amylases and proteinases and on the respiration rate (as indication of viability) of activated sludge is shown in Table 3.7. For this purpose HCl or NaOH was added to magnetically stirred sludge samples until the desired pH value was reached. After the treatment the pH values were readjusted to about 7. Part of the samples was used for the determination of DA and PA. Another part was washed etc. according to 2.6.4 before the oxygen uptake rate was determined. Table 3.7 shows that the amylases are very labile under acid conditions, which seems to confirm the presence of α-amylases. Generally, α-amylases are inactivated rapidly at low pH values, whereas glucoamylases and β-amylases are more stable under acid conditions.

3.6. PROPERTIES OF ACTIVATED SLUDGE-BOUND PROTEOLYTIC ENZYMES

3.6.1. *Location of the proteolytic enzyme system of sludge*

The proteolytic enzymes, like the amylolytic enzymes (3.5.1), were found to be bound to the activated sludge (7.2). In contrast to DA, no clear increase of PA was observed after ultrasonication of the sludge (7.2). Apparently all proteolytic enzymes of activated sludge are directly accessible to casein and other proteinaceous substrates.

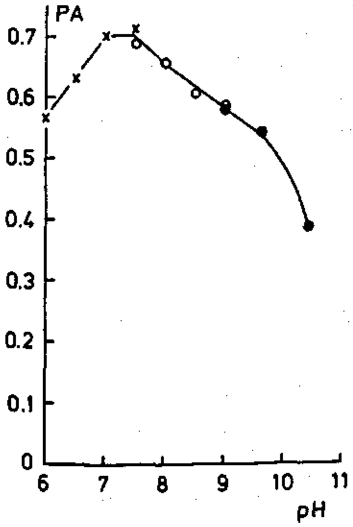

FIG. 3.14. Effect of pH on PA of activated sludge. The sludge had been loaded with starch at pH 7.0 during 64 days (SL = 0.3). During the experiment it was incubated for 3 hours at 25% of its original concentration. The buffers (0.1 M) used included: x, potassium phosphate; o, tris-HCl; ●, glycine-NaOH.

TABLE 3.8. Q_{10} values of proteolytic activities of starch activated sludges under standard conditions.

Planned loading of activated sludge (SL)	Number of days of starch loading	Q_{10} (20–30°C)
0.075	237	2.01
0.3	26	2.07
2.4	142	1.93
2.4	143	1.95

The extractability of proteolytic enzymes was about the same as that of amylolytic enzymes (7.2). The properties mentioned below reflect the bound condition of the proteolytic enzymes in situ.

3.6.2. *Effect of temperature on the proteolytic activity of activated sludge*

The effect of temperature on proteolytic activity of different activated sludges was measured between 20 and 30°C under standard conditions (Table 3.8). The Q_{10} values of the proteolytic activities were about 2; these values equalled those of dextrinizing activity (Table 3.6). The experiments concerning both types of enzymes, Tables 3.6 and 3.8, were carried out with the same sludges.

3.6.3. *Effect of pH on activity and stability of proteolytic enzymes of activated sludge*

Activated sludge was loaded with starch at a controlled pH value of 7.0. The PA of the sludge was estimated with different buffers as a function of the pH (Fig. 3.14). Tris buffer appeared to interfere with the Lowry protein determination (cf. REJ and RICHARDS, 1974). For this reason a standard curve was prepared by incorporating 0.1 M tris buffer (pH 7.5) in the casein standards used. Fig. 3.14 shows that the optimum pH was 7.0–7.5; the pH of the standard determination of PA is 7.5. The range of optimum values was narrow, compared with that of DA of sludge, grown at pH 7.0 (Fig. 3.13B). The decline of PA, at least at the alkaline side of the optimum pH, was less pronounced than that of DA. This indicates a greater stability of proteolytic enzymes at extreme pH values, as compared to that of amylolytic enzymes (Table 3.7).

3.7. SUMMARY

1. Exhaustive starch degradation by starch activated sludge and the cell-free ultrasonicate of this sludge resulted in hydrolysis of at least 75 and 90%, respectively, of the glycosidic bonds initially present.
2. The DA/SA ratio of the amylolytic enzyme system of different types of activated sludge and of the ultrasonicates of these sludges was found to be about 10 which is considerably lower than the ratio (24.6) of the fungal α-amylase Fungamyl 1600.

3. The concerted action of α-amylases and debranching enzymes is probably the most important mechanism of starch hydrolysis of starch-grown activated sludge. These enzymes are located outside the cytoplasmic membrane of the bacteria.
4. Low-molecular dextrins, maltotriose and possibly maltose were apparently degraded to an important extent by the same enzymes that attack whole starch. Enzymes, specifically hydrolysing these compounds were not clearly detected in starch-grown activated sludge or in the ultrasonicate of such sludge.
5. For the routine estimation of the amylolytic activity of activated sludge DA has several advantages compared to SA and was therefore employed.
6. The dextrinizing reaction rate of sludge remained constant until about 55 % of the initial iodine-stainable material was left. The initial reaction rate was strictly proportional to the enzyme concentration.
7. The presence of 500 mg of chloramphenicol per litre of sludge or the absence of nitrogen prevented the synthesis of amylases in the sludge when starch-grown activated sludge (SL = 0.3) was incubated at 25 °C in complete starch growth medium B_1 for periods up to at least 2 days. Growth seemed to be necessary for the synthesis of amylases in such sludge.
8. Toluene treatment of activated sludge generally brought about a moderate increase of DA and a slight decrease of PA; it accelerated the inactivation of dextrinizing enzymes during long periods of incubation.
9. The reaction rate of the proteinases of 'normal' activated sludge was found to remain constant until approximately 2 of the 5 g casein initially present per litre had been degraded to $HClO_4$-soluble products. This reaction rate was not exactly proportional to the sludge concentration.
10. Low concentrations of potassium phosphate (5 mM) stimulated the PA of sludge; higher concentrations had an inhibitory effect. Potassium phosphate had hardly any effect on PA when it was added during the proteolytic reaction. This suggests that the buffer affects the proteolytic enzymes only if no substrate is bound to the enzymes. Potassium phosphate in concentrations between 10 and 100 mM did not affect DA.
11. The amylolytic and proteolytic enzymes of activated sludge are completely bound to the sludge.
12. The Q_{10} values (interval 20–30 °C) of amylolytic and proteolytic activities of different activated sludges were found to be about 2. The effect of temperature (1–45 °C) on DA did not differ substantially in sludges grown in the laboratory and in an outdoor plant. However, at 60 °C the DA of the latter sludge was more labile than that of the former sludge.
13. The range of optimum pH values of starch activated sludge (cultivated at pH 7.0) was found to be wide with respect to DA and to fluctuate (between pH 5–8). As to PA the range of optimum pH values was narrow (pH 7.0–7.5). The optimum pH of DA shifted to a narrow range around pH 5.5 when the sludge was grown in starch medium at pH 5.0 for 11 days, but hardly shifted to higher values when the pH of cultivation was 8.8 for 70 days. This

suggests that bacteria producing amylases with a high optimum pH do not develop in activated sludge. The amylases of starch activated sludge, cultivated at pH 7.0, were very labile under acid conditions (pH 3–4).

4. LOADING OF ACTIVATED SLUDGE WITH STARCH

4.1. INTRODUCTION

In this chapter several aspects of the loading of sludge I with a polymer substrate like starch are reported. Acclimatization of activated sludge to a newly supplied substrate like starch includes the microbial synthesis of enzymes necessary for the attack of the macromolecular substrate. This enzymic acclimatization is achieved by one or more of the following mechanisms.

1) Induction of enzyme synthesis in some or in most of the micro-organisms present in the sludge (adaptation or physiological acclimatization).
2) Selection out of the existing microbial sludge population of organisms possessing the enzymes for starch degradation before starting the experiment (population shift or sociological acclimatization). Such enzymes are formed constitutively or possibly are induced by dextrins before the addition of the polymer.
3) Induction of enzyme synthesis in micro-organisms present following addition of starch and subsequent selection of these organisms (physiological acclimatization followed by sociological acclimatization).
4) Selection of regulatory mutants exhibiting higher enzyme activities, or requiring no external inducing substrate, or being insensitive to catabolite repression.

The different aspects of acclimatization of sludge to starch were studied intensively (4.4) and a population shift was analysed (8.3).

The method of counting bacteria and some preliminary observations are given in 4.2 and 4.3, respectively.

Also some aspects of (once acclimatized and) continuously operated starch activated sludge were studied: the conversion of starch (4.5), amylolytic activities (4.6.1) and numbers of total (viable) and (viable) amylolytic bacteria (4.6.2), all of them in relation to the loading with starch. In 4.6.3 the biomass fraction occupied by amylolytic enzymes in activated sludge was estimated and in 4.7 the effect of some unfavourable conditions of sludge cultivation on the production of amylolytic and proteolytic enzymes is dealt with.

For reasons of comparison some results of sludges without sludge retention (II) are given, especially regarding the occurrence of mixed or monocultures.

Other aspects of sludge (I) amylolytic enzymes, like their interaction with proteolytic enzymes and their location in activated sludge, are reported in other chapters (5.5 and 7.2).

4.2. COUNTING OF BACTERIA

Special attention has been paid to the enumeration of viable bacteria in sludge. Counting all living bacteria is impossible, as no culture technique meets the requirements of all bacteria present. The true number of living bacteria was approached by the application of carefully defined growth criteria and of cultural conditions resembling those in sludge. For this purpose serial sludge dilutions were incubated aerobically with low-concentrated complex liquid media (increasing complexity from medium A_1 to A_3; Table 2.2) at 25°C for a prolonged period in order to include also slowly growing organisms.

Activated-sludge extract or sewage was not included in the growth media. Several authors (Van Gils, 1964; Prakasam and Dondero, 1967a, 1967b; Pike et al., 1972) found that sewage agar in general was a poor medium for cultivation of aerobic heterotrophs of activated sludge. Prakasam and Dondero (1967a, 1967b) reported that an agar medium containing activated-sludge extract as the sole nutrient gave counts of activated-sludge bacteria generally higher than did other media. Pike et al. (1972), however, found lower numbers on rather rich media supplemented with activated-sludge liquor, but these counts were made after only 6 days at 22°C. According to Kato et al. (1971) basal media containing sludge extract or yeast extract yielded the highest viable cell count.

Numbers of starch- or casein-decomposing bacteria were also estimated with the dilution frequency method using liquid media. The spread-plate count method was rejected, as zones of starch hydrolysis of heavily grown parts or of large colonies often overlapped small colonies. Moreover, several isolated amylolytic bacteria showed no halo of hydrolysed starch around the (small) colonies, but gave only an area of slight degradation of the polymer under the colonies (see Plate 8.1). Starch was detected by flooding the plates with Lugol's iodine solution (containing iodine and potassium iodide in amounts of 3.33 and 10.0 g/l, respectively) or by evaporating a few drops of this solution in the cover of the Petri dish. Colonies of amylolytic bacteria growing poorly on starch as the only added carbon and energy source, could not be distinguished from strains growing on agar impurities. Moreover, amylolytic sludge bacteria requiring organic compounds would not grow on 'pure' agar starch media. For these reasons the counts were not made by the spread-plate method but by applying a statistical method (MPN; see 2.5.1) to the (tube) dilution frequency method for liquid media (multiple portion decimal dilution plantings), although this method is not precise.

As was pointed out in 2.5.1, the latter technique is based on diluting the sample to a point beyond which no infective units are present. A correct application of the method requires the presence of a homogeneous suspension. Clumping of bacteria badly interferes with this technique. To interpret the results obtained, it is generally safe to assume that it is purely a matter of chance whether or not a particular bacterial cell occurring in the homogeneous suspension will be present in a sample taken. This chance is inversely proportional to the dilution factor. Moreover it is assumed that growth will occur if at least one living organism is present in the sample.

Initially the numbers of total (viable) bacteria and (viable) starch-decomposing bacteria were estimated by incubating parallel series in tryptone-starch media A_1 or A_2 and starch media A_1 or A , respectively. In subsequent experi-

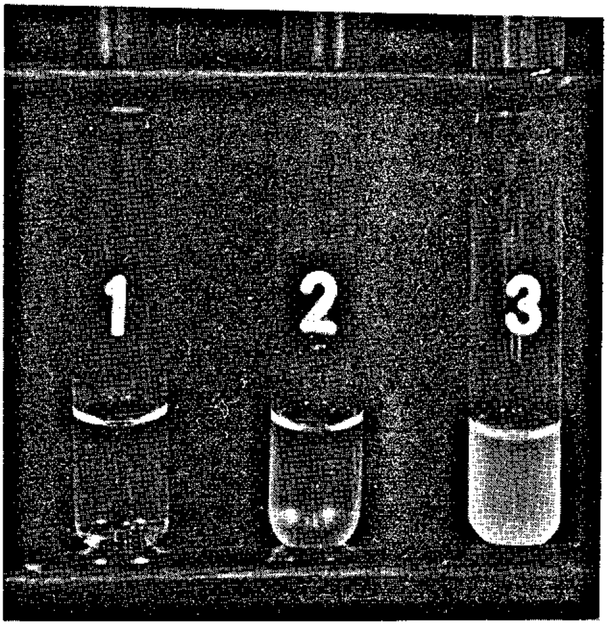

PLATE 4.1. Growth of bacteria from diluted activated sludge on starch medium A_3 under standard conditions. 1. Control without growth; 2. slight growth on yeast extract, trypticase soy broth and possibly glucose, but no utilization of starch; 3. dense growth on all components: starch was consumed.

ments total (viable) counts were made in the same set of tubes for the enumeration of bacteria growing at the expense of starch, glucose or casein (media A_3 and A_2). These media contain low concentrations (about $\frac{1}{10}$ of the main constituent) of non-selective compounds like yeast extract, glucose and trypticase soy broth, enabling bacteria to attain a density visible as a slight turbidity. In case of doubt, tubes were inspected microscopically. This was always done with the opalescent casein medium A_3. The microscopic control gave almost 100% certainty as to the occurrence of bacterial growth (all or none response). Utilization of the main constituents glucose, starch or casein resulted usually in dense growth (see Plate 4.1). Disappearance of glucose and starch was verified by (specific) reaction of part of the culture with Somogyi reagent (red precipitate after slight boiling indicates glucose) and Lugol's solution, respectively. The presence of casein was not verified.

Tubes of the starch series A_2 and A_3 showing scarce growth and no starch digestion after 15 days were often tested for the presence of glucose-consuming bacteria by aseptic addition of glucose to a final concentration corresponding to 1.2 g COD/l and subsequent incubation. These bacteria were assumed to be present if dense growth (without starch digestion) occurred within about 5 days. For the purpose of counting glucose-utilizing bacteria it was assumed that all starch-degrading bacteria counted were able to utilize glucose as well (Chapter 8).

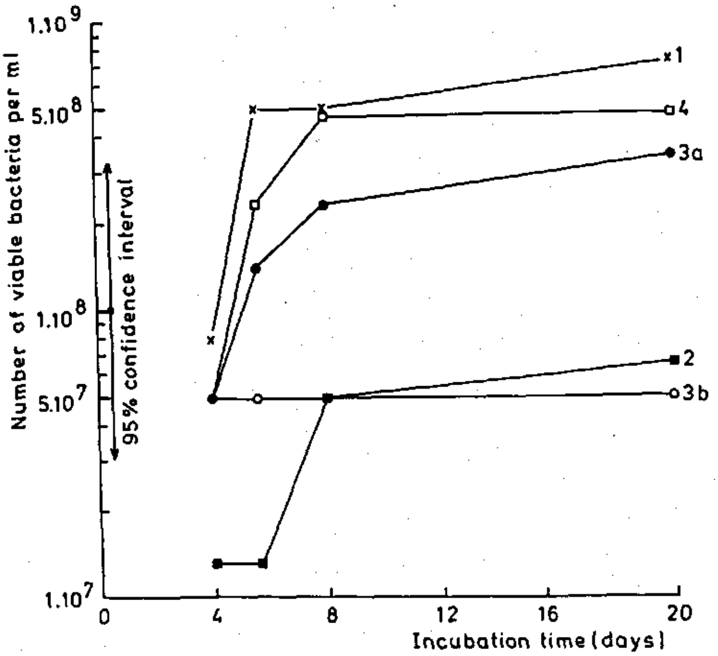

FIG. 4.1. Amylolytic and total (viable) counts (MPN-based) of bacteria in activated sludge grown for 52 days on maltose (SL = 0.3). Four different growth media were inoculated with serial sludge dilutions, 5 tubes per dilution. These tubes were incubated at 25°C without shaking and observed at intervals during 20 days. The final pH did not deviate more than 0.5 unit from the initial value. The 95% confidence interval is based on single data-points. Media: basic medium (Table 2.1), supplemented with potassium phosphate, pH 7.0, final concentration 15 mM, 295 mg $(NH_4)_2SO_4$ per litre and: 1 (x): yeast extract (100 mg/l), $(NH_4)_2SO_4$ omitted; 2 (■): starch (1.5 g COD/l); 3 (○ and ●): starch (1.5 g COD/l) and yeast extract (100 mg/l); 4(□): tryptone (1.125 g COD/l), starch (0.30 g COD/l), glucose (0.075 g COD/l) and yeast extract (100 mg/l). The bacterial growth in the higher dilutions of medium (3) was either dense (3b: starch utilizers) or slight (yeast extract, non-starch utilizers). Total (viable) counts (3a) in this medium were derived from all the tubes showing growth, and yeast extract – non-starch decomposers by subtracting the numbers of amylolytic organisms from the total (viable) counts.

In the experiment of Fig. 4.1 dilutions of a maltose-grown sludge were inoculated into tubes with 4 types of media, viz.: 1) yeast extract, 2) starch, 3) yeast extract and starch and 4) yeast extract and starch, supplemented with tryptone and glucose. After various periods the tubes were inspected for growth and the corresponding number of bacteria was calculated with the aid of MPN tables. Long incubation periods were necessary to permit development of all infective units present. In other experiments the number of tubes showing (good) growth had increased even after 10 days. Therefore, an incubation period of 15 (or 20) days was chosen as standard procedure; increased numbers were never found after this period. The viable counts estimated with three different media (1, 3, 4) containing yeast extract as common component, did not differ at the 5%-significance level; if they did so, they would have differed at least by a factor

5.41 (2.5.1). The same was true of the differences between the numbers of starch-decomposing bacteria in the presence (3b) and absence (2) of yeast extract. The data suggest that in this experiment yeast extract only accelerated the growth of bacteria on starch.

One set of tubes used for combined counts replaces two or three sets of tubes needed for parallel counting. Moreover, it considerably reduces, as compared with the parallel counting, the influence of both experimental errors and errors due to random fluctuations on the estimation of percentages of viable bacteria growing on a specific substrate.

When no growth was observed with the naked eye, several bacterial cells were sometimes observed microscopically in (every) field of view. This regularly concerned bacteria of the *Caulobacter* type which are unlikely to be counted on agar plates. These few bacteria were incidentally found to be responsible for the degradation of starch as was observed by the absence of iodine-stainable material in the used culture solution. Obviously these bacteria degraded starch in e.g. 15 days without substantial growth in this medium. They were noted as viable amylolytic bacteria. Doubt on the nature of bacterial growth sometimes existed, e.g. when cell density in a starch yeast extract medium was moderate and the starch was degraded partly. The presence of viable amylolytic bacteria was deduced only when a substantial loss of iodine-stainable material was observed.

As activated sludge is a dynamic system consisting of living and dead material, we were also interested in numbers of dead compared to living bacteria. As a first approach the assumption, as made in Water Pollution Research (1970), was used: $1 - 5 \times 10^9$ bacteria have a dry weight of 1 mg. This corresponds satisfactorily with microscopic estimates of total bacterial cell numbers in homogenized sludge (mixed liquor) from sewage works and experimental plants viz.: 1 mg of dried sludge solids contains $0.5 - 4 \times 10^9$ cells (Wat. Poll. Res., 1970). In the present study an estimate of 1.4×10^9 cells per mg dry wt of activated sludge was made (5.3).

4.3. PRELIMINARY OBSERVATIONS

4.3.1. *Effect of loading of Zeist activated sludge with starch or maltose on the percentages of amylolytic bacteria*

To gain an impression of the relative occurrence of amylolytic bacteria in activated sludge and of the rate of possible acclimatization of the sludge population upon the addition of starch, an experiment was carried out with activated sludge withdrawn from the Zeist plant on 4-7-1973. After one week of storage at 4°C, the sludge was brought into two laboratory reactors (Fig. 2.1) and loaded with maltose and starch, respectively, in amounts corresponding with 0.75 g COD/day.l. The average sludge concentration approached 2 g/l which was less than the planned 2.5 g/l. Maltose was preferred to the monomer glucose as the latter might promote the bulking growth of *Sphaerotilus* species. MPN-based counts with separate media for the total (viable) count and the amylolytic count

TABLE 4.1. MPN-based total (viable) and amylolytic bacteria in Zeist sludge before and after loading with maltose or starch for 12 and 40 days. Viable counts were made in tryptone-starch-maltose medium A_1-TSM, counts of amylolytic bacteria in starch medium A_1-S; 5 tubes per dilution were used. Incubation occurred for 10 days at 25°C.

Days of loading (SL = c. 0.4)	Sludge substrate			
	Maltose		Starch	
	Number/10^{-7} ml in media			
	A_1-TSM	A_1-S	A_1-TSM	A_1-S
0 (= Zeist sludge)	13	1.3(10)	13	1.3(10)
12	43	4.9(11)	280	180 (64)
40	220	2.8(1.3)	64	49 (77)

Figures in parentheses denote amylolytic bacteria as percentages of total (viable) bacteria.

were made after 0, 12 and 40 days of loading. The Zeist sludge was stored in the refrigerator for 2 weeks before counting.

The total numbers of viable bacteria appeared to increase upon loading of Zeist activated sludge with either starch or maltose (Table 4.1). Amylolytic bacteria in

TABLE 4.2. DA_{spec} of various sludges I cultivated in laboratory reactors and outdoor plants.

Activated sludge	Sludge loading (SL: g COD per g sludge per day)	Hydraulic space load (D: day^{-1})	DA_{spec} (g starch per g sludge per h)
Lab; starch; pH 7.0; 20°C	0.3	1.0	2.0–2.5[1]
Lab; starch; pH 8.8; 20°C	0.3	1.0	6.0–7.5[2]
Lab; Na-lactate[3]; pH 8.5–8.9; 25°C	0.3	0.26	< 0.1
Lab; glucose; pH 7.0; 20°C	0.3	1.0	0.11–0.22
Lab; maltose; pH 7.0; 20°C	0.3	1.0	0.2–0.5 (0.1–0.7)
Lab; artificial dairy waste; pH 7–8; 25°C (fill and draw system)[4]	0.15–0.25	0.88	0.22 and ≥0.5
Outdoor; Bennekom (May-Oct./Nov. '74–'76)	c. 0.063	0.33–0.5	0.54[5] (0.40–0.67)
Outdoor; Zeist (May-Oct./Nov. '74–'76)	c. 1.5	c. 4	1.08[5] (0.96–1.30)

[1] See section 4.6.1; [2] see section 4.7; [3] maltose activated sludge was fed continuously during only one week with sodium lactate, NH_4Cl (C/N = 7.2), basal salts (including 8 mM potassium phosphate) and Na_2SO_4. Effluent was removed by centrifugation once a day; [4] ADAMSE (1974). DA was estimated twice (1974 and 1976); [5] arithmetical average of 7 and 8 values, respectively, estimated during the summer periods.

the original Zeist sludge, counted in starch mineral salts medium, amounted to about 10% of the viable count in tryptone starch maltose medium. Loading with maltose slightly decreased this percentage, but loading with starch during 12 and 40 days gave an increase to roughly 64 and 77%, respectively. These percentages would possibly have been higher if growth-promoting substances like yeast extract had been present in the media used for counting (without microscopic control doubtful growth occurred in the tubes).

The results indicate that acclimatization of sewage-fed activated sludge to starch loading is accompanied with a population shift. Within 12 days, the absolute numbers of amylolytic bacteria increased many times.

4.3.2. *Amylolytic activities of various activated sludges*

DA_{spec} of starch-fed sludge I was found to be considerably higher than DA_{spec} of sludges I grown on other substrates (Table 4.2). Only in lactate-grown sludge, DA_{spec} was hardly detectable; within 5 days of lactate feeding it decreased from 0.68 (activity of maltose sludge, cultivated at pH 7.0) to less than 0.1. In all of the other sludges clearly detectable DA_{spec} values were observed, especially in sewage-fed sludges from outdoor plants. Sewage is likely to contain some amylaceous compounds. Comparison of two extensively aerated activated sludges (Tables 4.2 and 4.4) shows that the DA_{spec} of the sewage-fed Bennekom sludge (0.54) was even hardly lower than that of sludge supplied with starch as sole carbon and energy source (0.66). That the activity of the higher-loaded Zeist sludge was higher than that of Bennekom sludge, agrees with the relation found between SL and DA_{spec} (Table 4.4 and Fig. 4.8). The production of amylolytic enzymes in glucose and especially maltose-fed sludge is attributed to an inducing effect exerted by these sugars and/or by contaminating dextrins. Such an explanation cannot be given for the sludge fed daily with whey and skim-milk (3:1; C/N = 13) as a shock loading (ADAMSE, 1974). In spite of the presumed absence of α-glucosides in these substrates, clearly measurable DA_{spec} values were observed. Polysaccharides were accumulated by the cells after the shock loading (ADAMSE, 1974). Possibly glycogen-like polysaccharides, released to the sludge liquid, served as an inducer of DA synthesis.

The relation between DA_{spec} of activated sludge and the presence of amylaceous compounds in the substrate, and the apparent absence of DA in lactate-grown sludge (Table 4.2), suggest that extracellular amylases generally play a role only in the degradation of starch-like compounds of the influent but apparently are not involved in the degradation of bacterial polysaccharides. No activated sludge could be grown in which PA was undetectable (Chapter 5). This is indicative of the different functions of amylolytic and proteolytic enzymes in sludge metabolism.

4.3.3. *Effect of shock loadings on starch removal by starch activated sludge*

To know if DA may be a limiting factor in the purification of waste water, two experiments were performed on the effect of 'shock' conditions on starch degradation by starch-grown sludge I.

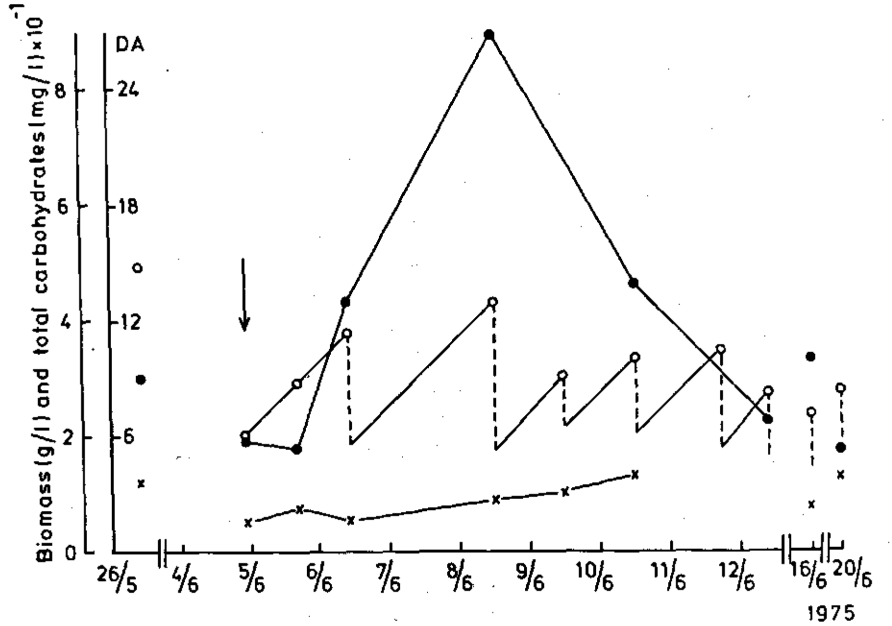

FIG. 4.2. Effect of quantitative shock loading of continuously fed starch sludge I (SL = 0.3 during 36 days) on amylolytic activity and conversion of carbohydrates. The arrow denotes the increase of loading from 0.75 to 3.0 g starch COD/l.day. ○, Biomass (dry wt) in reactor before discarding part of it; ●, DA of reactor contents before partial disposal of sludge; x, total carbohydrates, expressed as glucose (equivalents), in membrane-filtered sludge liquid (2.6.5), supplied with $HClO_4$ (70%), one drop per 5–10 ml.

In the first experiment activated sludge fed with 0.75 g starch COD/l.day during 36 days was submitted to a fourfold increase of starch, phosphate and ammonium sulphate concentrations in the influent stream (quantitative shock loading). From the measurements of carbohydrates in the sludge filtrate it can be concluded that the added starch was almost completely converted by sludge treatment (Fig. 4.2). Increased loading with starch did hardly affect the carbohydrate content of the liquid. As DA of the sludge system, measured for the first time at 18 h after increased loading, had not risen, it can be stated that the existing amylolytic enzyme activity had been sufficient to degrade the 4 times increased amount of starch. Nevertheless, DA and DA_{spec} showed a pronounced rise upon continued high loading with starch.

During the second experiment (no results shown), two starch activated sludges (SL = 0.3, pH 7.0) were submitted to a sudden drop of the pH to 5.5 and subsequently 5.0, and a sudden rise to pH 8.5–9.0, respectively (toxic shock loading). After 16 days the pH values of the two sludge systems were reversed to 8.0 and 5.5, respectively. The drop of pH caused a clear decrease of DA_{spec}, in the case of alteration from pH 9 to 5.5 even from 3.30 to 0.65. In spite of this pronounced reduction of DA_{spec}, the carbohydrate content of the supernatant of the centrifuged sludge remained approximately at the same low level, showing that even the strongly reduced DA_{spec} brought about adequate elimination of the substrate.

From the results of both experiments it is concluded that the amylolytic activity of moderate-loaded starch activated sludge at continuous operation is not limiting but apparently far in excess of the activity required for adequate purification.

4.3.4. *Settleability and floc structure of sludges I and II*

Although we were primarily interested in the biochemical performance of sludge system I and not in the overall efficiency, some remarks are made about the settleability of the sludge, determined according to Standard Methods (1975).

The settleability of the Zeist sludge (e.g. of 4/7/'73) was good. However, after 5–7 days of loading of this sludge with starch in the laboratory reactor, as described in 4.3.1, the sludge settled no more, in contrast with the maltose sludge. Moderate-loaded starch sludge never showed any substantial settling, even after years of loading. The settleability of moderate-loaded maltose (and glucose) sludge was less poor, but subject to variation. At SL = 0.075 the settleability of the starch sludge was also almost nil. Only (very) high-loaded starch sludge did often settle slightly, although the supernatants usually were turbid due to suspended bacteria.

The moderate-loaded starch sludge usually contained large amounts of filamentous bacteria (Plate 4.5) that prevented settling of the flocs. However, also when the threads were small in number, almost no settling occurred. This was caused by large amounts of sludge-bound slime that was always produced by the bacteria in this sludge. Rods embedded in slime were frequently observed in starch sludge (Plate 4.6, rods near threads; Plate 4.7, rods on the outside of a floc). Generally, the flocs of all laboratory-grown carbohydrate-fed sludges were loosely packed (see e.g. Plate 4.4). The very low-loaded starch sludge was characterized by great amounts of dead cells and cell debris (Plates 4.2 and 4.3) and a dark-brown colour (Plate 2.1). The very high-loaded sludge contained large numbers of viable bacteria (Plates 4.7 and 4.8) and had a yellow or pink colour (Plates 2.1 and 2.2).

Sludge II (SL = 0.3–0.1) was generally characterized by suspended bacteria sometimes forming loose aggregates of cells. The most striking example was a branched *Nocardia*-like bacterium growing in suspension and completely predominating the microflora during 500 days of starch loading (Plate 4.10). However, in the few flocs present other types of micro-organisms occurred (Plate 4.9). The *Nocardia*-like bacterium was surrounded by a heavy slime layer (Plate 4.11).

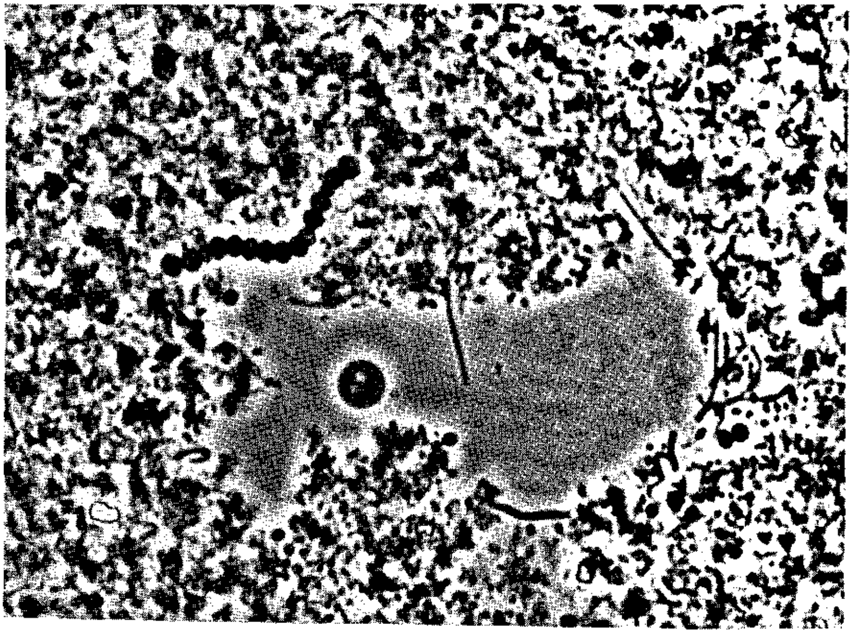

PLATE 4.2. Floc structure of sludge 1 after 351 days of starch loading at SL = 0.075, 1250 ×.

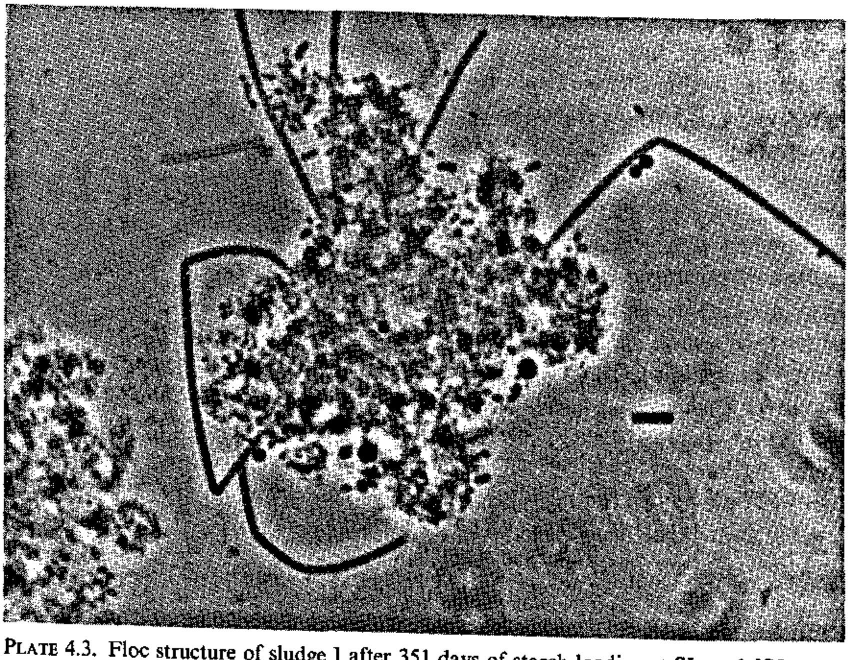

PLATE 4.3. Floc structure of sludge 1 after 351 days of starch loading st SL = 0.075, 1250 ×.

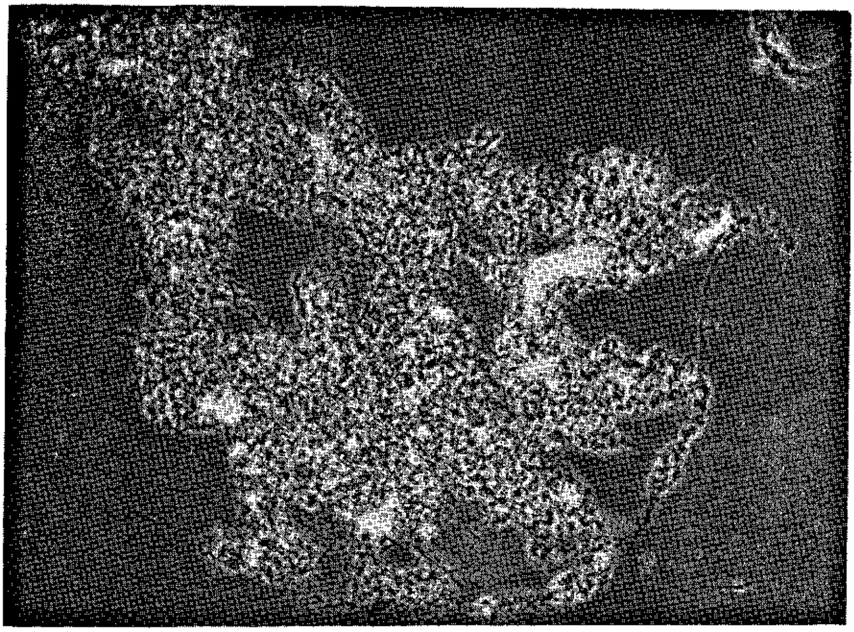

PLATE 4.4. Floc structure of sludge I after 41 days of starch loading at SL = 0.3, 325 ×.

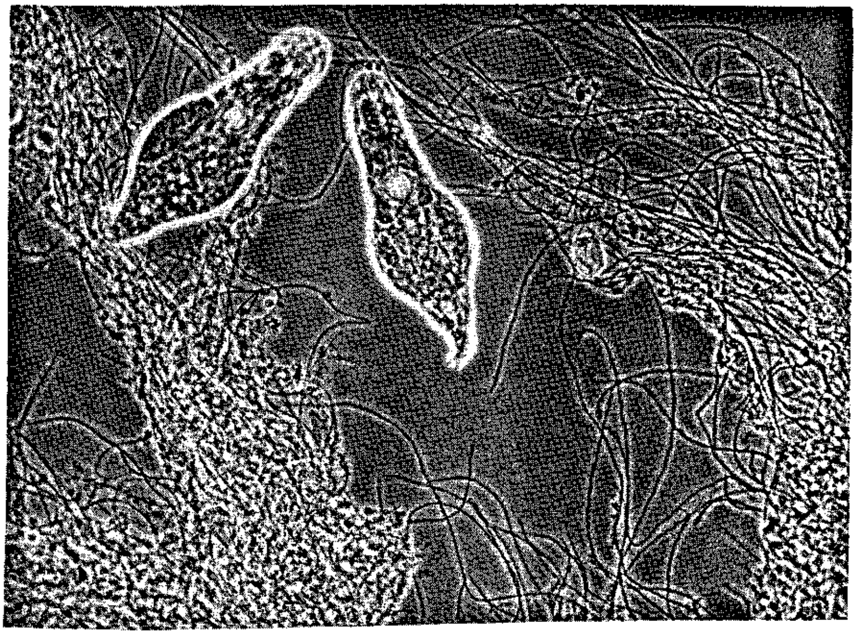

PLATE 4.5. Floc structure of sludge I after 84 days of starch loading at SL = 0.3, 325 ×.

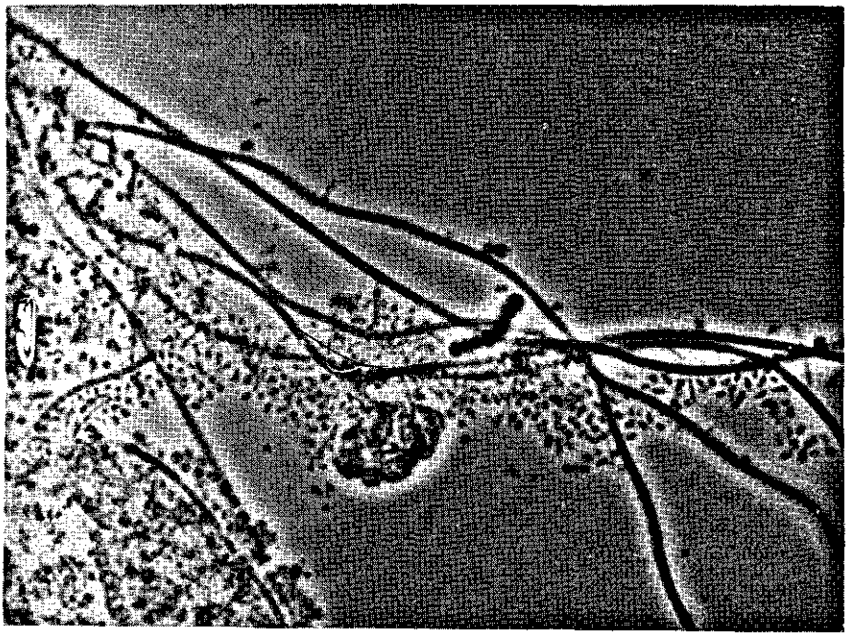

PLATE 4.6. Floc structure of sludge I after 84 days of starch loading at SL = 0.3, 1625 ×.

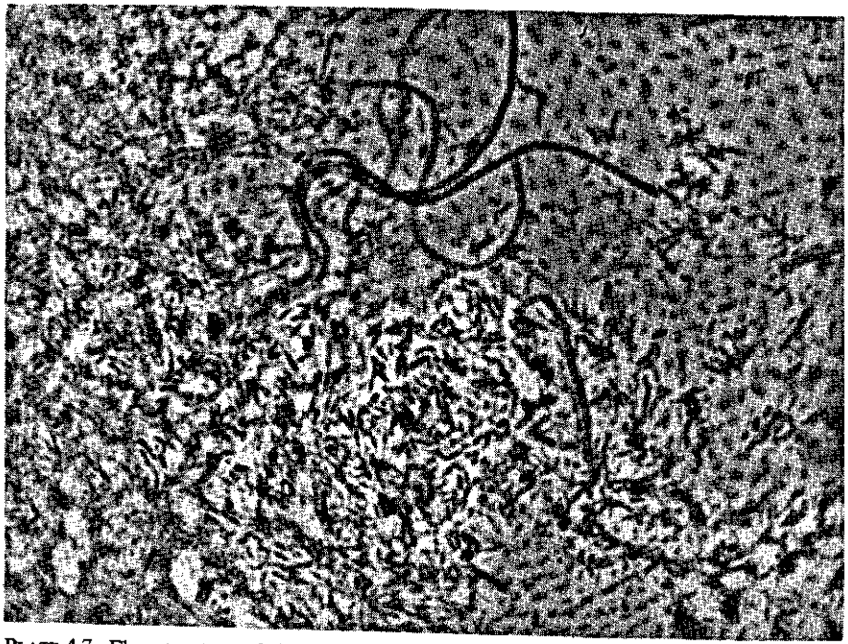

PLATE 4.7. Floc structure of sludge I after 269 days of starch loading at SL = 2.4, 1250 ×.

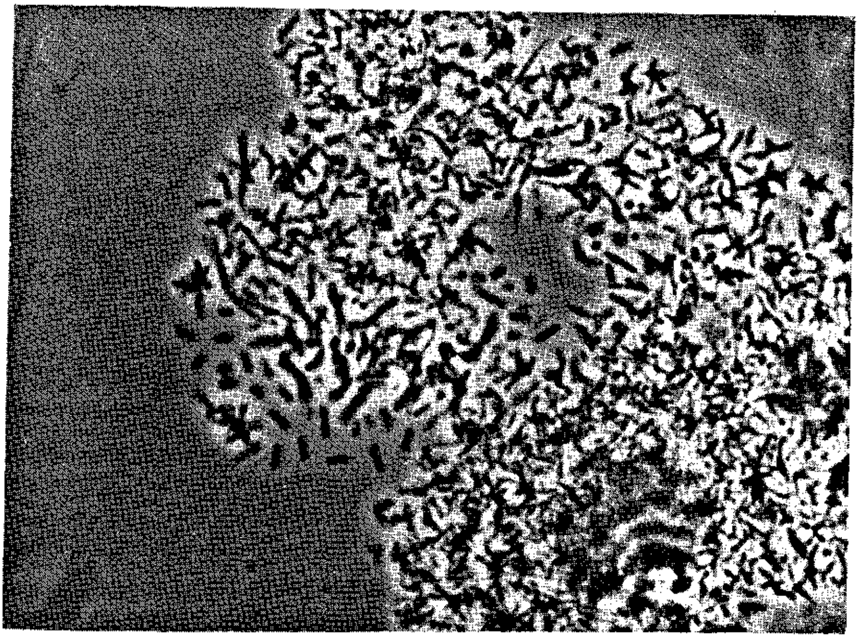

Plate 4.8. Floc structure of sludge I after 269 days of starch loading at SL = 2.4, 1250 ×.

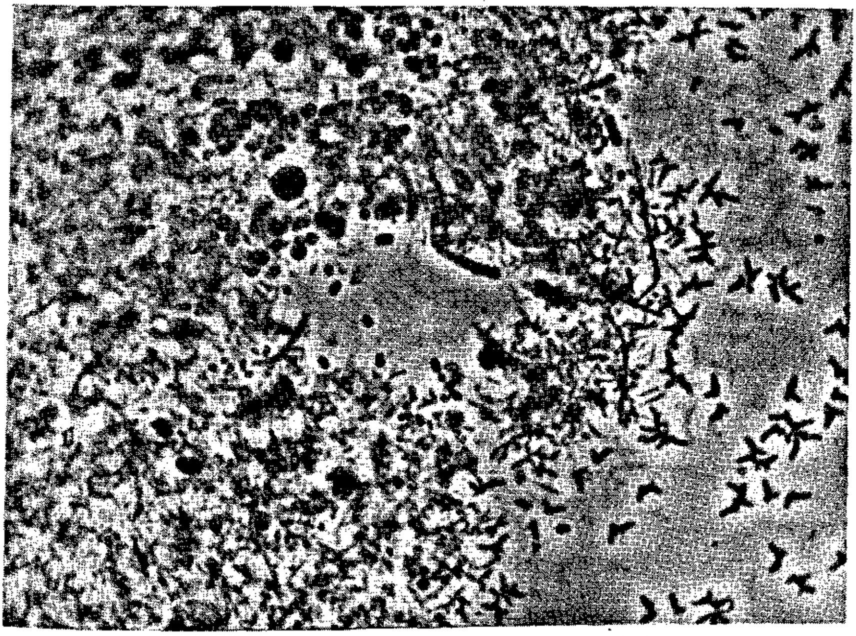

Plate 4.9. Composition of flocculated and suspended biomass of sludge II after 62 days of starch loading at SL = c. 0.15 and D = 0.05 day^{-1}, 1250 ×.

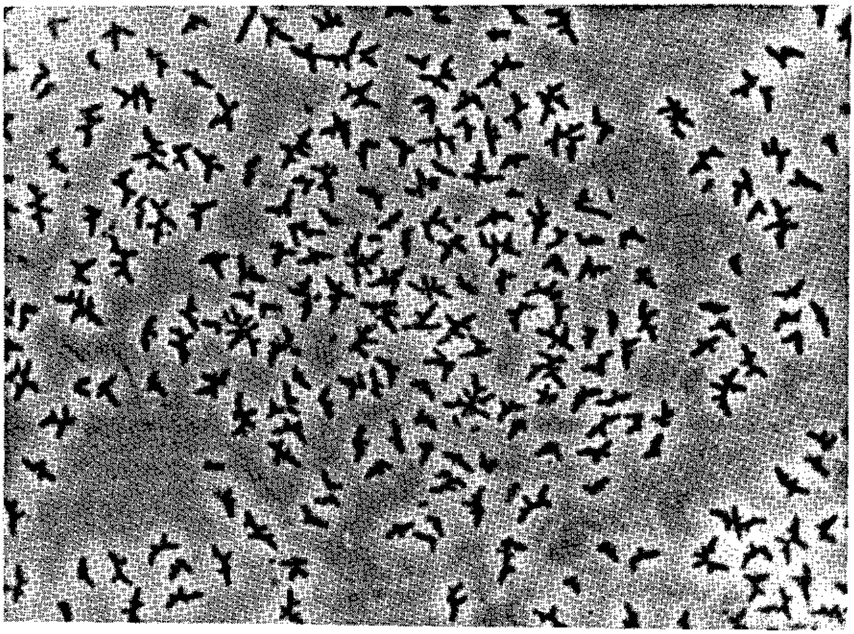

PLATE 4.10. Composition of suspended biomass (*Nocardia*-like bacteria) of sludge II after 62 days of starch loading at SL = c. 0.15 and D = 0.05 day^{-1}, 1250 ×.

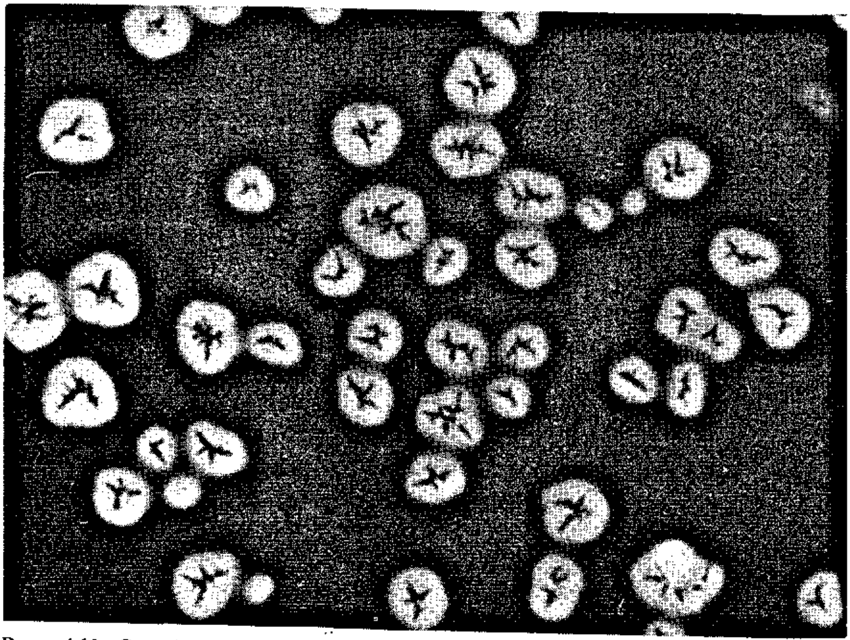

PLATE 4.11. Capsules around *Nocardia*-like bacteria in sludge II (see Plate 4.10), demonstrated by dispersing sludge cells in Indian ink, 1250 ×.

4.4. QUALITATIVE SHOCK LOADING OF MALTOSE AND GLUCOSE ACTIVATED SLUDGES WITH STARCH

The acclimatization of continuously loaded maltose- and glucose-grown activated sludges to starch was studied by replacing the maltose or glucose reservoir with a starch reservoir (qualitative shock loading). The new substrate was slowly introduced in order to imitate qualitative substrate changes in outdoor plants. Under such conditions and assuming that the new substrate is not metabolized, the increase of its concentration in a complete mixing reactor is described by the equation: $S_1/S_0 = 1 - e^{-tQ/V} = 1 - e^{-t/t_h}$ (S_0 and S_1: substrate concentrations in influent and reactor, respectively; Q: flow rate of influent; V: working volume of reactor; t: time after introduction of new substrate in influent; t_h: reactor mean hydraulic retention time). As dextrins, maltose and glucose are end products of extracellular starch hydrolysis, the acclimatization of the sludge requires only the production of amylolytic enzymes, and, possibly, of dextrine-permeases.

In order to find a correlation between purification and amylolytic activity, qualitative shock loadings were applied in five independent experiments (A-E) in which the initial dextrinizing activities were different. In all experiments growth, dextrinizing activity and degree of carbohydrate conversion were followed with time (Fig. 4.3A-E). In experiments B and E in addition total (viable) numbers and amylolytic bacteria were estimated (Figs. 4.4 and 4.5), whilst in experiment B also the population shift was analysed (8.3). In experiment E starch and dextrins in sludge filtrates were estimated with iodine reagent (Fig. 4.3E). Growth was measured as the increase of biomass in the reactor, corrected for loss of biomass by sampling. During the first periods of starch loading almost no sludge washed out through the spongy filter element. However, after one or more weeks of starch loading, sludge frequently began to wash out; no corrections were made for this loss of biomass (Fig. 4.3E).

Although two of the experiments were performed under less ideal conditions (C was started with starved sludge and D with pH-shocked sludge), a general trend was observed in these five experiments, as shown in the following survey. 1) Almost complete removal of carbohydrates occurred from the start of experiments A and B, but not of C, D and E. This can be correlated with the initial amylolytic activity which in the first 2 experiments was higher than in the last 3. The initial DA_{spec} in experiments A and B was 0.82 and 0.52, and in experiments C, D, and E 0.33, 0.23 and 0.13, respectively. Therefore, the lowest DA_{spec} of sludge giving adequate removal of carbohydrates (the critical or purification-limiting DA_{spec}) might be expected to approximate values between 0.3 and 0.5. To obtain more exact data concerning this critical value, the results of experiment E have been used. The very low initial amylolytic activity of this sludge, which was apparently responsible for the poor removal of the added starch during the first period of the experiment, slowly rose with time until at 135 h a value was obtained ($DA_{spec} = 0.25$, corresponding with $DA = 0.65$), at which carbohydrate removal was approaching completion. Complete elimi-

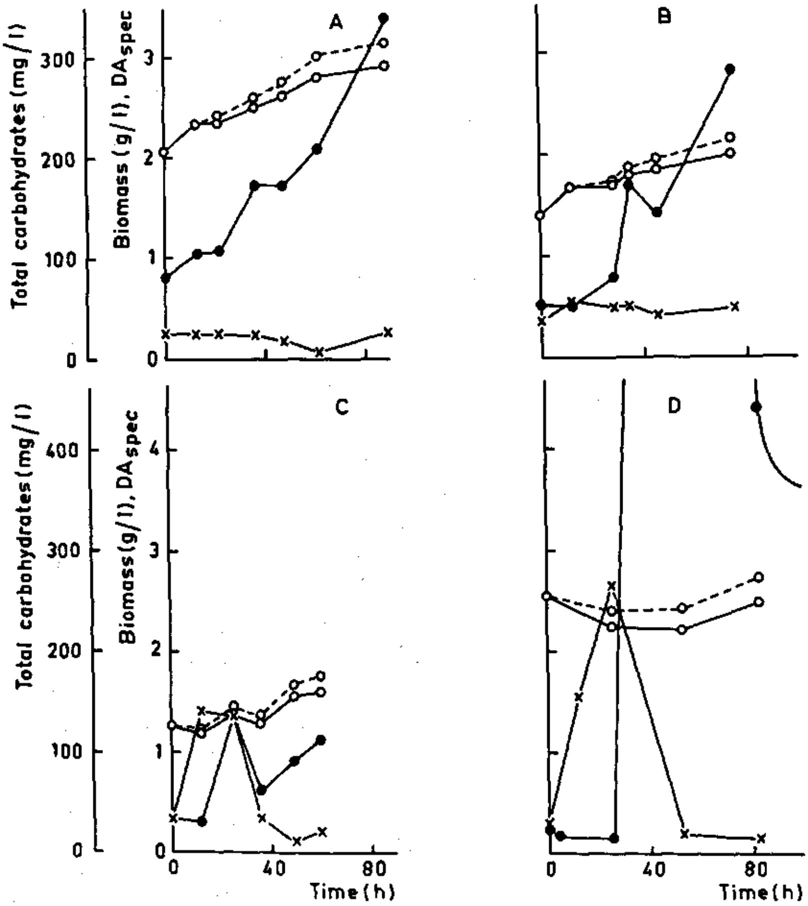

FIG. 4.3A-D. Effect of the addition of starch to maltose-grown activated sludge (qualitative shock loading) on amylolytic activity, removal of carbohydrates and growth of sludge. The loading amounted to 0.75 g COD (0.633 g starch, 0.704 g glucose equivalents) per day per litre; it started at t = 0 h. Before the loading with starch, the sludges had successively been loaded at VL = 0.75 g COD/l.day as follows: A, maltose 17, no substrate 5, starch 5 and finally maltose 8 days; B, starch 41 and subsequently maltose 84 days; C, starch 55, maltose 17 and finally during 5 days no substrate; D, Zeist activated sludge, maltose 55 days. Before and after starting with starch loading, sludge D endured several pH shocks. The initial DA values in the 4 reactors were as follows: A, 1.70; B, 0.73; C, 0.42 and D, 0.58. ●, DA_{spec}; ○, biomass, dry wt (the broken lines denote biomass values cumulatively corrected for sampling losses); x, total carbohydrates (as glucose equivalents) in supernatant of centrifuged sludge samples, provided with $HClO_4$ before centrifugation.

nation occurred at 180 h, when the amylolytic activity had only slightly risen (DA_{spec} = 0.30 and DA = 0.79). As the amylolytic activity after 135 h had been estimated with toluene-treated (1 % v/v) sludge (which gave somewhat increased DA; see 3.3.6), it was corrected for this effect. The critical DA_{spec} was calculated (as the average of both activities) to be 0.26 (corresponding with a DA of 0.69 at a sludge content of 2.63 g/l).

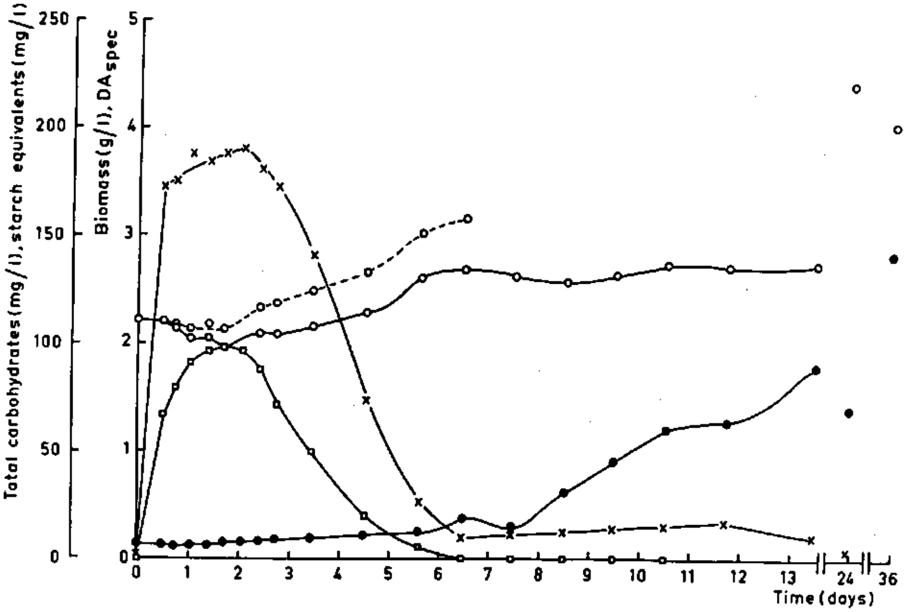

FIG. 4.3E. Effect of the addition of starch to glucose-grown activated sludge (qualitative shock loading) on amylolytic activity, removal of starch and dextrins and of total carbohydrates and growth of sludge. The sludge had been loaded with 0.75 g glucose COD/l.day during 287 days before loading with starch started at $t = 0$ h. The initial DA value in the reactor was 0.28. During the first week toluene was added to the DA assay mixture (final concentration of 1 %, v/v). ●, DA_{spec}; ○, biomass, dry wt (the broken line denotes biomass values cumulatively corrected for sampling losses); x, total carbohydrates as glucose equivalents; □, starch-extinction equivalents as mg starch equivalents/l. The last-mentioned two components were measured in membrane-filtered sludge liquid, supplied with $HClO_4$ after filtration.

The DA_{spec} and DA values of the A and B sludges at $t = 0$ h were considerably higher, those of sludges D and E were lower than the critical amylolytic activities derived from experiment E. In spite of the fact that DA_{spec} of sludge C at $t = 0$ h was higher than this critical value, the initial carbohydrate removal in this experiment was inadequate. This was due to the considerably lower initial sludge content of reactor C (1.27 g/l) compared to that of reactor E (2.63 g/l after one week of loading) resulting in a DA value of 0.42; this is clearly lower than the above-mentioned critical DA of 0.69. These results show that adequate purification in a reactor depends on both DA_{spec} and sludge concentration, i.e. on DA.

2) The complete removal of carbohydrates in experiments A and B was accompanied with a ready rise of DA (DA_{spec} × biomass concentration) and biomass. The increase of DA usually continued for 3–5 days (not shown). The systems with inadequate carbohydrate removal (C, D and E) initially showed a constant or a slightly decreasing DA and biomass, after 1–2 days followed by a sharp rise of DA (experiments C and D); in the case of E only a slow rise was

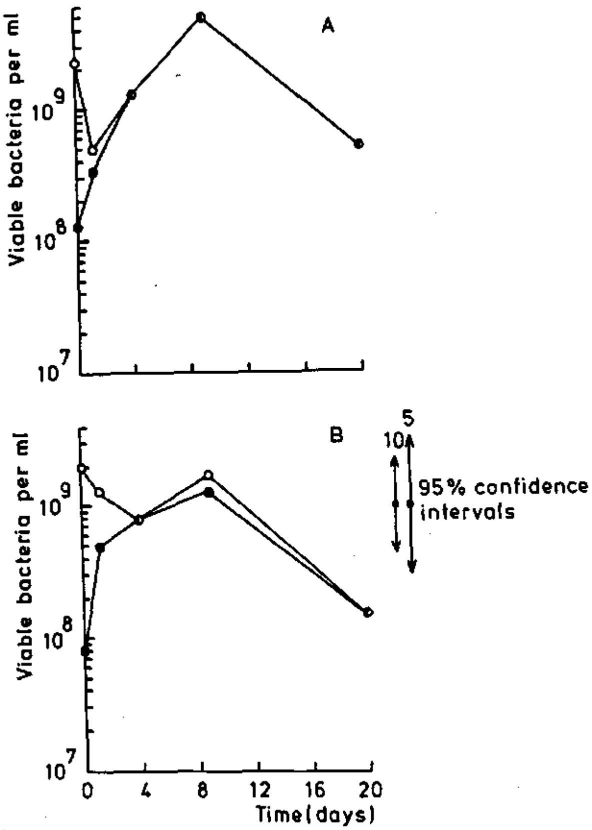

FIG. 4.4. Effect of qualitative shock loading with starch on total (viable) and amylolytic bacteria in maltose-grown activated sludge. The MPN-based counts were made during experiment B (Fig. 4.3B). Five tubes were used per dilution except for the counts on $t = 0$ and $t = 20$ days of loading when 10 tubes per dilution were used. The arrows indicate 95% confidence intervals for 10 and 5 tubes per dilution; they are based on single data-points. Two sets of tubes, both used for combined counts, were incubated at 25°C during 20 days, viz. A, tryptone-starch-yeast extract medium A_2 and B, starch-yeast extract medium A_2. ○, Total viable count; ●, amylolytic bacteria in the reactor.

observed. The high peak of amylolytic activity of sludge D may have been due to a pronounced rise of the pH favouring the synthesis of amylases.

3) The non-utilized carbohydrates in sludge systems with an initial DA below the critical value, largely and likely completely consisted of iodine-stainable material (Fig. 4.3E). Reducing sugars as estimated with Somogyi-Nelson reagent (2.6.6) could not be detected. This means that in such sludge systems the initial stages of starch hydrolysis are the limiting step of carbohydrate removal; lower dextrins and sugars are probably readily taken up by the sludge organisms.

4) The bacterial population of maltose-grown sludge B (Fig. 4.3B) with DA above the critical value consisted for only 4–6% of amylolytic organisms. Upon loading with starch a ready increase of the latter bacteria occurred so that after

29 h approximately half of the viable bacteria and after 4 days about the entire population consisted of starch-degrading cells (Fig. 4.4). Upon continued starch loading this high percentage of amylolytic bacteria in sludge was maintained indefinitely (Table 4.5). The trends of the countings with different media were similar (Figs. 4.4A and B).

The results obtained demonstrate that the acclimatization of the maltose-grown activated sludge to starch was not due to adaptation of an existing population to the newly introduced substrate, but to a population shift (4.1). The almost complete replacement of the non-amylolytic bacteria by amylolytic organisms shows that the latter bacteria, at least under conditions of complete starch removal, did not excrete starch degradation products which would favour the growth of non-amylolytic organisms. From the point of view of substrate elimination there was no obvious need for the observed population shift as the initial low percentage of starch-degrading bacteria brought about an immediate and complete removal of the added starch (Fig. 4.3B).

The curves of Fig. 4.4 permit the calculation of average generation times ($\bar{g}$) of the amylolytic bacteria, using the equation: $\bar{g} = t/n = (t-t_0) \log 2/(\log N_t - \log N_0)$ (n = number of cell divisions; t = growth period (h); N_0 and N_t = numbers of amylolytic cells counted at t_0 and after t hours, respectively). The $\bar{g}$ values

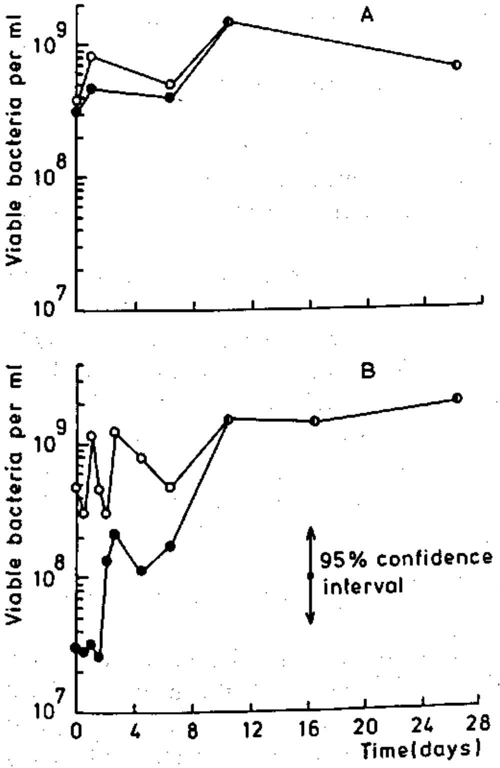

FIG. 4.5. Effect of qualitative shock loading with starch on total (viable), amylolytic, and glucose-utilizing bacteria in glucose-grown activated sludge. The MPN-based counts were made during experiment E (Fig. 4.3E). Ten tubes per dilution were applied. Two sets of tubes, both used for combined counts, were incubated at 25 °C during 20 days, viz. A, glucose-yeast extract medium A_3 and B, starch-yeast extract medium A_3. ○, Total viable count; ●, glucose-utilizing (A) or starch-utilizing (B) bacteria. The 95% confidence interval is based on single data-points.

during the first 29 h, calculated for curves A and B, are 21.5 and 11 h, respectively. These $\bar{g}$ values are low as compared with the doubling times calculated for bacteria in starch activated sludge under presumed steady-state conditions (roughly 3 days; see 5.3). The calculated average generation times probably are an overestimate, since no correction has been made for death of amylolytic bacteria. Since g increases with time, it is furthermore reasonable to assume that the initial g values were lower than the average values calculated for the first 29 h.

The percentage of amylolytic bacteria in the glucose-grown sludge (Fig. 4.3E) at the onset of starch loading (viz. 6%) was the same as that in the maltose-grown sludge. Nevertheless, the response of these bacteria to the added starch was much less pronounced (Fig. 4.5B). The numbers of the amylolytic bacteria remained at the same level during at least 38 h after starch loading. Hereafter an increase of these bacteria started which resulted in the complete replacement of non-starch-degrading by starch-decomposing bacteria during the time interval 7–10 days.

In agreement with the retarded rise of the amylolytic bacteria after the addition of starch, biomass also increased only after some retardation (Fig. 4.3E). DA, which originally was far below the critical value, remained at this low level for the first few days of starch nutrition, whereupon a small rise occurred followed at 7–8 days by a much more pronounced increase which coincided with the (pronounced) increase of starch-degrading bacteria until about 100% of the viable count. At this stage, starch removal by the sludge was practically complete (Fig. 4.3E). During the entire period of starch loading, a large majority of the bacteria was able to utilize glucose (Fig. 4.5A).

The relatively slow increase of amylolytic bacteria and of DA in glucose-grown activated sludge upon starch loading is difficult to explain. It might be due to the presence of types of amylolytic bacteria growing more slowly than those occurring in the acclimatizing maltose sludge.

A second, more obvious suggestion concerns the somewhat lower number of amylolytic bacteria which was apparently responsible for the DA of glucose-grown sludge being far below the critical level. Owing to the low DA, accumulation of dextrins occurred (Fig. 4.3E) which presumably enabled the growth of non-amylolytic, besides amylolytic, bacteria. When the latter bacteria had succeeded in rising DA above the critical value, starch was entirely consumed by the amylolytic bacteria resulting in a ready increase of the number of these bacteria to 100% of the population and a considerable rise of DA.

4.5. STARCH CONVERSION BY CONTINUOUSLY OPERATED STARCH SLUDGES

Sludges with (I) and without retention of flocculated biomass (II) were acclimatized to different starch loadings (2.3.3 and 2.4.2). When this had been achieved as to carbohydrate removal i.e. when steady-state conditions (4.6) had apparently been attained, samples were withdrawn from the reactors during

TABLE 4.3. Relation between sludge loading and carbon compounds in cell-free liquid of continuously operated starch sludge.

Sludge system	SL^1 (g starch COD per g sludge per day	D (day^{-1})	Influent (g starch COD^2 per litre)	Average concentration (mg/l) in cell-free liquid				
				Total carbohydrates (glucose equivalents)^2	COD	TOC^3	Iodine-stainable material (starch equivalents)^4 +HClO$_4$	−HClO$_4$
I, retention of biomass	0.075	1.0	0.188	2.3 (18; 1.0; 98.7)	12.0^6 (3; 4.2)	2.6^7 (3; 0.8)	0.0 (1)	–
	0.3	1.0	0.75	6.9 (24; 3.3; 99.0)	–	–	0.7 (4; 0.3)	0.5 (2; 0.2)
	1.2	1.0	3.0	13.6 (15; 7.5; 99.5)	–	–	–	–
	2.4	3.0	2.0	11.9 (19; 3.7; 99.4)	57 (5; 19)	20 (3; 5)	1.7 (9; 0.7)	0.5 (2; 0.2)
II, no retention of biomass	0.121	0.05	15.0	99^5 (3; 24; 99.7)	115 (2; 13)	42 (1)	–	–
	2.12	1.0	3.0	37 (4; 5; 98.7)	–	–	8.2 (3; 7.2)	0.9 (2; 0.3)

Note: The figures in parentheses indicate the number of measurements, the sample standard deviation and the average conversion percentage, respectively; the standard deviation reflects fluctuations of the sludge system (as to the concerned component) during time as well as fluctuations due to the method of estimation of this component. The periods of loading during which components were measured (100–460 days) were much longer that the short intervals within these periods during which measured values sometimes showed some correlation.

[1] See Table 2.3; [2] 1 g glucose is equivalent to 1.065 g COD; [3] measured with T (total) O (organic) C (carbon) analyser; [4] estimated in the supernatant of centrifuged sludge to which HClO$_4$ was added immediately after sampling (+HClO$_4$) and in the supernatant of sludge ice-cooled immediately after sampling (−HClO$_4$); [5] determined with the phenol-sulphuric acid method (according to HODGE and HOFREITER, 1962); [6] averages of values of unaltered samples and of values of samples concentrated four times by vacuum evaporation at 38 °C. The latter treatment apparently gave a loss of $\frac{1}{3}$–$\frac{1}{4}$ of COD; [7] measured only in the concentrated fraction.

periods of 100–460 days and their cell-free liquids analysed, usually for total carbohydrates. Since under steady-state conditions no correlation could be established between the amylolytic activity of the sludge and the concentration of total carbohydrates in the sludge liquid (see e.g. Figs. 4.2 and 4.3E, after 1 week of loading), it was of less interest to report the rather unpredictably fluctuating values observed in the course of the experiments. For that reason average values of the total carbohydrates and of some other components are reported; the calculated sample standard deviations give an impression of the fluctuations (Table 4.3).

Except for the low-loaded open continuous system II and for the very high-loaded sludge system I, the sludge loading was varied only by changing the substrate concentration in the influent. Variation of substrate supply by variation of the hydraulic space loading (*D*), as occurs in plants treating domestic sewage and in chemostats with pure cultures for estimating growth characteristics, was adversely affected by frequent clogging of the filter element. Table 4.3 shows that the concentrations of the different components of the sludge-free liquid were low for the whole range of loadings applied to sludge I. Although the concentrations in the liquids of non-recycled sludge II were higher, the average performance of carbohydrate conversion (99.7 and 98.7% at SL = 0.121 and 2.12, respectively) was slightly lower than that of recycled sludge only at SL = 2.12. Sludge-free liquids of low-loaded open systems II ($D = 0.1$ or 0.05 day^{-1}, influent concentration 10 or 15 g starch COD/l, respectively) always contained substances interfering with the anthrone method. For this reason, total carbohydrates were estimated with the phenol-sulphuric acid method.

Little is known about the nature of the phenol-sulphuric-acid-positive and anthrone-positive material detectable in liquids of continuously operated starch sludges. Treatment with iodine reagent revealed that the content of iodine-stainable dextrins was very low, if detectable at all. The very small (blank-corrected) extinctions (found at 623 nm) may have been due to interfering substances, particularly when $HClO_4$ had been added to the sludge after sampling. Calculated estimates of content of higher dextrins are given in 6.5. The low level of iodine-stainable dextrins indicates that the amylolytic activity was not limiting the process of purification at continuous operation. This is affirmed by the observation that during about 200 days of loading (SL = 0.3) the average concentration of anthrone-positive material in the liquid of maltose and glucose sludge (viz. 4.7 mg glucose equivalents per litre; $n = 9$) was not clearly different from that in starch sludge liquid (5.2 mg/l; $n = 11$).

Estimation of reducing sugars with the Somogyi-Nelson method failed due to the interference of sludge-free liquids with this method.

The anthrone-positive material presumably consisted only for a minor part of residual non-converted carbohydrates of the substrate as can be concluded from the following experiment. When starch activated sludge (I, SL = 0.3) and non-recycled low-loaded starch sludge (II) were starved and aerated for several days, no clear decrease of the anthrone-positive material was found. Additional evidence concerning the above-mentioned hypothesis came from the experiment with glucose sludge whose liquid contained about 5 mg anthrone-positive material per litre. Such a value would be hardly compatible with the estimated saturation constant of this sludge for glucose (about 1–3 mg/l) accompanied with the high rate of uptake of glucose (6.4). Calculated estimates of the content of lower dextrins are reported in 6.5.

The COD and total organic carbon estimates of the liquid of sludge I were much higher than the corresponding values of total carbohydrate. This suggests that non-carbohydrate organic matter of the sludge I liquid accounted for the majority of components which apparently were produced by the sludge, like the majority of anthrone-positive compounds. All these compounds were likely metabolic or cell-leakage products which were not directly derived from the substrate (e.g. soluble slimy components of normally firmly cell-bound slime). The accumulation of inert or slowly-degradable substances, amounting to several per cents of the COD of the influent, is in fact limiting purification.

4.6. AMYLOLYTIC ACTIVITIES AND AMYLOLYTIC BACTERIA IN CONTINUOUSLY OPERATED STARCH SLUDGES

In the beginning of this study it was supposed that at continuous operation of starch activated sludge at a distinct sludge loading, an equilibrium would be attained with respect to such parameters as amylolytic activity and number of total (viable) and (viable) amylolytic bacteria in the sludge. Investigation of bacteria and enzyme activities at different loadings would give information about the proportion of the bacterial population by which starch is degraded and consumed and about the relation between amylolytic activity of the sludge and number of amylolytic bacteria. To decide whether steady-state conditions occur in starch activated sludge, different criteria may be used, viz. constancy as to starch removal, DA_{spec}, numbers of total (viable) and amylolytic bacteria and composition of the bacterial population. With regard to the first criterion steady state is indeed approached (about 99% conversion of carbohydrates) within some days of loading with starch (4.4 and 4.5); with respect to the last-mentioned criterion no indication was obtained that the composition of the bacterial population becomes stable under the conditions described in 2.4.2 (Chapter 8). Whether steady state is attained with respect to the two other criteria mentioned, is discussed below (4.6.1 and 4.6.2).

4.6.1. *Amylolytic activities of starch sludges I and II*

Sludges were grown for periods up to over one year under the conditions described in 2.4.2. The carbon compound was starch which was added at different sludge loadings (Table 2.3) to sludge with retention of flocculated biomass (I) (Fig. 4.6A). The results of a similar experiment with low-loaded sludge without retention of biomass (II) are shown in Fig. 4.6B. The highest planned SL of sludge I was 32 times as high as the lowest SL employed (extended aeration as occurs in oxidation ditches). As a consequence the entire range of operations practised for treatment of domestic sewage, was covered. In fact, the real SL values varied to some extent during the experiments (2.4.2).

From the results of the experiment with differently loaded starch activated sludges (I; Fig. 4.6A) it can be seen that DA$_{spec}$ showed a pronounced rise with SL increased from 0.075 until 2.4. The fluctuations of the single DA$_{spec}$ values were extremely large at high loadings (SL = 2.4 and 1.2). At SL = 0.24 the fluctuation was moderate and at SL = 0.075 it was small. The quotients of maximum and minimum DA$_{spec}$ values for the 4 loadings (2.4, 1.2, 0.24 and 0.075) were calculated to be as follows: 53, 30, 4.8 and 2.7, respectively. It is

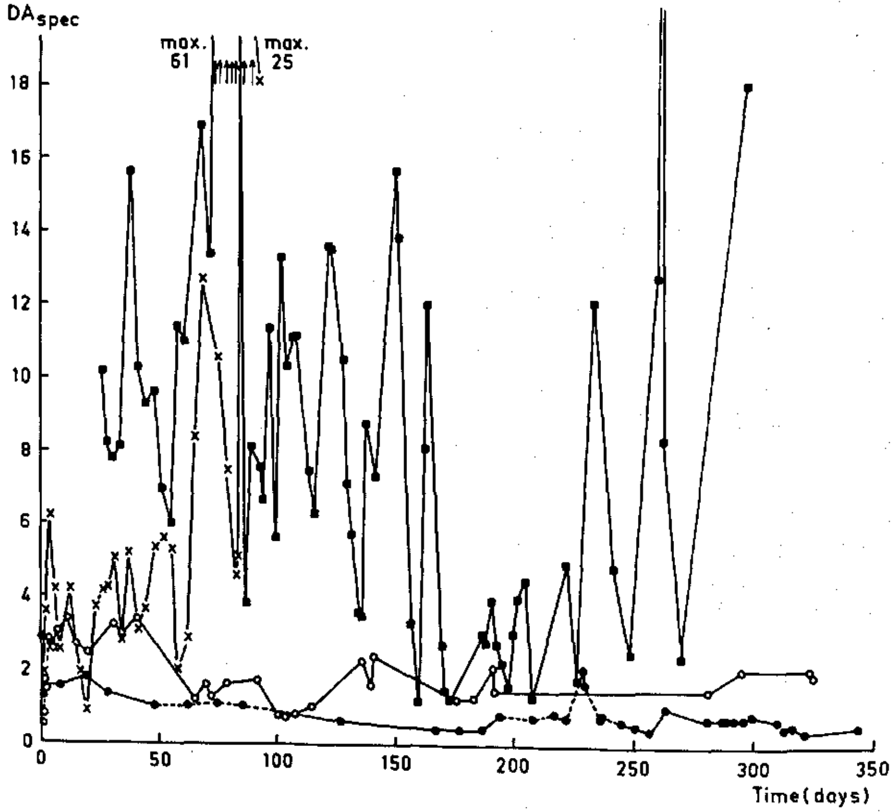

FIG. 4.6A. DA$_{spec}$ values during continuous loading with starch of sludge (with retention, I) at pH 7.0 and 20°C. ●, O, x and ■: SL = 0.075, 0.24, 1.2 and 2.4 g COD per g of sludge per day, respectively (preloading of the sludges had been as follows: ● and x, starch, SL = 0.3, during 36 days; ■, starch, SL = 1.2, 94 days; O, maltose, SL = 0.3, 84 days). The broken parts of the line (SL = 0.075) indicate that the dry wt of the sludge was less than half the planned 2.5 g/l. The DA values estimated during these periods and during the first 125 days of loading were not used for calculations, as were the DA values observed during the first 3 days of SL = 0.24.

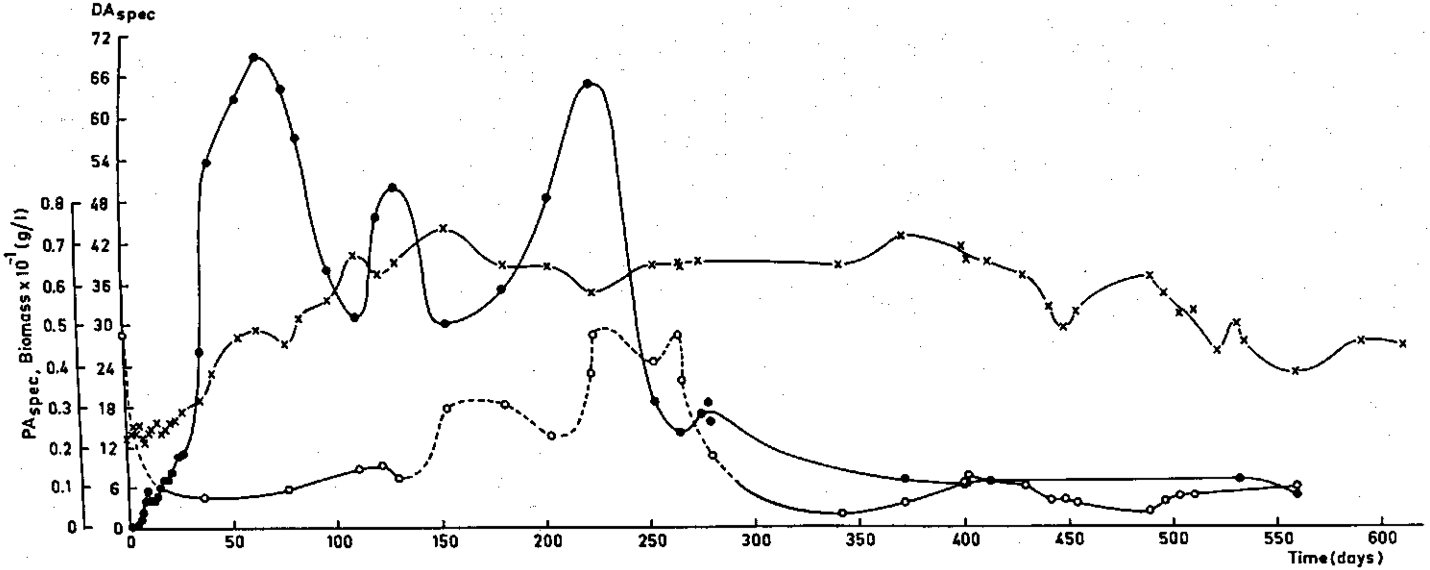

FIG. 4.6B. DA$_{spec}$, PA$_{spec}$ and biomass concentration during continuous loading of sludge (without retention, II) with 0.75 g of starch COD per litre per day at pH 7.0 and room temperature (D = 0.05 day^{-1}). The sludge had been preloaded as sludge I during 290 days with 0.3 g glucose COD per g sludge per day. The broken line (150–280 days) indicates a period of enhanced proteolytic activity, likely due to the presence of algae; darkening of the reactor eliminated this effect. These high values were not used for calculations. ●, DA$_{spec}$; ○, PA$_{spec}$; x, biomass.

striking that the pronounced fluctuations of DA_{spec} at high sludge loadings were due to extremely high maximum values; the minimum DA_{spec} values at these high loadings deviated only slightly from the minimum values at low loadings (see also Fig. 4.7). Although variation in biomass is expected to affect DA_{spec}, it is not the cause of the large fluctuations of this activity. This is concluded from the relatively small deviations of the concentration of biomass from the planned 2.5 g/l and from the fact that variation of biomass was smallest at high SL when the fluctuations of DA_{spec} were largest.

During the first 62 days of loading at SL = 1.2, DA_{spec} fluctuated around an average value of about 3.5 to 4. The high values of 18–25 after 85–93 days coincided with an outburst of branched *Nocardia*-like bacteria growing in suspension. These bacteria were found to be also responsible for the extremely high DA_{spec} values in low-loaded starch sludge II (see Fig. 4.6B and Plate 4.10).

Starch sludge I at SL = 2.4 was even more unstable and unpredictable than sludge at SL = 1.2 with respect to DA_{spec} observed over long periods (compare e.g. the values up to 60 between 76 and 83 days of loading with those of 1.2 to 5 between 170 and 227 days). This extreme variability of DA_{spec} may be attributed to rapid shifts in the population composition of this dynamic system, associated with rapidly changing numbers of amylolytic bacteria and amounts of amylolytic enzymes per amylolytic cell. The competition between cells in suspension and those in flocs, resulting in a varying ratio of biomass in suspension-biomass in flocs (Table 7.5), may have contributed to these rapid shifts. The relatively small differences between the mean retention times of suspended and more slowly growing bacteria within flocs, viz. about 8 h (= D^{-1}) and about 1–2 days, respectively, may allow the suspended bacteria to remain in the reactor and to compete to some extent with the bacteria within the flocs (Table 7.5). Such small differences in retention time were also maintained at SL = 1.2.

The results of Fig. 4.6A clearly show that DA_{spec} increases with SL. Although it is not recommendable to calculate mean DA_{spec} values at the high SL values in view of the widely dispersed activities and the unpredictable fluctuations, these

TABLE 4.4. Averages of DA_{spec} values of starch sludges I (Fig. 4.6A).

SL (g COD per g sludge per day)	0.075–0.10	0.24	1.2	2.4
Number of observations	20	29	31	69
Arithmetical average:				
DA_{spec}	0.661(0.191)[1]	1.98(0.82)[1]	6.28(5.72)[1]	10.03(10.41)[1]
Logarithmic average:				
$D\tilde{A}_{spec}$	0.635	1.80	4.72	7.02
Median DA_{spec}	0.657	1.95	4.25	8.11

[1] Sample standard deviation of the measured DA_{spec} values.

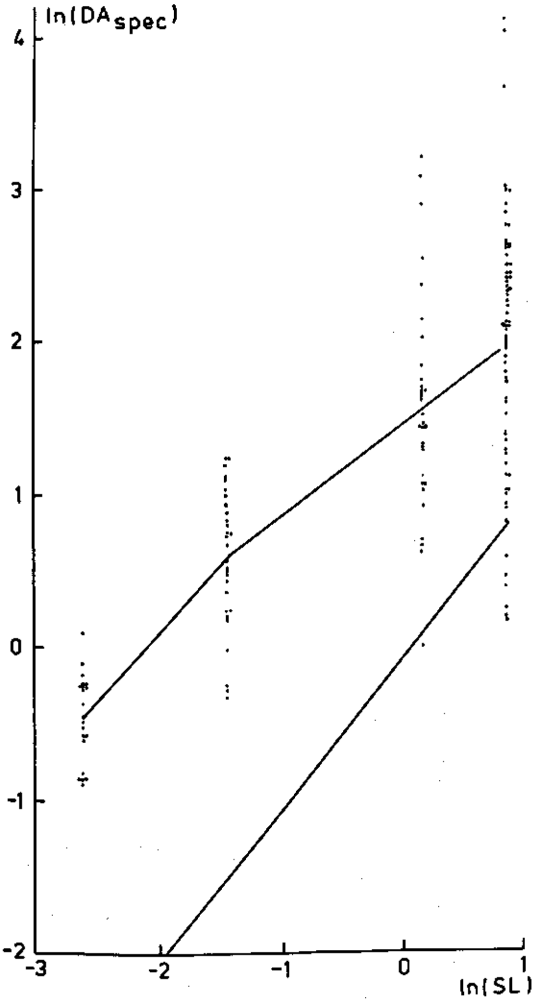

FIG. 4.7. Relation between the logarithm of SL (g starch COD per g of sludge I per day) and the logarithm of DA$_{spec}$ values (data of Fig. 4.6A). The curve represents the logarithmic mean of the DA$_{spec}$ values. The straight line represents the critical ln (DA$_{spec}$) values, valid for a biomass concentration of 2.5 g/l; below these activities inadequate starch degradation and consequently inadequate purification can be expected (see 6.5).

calculations were made because the mean values were needed for other purposes. Due to the occurrence of extremely high DA$_{spec}$ values at SL = 1.2 and 2.4, the arithmetically averaged DA$_{spec}$ values are misleading at these loadings. Table 4.4 shows that the sample standard deviation is approximately equal to the $\overline{DA}_{spec}$ at these high loadings and that $\overline{DA}_{spec}$ differs considerably from the median DA$_{spec}$. (The median is an alternative to the mean as a measure of central tendency; it is that value for which 50% of the observations, when arranged in order of magnitude, lie on each side or with other words: it is the middle value of a random test. It is hardly or not influenced by an exceptional value and is therefore useful in the case of skewed distributions of relative frequencies).

When the relative frequencies of DA$_{spec}$ at SL = 1.2 and 2.4 are plotted over categories of ln (DA$_{spec}$) instead of over categories of DA$_{spec}$, the distribution

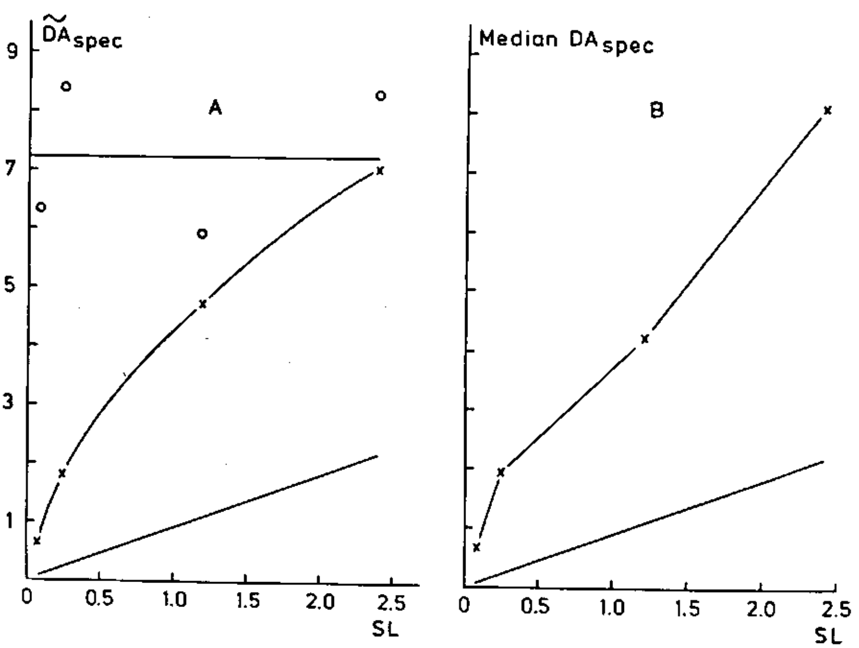

FIG. 4.8. Relation between loading of activated sludge with starch and logarithmic mean of the measured specific dextrinizing activities (Fig. 4.8A) or the median DA_{spec} (Fig. 4.8B) (symbol x). Data were obtained from Fig. 4.6A. The lower straight line represents the critical DA_{spec} values, valid for a biomass concentration of 2.5 g/l (see 6.5). The upper straight line (symbol O) represents imaginary $\widetilde{DA}_{spec}$ values, if $k_i = 0$ and $a^* = 0$ (see 5.5).

resembles more that of Gauss. Some symmetry of the ln (DA_{spec}) values around the logarithmic mean of the DA_{spec} values is observed (Fig. 4.7) and this logarithmic mean ($\widetilde{DA}_{spec}$) approaches the median DA_{spec} better than $\overline{DA}_{spec}$ does (Table 4.4 and Fig. 4.8). Even on this logarithmic scale the sample standard deviation increases to a large extent with SL (Fig. 4.7). For further calculations the logarithmic mean was used, as it was done with bacterial counts (see e.g. 2.5.1 and Table 4.5).

The development of DA_{spec} in low-loaded sludge without retention of flocculated biomass (II) was different from that in sludge I (Fig. 4.6B). Although the ratio of the estimated extremely high maximum and minimum DA_{spec} values was greater than that of equally loaded sludge I and although DA_{spec} did not become constant, at least during the first year, it can be seen that the course of DA_{spec} was much smoother. Only 3–5 peaks but no sudden changes were observed during a period of 560 days; the system was less dynamic than activated sludge. Several weeks after starting starch loading a branched *Nocardia*-like bacterium predominated the whole population and this continued during 500 days. The initial increase of DA_{spec} was accompanied with an increase of biomass, due to the growth of the *Nocardia*-like bacterium until an equilibrium was reached after 110 days (5.5 times the retention time of the sludge). The organism lost its predominant position after 500 days; biomass decreased simultaneously. The bacterium, surrounded by a heavy and fixed slime layer, grew, also in batch culture, only in the suspended form and during this experiment it was usually the only organism occurring outside the flocs (see

TABLE 4.5. Amylolytic activities and amylolytic and total (viable) bacteria (estimated in the same samples) of starch sludges with retention of flocculated biomass (I) (Fig. 4.6A).

SL	Days of loading	DA_{spec}	Total (viable) bact. per 10^{-7} mg	Amylolytic bact. per 10^{-7} mg	Glucose-util. bact. per 10^{-7} mg	Dextr. act. per amylol. cell[1]
0.075	121		7.77	4.24(55)	5.27(68)	
	124	0.620	2.19	1.91(87)	2.05(94)	325
	132		2.46	1.89(77)	2.07(84)	
	135		5.02	1.86(37)	2.30(46)	
	138		3.15	1.59(51)	1.85(59)	
	142		5.64	1.57(28)	1.69(30)	
	287	0.783	9.25	5.45(59)	5.45(59)	144
	289	0.792	5.53	5.53(100)	5.53(100)	143
	296	0.794	8.47	8.47(100)	8.47(100)	94
	310	0.782	12.09	9.20(76)	10.67(88)	85
	313	0.546	4.61	2.96(64)	2.96(64)	185
	316	0.597	4.38	1.61(37)	2.32(53)	371
	321	0.404	3.80	1.70(45)	2.23(59)	238
	$n = 13$		$\overline{\ln d} = 1.625$ $s = 0.512$ $\tilde{d} = 5.1$	$\overline{\ln d} = 1.086$ $s = 0.668$ $\tilde{d} = 3.0(58)$	$\overline{\ln d} = 1.209$ $s = 0.622$ $\tilde{d} = 3.4(66)$	$\overline{\ln \text{rat.}} = 5.163$ $s = 0.539$ rãt. = 175
0.3^2	12	5.90	200	129 (64)		46
	40	3.30	32.7	25.0 (77)		132
	4	2.84	36.7	36.7 (100)		77
	4	2.84	60.5	60.5 (100)		47
	9	3.40	58.6	44.8 (76)		76
	9	3.40	169	169 (100)		20
	20	2.43	12.0	12.0 (100)		203
	34	2.98	27.3	27.3 (100)		109
	78	1.67	21.5	17.1 (79)		98
	78	1.67	20.4	20.4 (100)		82
	11		55.4	55.4 (100)		
	(11)		(54.4)		54.4 (100)	
	17		42.5	42.5 (100)		
	27		39.7	39.7 (100)		
	(27)		(12.5)		12.5 (100)	
	202	2.07	27.2	22.0 (81)		94
	$n = 14$		$\overline{\ln d} = 3.737$ $s = 0.769$ $\tilde{d} = 42.0$	$\overline{\ln d} = 3.635$ $s = 0.737$ $\tilde{d} = 37.9\ (90)$		$\overline{\ln \text{rat.}} = 4.343$ $s = 0.614$ rãt. = 77

TABLE 4.5. Continued.

SL	Days of loading	DA_{spec}	Total (viable) bact. per 10^{-7} mg	Amylolytic bact. per 10^{-7} mg	Glucose-util. bact. per 10^{-7} mg	Dextr. act. per amylol. cell[1]
1.2	34	2.78	60.2	35.5 (59)	60.2 (100)	78
	37	5.20	53.1	36.3 (68)	46.9 (88)	143
	41	3.03	218	218 (100)	218 (100)	14
	44	3.63	79.9	33.7 (42)	79.9 (100)	108
	48	5.33	154	71.3 (46)	82.2 (53)	75
	$n = 5$		$\overline{\ln d} = 4.575$ $s = 0.612$ $\tilde{d} = 97.0$	$\overline{\ln d} = 4.066$ $s = 0.799$ $\tilde{d} = 58.3$ (60)	$\overline{\ln d} = 4.424$ $s = 0.584$ $\tilde{d} = 83.4$ (86)	$\overline{\ln \text{rat.}} = 4.190$ $s = 0.910$ $\widetilde{\text{rat.}} = 66$
2.4	27	9.20	113	99.6 (88)	99.6 (88)	92
	30	7.75	284	223 (79)	284 (100)	35
	38	15.67	237	198 (84)	217 (92)	79
	41	10.25	73.1	73.1 (100)	73.1 (100)	140
	44	9.24	195	195 (100)	195 (100)	48
	48	9.63	247	202 (82)	247 (100)	48
	52	6.95	311	154 (50)	311 (100)	45
	115	6.86	162	162 (100)	162 (100)	42
	122	13.61	80.6	80.6 (100)	80.6 (100)	169
	193	2.76	59.9	54.5 (91)	54.4 (91)	51
	195	2.22	88.3	49.1 (56)	68.9 (78)	45
	222	4.98	92.4	92.4 (100)	92.4 (100)	54
	227	1.78	146	73.4 (50)	82.3 (56)	24
	$n = 13$		$\overline{\ln d} = 4.940$ $s = 0.559$ $\tilde{d} = 140$	$\overline{\ln d} = 4.723$ $s = 0.533$ $\tilde{d} = 113$ (81)	$\overline{\ln d} = 4.853$ $s = 0.604$ $\tilde{d} = 128$ (92)	$\overline{\ln \text{rat.}} = 4.055$ $s = 0.546$ $\widetilde{\text{rat.}} = 58$

MPN-based counts were made in combined-substrate tubes with starch-yeast extract medium A_3 (10 tubes per dilution). Glucose was added to tubes with slight growth after about 15 days of growth (4.2). Numbers of bacteria were computed at sludge dry wt. Figures in parentheses denote amylolytic bacteria as % of total numbers of bacteria. n = number of estimates; d = MPN-based estimate of density of bacteria; s = sample standard deviation (of ln); rat. = ratio; $\overline{}$: superscript denoting arithmetical mean; $\tilde{}$: superscript denoting logarithmic mean.

[1] 10^{-10} mg SE/amylolytic cell.h; [2] the results are derived from different experiments. Part of the counts was made with 5 tubes per dilution, starch-yeast extract medium A_2 and tryptone-starch-yeast extract medium A_2.

Plates 4.9 and 4.10). Within the flocs several other bacteria were observed, but they formed a small minority compared to the suspended organism. The whole sludge looked like a very slimy homogeneous liquid (almost) without visible flocs. After 550–600 days of continuous loading, visible flocs reappeared and settled after prolonged periods.

In all experiments in which activated sludge was submitted (from $t = 0$) to starch loading without retention of flocculated biomass (VL = 0.75 or

1.00 g starch COD/l.day; D = 0.05 or 0.10 day^{-1}, respectively) sludge II tended to develop a monoculture of starch-degrading bacteria occurring in suspended form. However, different monocultures developed in different experiments and even in the same experiment after different periods of time. In parallel systems (SL = 0.3) with retention of biomass (I) no monocultures of suspended starch-degrading bacteria developed; the majority of the biomass was present in flocs.

4.6.2. *Amylolytic and non-amylolytic bacteria and their relation to amylolytic activities in various activated sludges*

The data of Table 4.4 (see also Fig. 4.8) have shown that DA_{spec} of sludge I increased with SL. The question arises whether this correlation was due to increasing numbers of amylolytic bacteria, to promoted induction or stimulation of the synthesis of enzymes or to other factors. Another question to be answered is to which extent in continuously operated (starch) activated sludges the degradation products of starch are utilized by amylolytic bacteria. As to SL = 0.3, some data on percentages of amylolytic bacteria have been given in 4.4.

To obtain more detailed results, numbers of amylolytic and total viable bacteria and DA_{spec} values were estimated in activated sludges grown with starch ammonium medium at different SL values (experiments shown in Fig. 4.6A; for SL = 0.3 other experiments are included). After attaining apparent steady-state conditions (which for DA_{spec} at SL = 0.075 were attained after a prolonged period), estimates were made at irregular intervals.

As shown in Table 4.5, numbers of viable bacteria sharply increased with SL. At an SL value of 0.075 on the average only about 5×10^7 viable bacteria were present per mg dry activated sludge; at SL = 2.4 this number exceeded 10^9 which is supposed to approach the maximum number possible (5.3).

The average proportion of amylolytic bacteria was found to be about 80 to 90% of the viable count over the whole range from moderate to even very high loadings. The percentage of 60 at SL = 1.2 was less accurate, because of the small numbers of observations at this SL value. At SL = 0.075 the percentage of amylolytic bacteria was clearly lower, viz. about 60. At all these loadings at most a small percentage of bacteria was attributed to non-amylolytic, glucose-utilizing organisms which were expected to utilize degradation products of starch in situ. However, most of the bacteria utilized starch only by the action of their own amylolytic enzymes; they apparently did not excrete degradation products of starch which might have been utilized by non-amylolytic organisms. This could have happened under conditions of incomplete starch removal (4.4).

In spite of the difficulties met with in counting, especially at very low loadings (4.2), it appears to be allowable to state that on the average only about 2/3 of the viable bacteria present in starch activated sludge at SL = 0.075 were able to grow on glucose (or starch) in the presence of some yeast extract etc. in the counting medium A_3. This sludge consisted largely of dead material; a (planned) sludge concentration of 2.5 g per litre could not be maintained (2.4.2). The remaining 1/3 of the 'living' bacteria apparently could not grow in situ on the

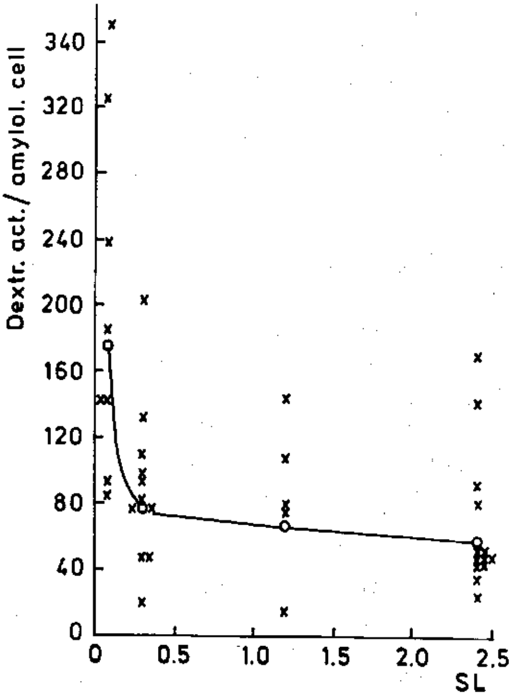

FIG. 4.9. Relation between amylolytic activity per (viable) amylolytic bacterium (Table 4.5) and loading of activated sludge with starch. x, Estimates of dextrinizing activity per (viable) amylolytic bacterium (10^{-10} mg SE/amylolytic cell.h); o, logarithmic averages of these estimates.

reactor influent, but required a different, presumably more complex substrate. These bacteria probably grew in activated sludge on decaying cell material and were unable to utilize glucose or starch ('entirely cryptic growth'; POSTGATE and HUNTER, 1962).

The last column of Table 4.5 (see also Fig. 4.9) contains the dextrinizing activity per amylolytic cell (10^{-10} mg SE/amylolytic cell.h) calculated as ratio of DA_{spec} to numbers of amylolytic bacteria per mg of sludge. In spite of the apparently large differences between the ratios at a distinct SL, the average enzyme level per viable amylolytic cell did not show clear differences over the range SL = 0.3 to 2.4. At SL = 0.075, however, the average dextrinizing activity calculated per viable amylolytic cell was roughly 3 times higher than that at SL = 2.4. This increased activity was likely not the result of an increased derepression, since such a mechanism, if operative at all in (partially viable) activated sludge, would function to the same extent at higher loadings at which the concentration of carbohydrate in the sludge liquid was also low. The increased dextrinizing activity per viable amylolytic cell at low SL values was thought to be due to the amylolytic activity associated with non-viable cells and cell debris. The contribution of this activity was particularly clear when the number of viable cells was very low, i.e. at extremely low SL.

Whether the sharp fluctuations of DA_{spec} of activated sludge at continuous operation (Fig. 4.6A) have to be attributed to variations of number of amylolytic bacteria and/or variations of amount of enzyme present per viable amylolytic cell is difficult to decide even at very high loadings (Fig. 4.10). This is due to the poor repeatability of the MPN-based counts and thus also of the enzyme levels per cell. Since no exclusive correlation was found as to one of the two possible relations, it is suggested that both variables affect DA_{spec} (Fig. 4.10A and 4.10B).

An estimation of the amylolytic enzyme activity per viable amylolytic cell in different sludges I grown without starch is given in Table 4.6. The percentage of amylolytic bacteria in maltose (and glucose) sludge (SL = 0.3; pH 7.0) usually amounted to 4–10%, in Zeist sludge 10–13% (estimated twice, in 1973 and 1975). The values of amylolytic activity per amylolytic cell in the laboratory-grown sludges were about the same as those in starch sludges at SL values of 0.3 or higher and at pH 7.0. The Zeist sludge, however, had a relatively high amylolytic enzyme activity per amylolytic cell. No explanation of this deviating result can be given. As to laboratory-grown activated sludges loaded with different carbohydrates, it can be concluded that the differences in DA_{spec} depended on differences in number of amylolytic bacteria rather than on differences in amylolytic activity per amylolytic cell. This is a confirmation of the earlier statement that enzymic acclimatization of activated sludge proceeds by population shift and not by enzyme induction in an existing population (4.4).

4.6.3. *Estimation of amylolytic enzymes as % (wt/wt) of sludge biomass*

Attempts were made to estimate roughly from the data of 4.6.1 and 4.6.2 the percentage of sludge material and that of an amylolytic bacterium occurring as

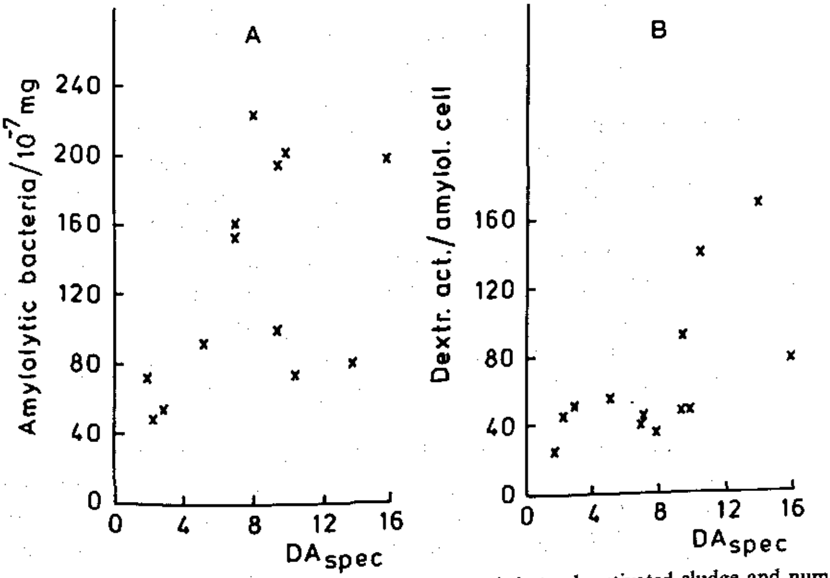

FIG. 4.10. Relation between DA_{spec} of very high-loaded starch activated sludge and number of amylolytic bacteria (A) or dextrinizing activity per (viable) amylolytic bacterium (10^{-10} mg SE/amylolytic cell.h) (B). Data were obtained from Table 4.5.

TABLE 4.6. Amylolytic activities and amylolytic and total (viable) bacteria (estimated in the same samples) of sludges with retention of flocculated biomass (I).

Activated sludge	Days of loading	DA_{spec}	Total (viable) bact. per 10^{-7} mg	Amylolytic bact. per 10^{-7} mg	Glucose-util. bact. per 10^{-7} mg	Dextr. act. per amylol. cell[1]
Maltose sludge	84	0.52	139[2]	5.64[2] (4.1)		92
(SL = 0.3)	84	0.52	159[3]	9.29[3] (5.8)		56
Glucose sludge	287	0.13	21.5[4]	1.35[4] (6.3)		96
(SL = 0.3)	287	0.13	17.0[5]		14.3[5] (84)	
Zeist sludge (17/10/'75)	–	1.11	13.0[4]	1.63[4] (12.6)		681

MPN-based counts were made in combined-substrate tubes (10 per dilution).

[1] 10^{-10} mg SE/amylolytic cell.h; [2] starch-yeast extract medium A_2; [3] tryptone-starch-yeast extract medium A_2; [4] starch-yeast extract medium A_3; [5] glucose-yeast extract medium A_3. Figures in parentheses indicate percentages of total numbers of bacteria.

(active) amylolytic enzymes. Data on the maximum specific enzyme activities (i.e. activity per mg of protein of amylase) are not available, since the sludge amylolytic enzymes are difficult to purify. They consist of an enzyme mixture produced by and bound to a very heterogeneous population. For this reason data on α-amylases were taken from literature.

BERNFELD (1951) reported saccharifying activities of 4000, 6400, 2350 and 3200 mg maltose (equivalents)/mg enzyme N.3 min, at 20°C and at optimum pH for 5 pure crystalline α-amylases of pig pancreas, human saliva, human pancreas, barley malt and *Bac. subtilis*, respectively. The specific activities of the α-amylase of *Ps. saccharophila* (MARKOVITZ et al., 1956) and of the maltotetraose-liberating exo-amylase of *Ps. stutzeri* (ROBYT and ACKERMAN, 1971) adjusted to the conditions of BERNFELD's experiments, are within this range. The average saccharifying activity of amylases of starch sludge is therefore assumed to be 4 g maltose/mg enzyme N. 3 min at 20°C and optimum pH, i.e. $4 \times 20 \times 2/6.25 \times 1.90 =$ about 13.5 g glucose/mg protein. h at 30°C.

The commercial preparation Fungamyl 1600 (containing 30% protein and having an SA of about 0.122 g glucose/mg preparation.h at pH 7.0 and 30°C) was calculated to have an SA of 1.07 g glucose/mg protein.h at 30°C and optimum pH, (4.9; data reported in Novo enzyme information, Sept. 1971). This suggests that only a small fraction of the protein represents active α-amylase.

Using the above-mentioned specific activity of SA and a DA/SA ratio of 10 for sludge amylases (Table 3.1), the logarithmically averaged DA_{spec} values of starch activated sludges loaded at SL = 0.075, 0.24, 1.2 and 2.4 (Table 4.4) correspond with 0.0047, 0.013, 0.035 and 0.052 mg amylase/g dry biomass, respectively. Assuming an average DA_{spec} of extremely high-loaded starch sludge of about 10, an average amount of 0.074 mg amylase/g biomass will be found. The lowest (0.404) and highest (61) DA_{spec} ever estimated in the present study in starch sludge I correspond with 0.0030 and 0.45°/₀₀ enzymes, respectively. All these percentages are in fact higher when the amounts of amylase are related to the viable amylolytic bacteria which produced the enzymes. If it is assumed that the average dextrinizing activity is about 55×10^{-10} mg SE per viable amylolytic bacterium per h (Fig. 4.9) and that 1 mg of dry biomass contains 1.4×10^9 bacteria (5.3), viable amylolytic bacteria will contain on the average 0.057°/₀₀ amylases (calculated on biomass). However, the 'total' amylolytic activity of sludges is higher than that estimated under standard conditions (i.e. without any pretreatment of the sludge). This was concluded from the observation that upon ultrasonic treatment the amylolytic activity rose to values maximally twice the activity of untreated samples.

Literature data relating amounts of amylolytic enzymes to total cell protein are scarce. KLEIN (1963) reported that as to cells (of *Pseudomonas saccharopila*), actively growing on starch, α-amylase represents about 0.03% of the total protein synthesized (experiments of at most 3 h). As to cells after an overnight incubation in a complete medium, the percentage was about 0.1%, calculated from the incorporation of C^{14}-L-aspartic acid into amylase and cellular proteins. NINOMIYA et al. (1976) reported that *Bacillus amyloliquefaciens*, strain KA 63, excreted large amounts of α-amylase into the medium, viz. 6% (personal communication 1977).

These data suggest that the 'total' amount of amylase, associated with an amylolytic bacterium in continuously operated starch activated sludge, approaches that excreted by *Pseudomonas saccharophila* into a starch medium.

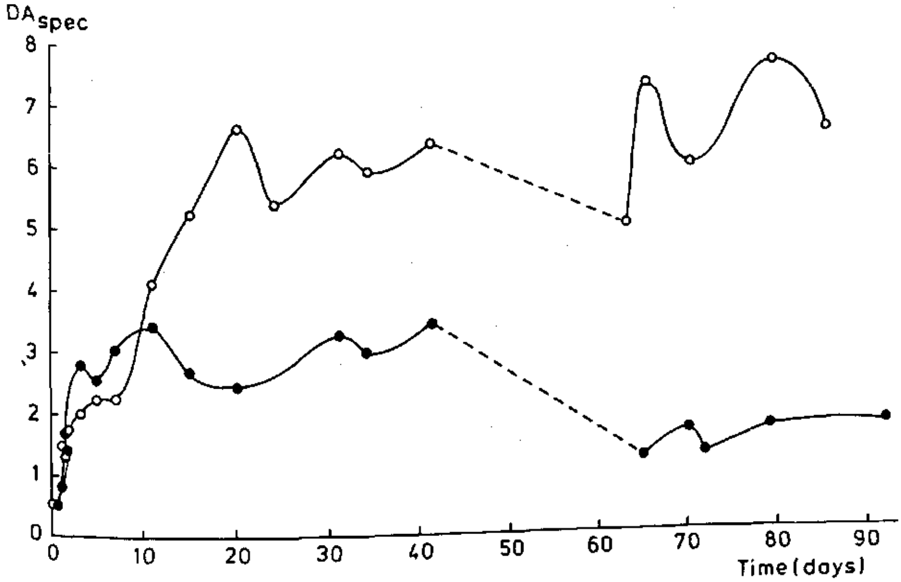

FIG. 4.11. Effect of pH on the development of standard DA_{spec} (pH 7.0) in starch activated sludge (average SL about 0.25; 20°C). The sludge had been loaded with starch during 41 days and subsequently with maltose during 84 days whereupon starch loading was resumed (at $t = 0$). Broken lines denote absence of determinations. ●, pH 7.0; ○, pH 8.8.

4.7. EFFECT OF pH AND TEMPERATURE ON THE DEVELOPMENT OF DA_{spec} AND PA_{spec} IN ACTIVATED SLUDGE

From the data of Tables 4.5 and 4.6 it can be seen that the average amylolytic activity per viable amylolytic cell is roughly equal in various laboratory-grown activated sludges with SL values of 0.3 or higher. As all these sludges were grown at pH 7.0 and at 20°C, the effect of different pH values and temperatures on DA_{spec} was studied in two separate experiments. In one of these experiments the effect of temperature variation upon PA_{spec} was included.

To study the effect of pH on the development of DA_{spec} in activated sludge, an experiment was carried out with 2 reactors containing activated sludge loaded with starch at 20°C and submitted to pH 7.0 and 8.8, respectively. The original sludge had been loaded with maltose during about 3 months and it was distributed over the 2 reactors when starch was supplied (at $t = 0$). Fig. 4.11 shows that after the usual initial increase of DA_{spec} two clearly different levels of DA_{spec} were attained in the 2 reactors. The fluctuating values of DA_{spec} may be ascribed, at least partly, to the irregular loading due to discarding about half of the sludge every 10–14 days. The logarithmically averaged DA_{spec} of the sludges with pH 7.0 and 8.8 was 2.3 ($n = 14$) and 6.2 ($n = 11$), respectively, when

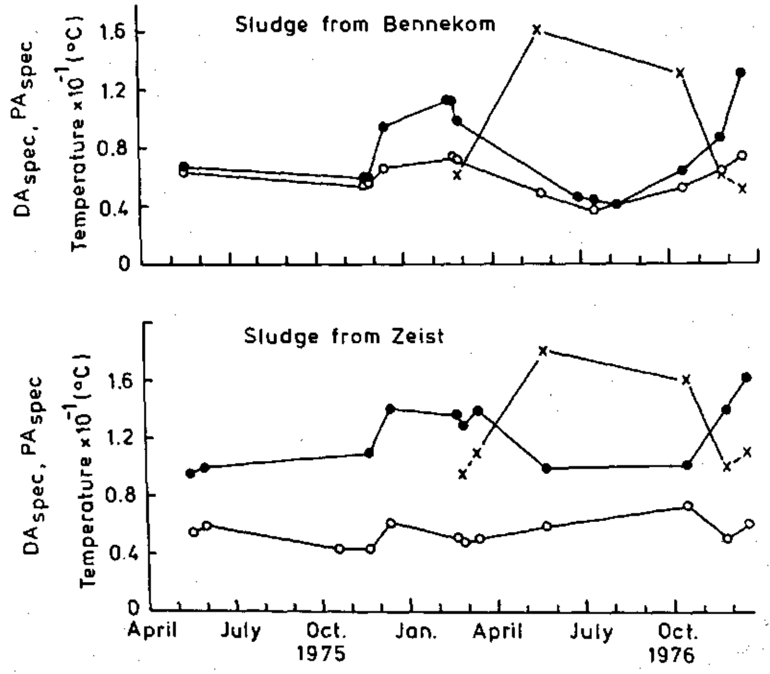

FIG. 4.12. Effect of season (temperature) on the development of dextrinizing and proteolytic activities of activated sludge in 2 outdoor plants; Zeist sludge: average dry wt ± sample standard deviation during 1974–1976: 2.44 ± 0.65 g/l, $n = 14$; Bennekom sludge: 3.66 ± 0.95 g/l, $n = 15$. ●, Standard DA_{spec} (30°C); ○, standard PA_{spec} (30°C); x, temperature (°C) of activated sludge.

measured under standard conditions (at pH 7.0). When the latter sludge was measured under the conditions of the experiment (pH 8.8), DA_{spec} was lower (cf. pH optimum curve in Fig. 3.13C) but it was still considerably higher than DA_{spec} (pH 7.0) of sludge grown at pH 7.0. The reduction of DA_{spec} at pH 8.8 as compared to DA_{spec} at optimum pH 7.0 was apparently compensated (in fact overcompensated) in the sludge grown at pH 8.8 by the production of additional amounts of amylolytic enzymes. It is improbable that this was brought about only by the presence of higher numbers of bacteria, so that it must be assumed that at a high pH the amount of amylolytic enzymes per viable amylolytic cell was higher.

That also in outdoor plants growth conditions may affect enzyme activities of activated sludge was shown for standard DA_{spec} and PA_{spec} (measured at 30 °C, Fig. 4.12). The seasonal effect noted has undoubtedly been due to temperature differences. The changes of enzymic activity, which was high in winter, low in summer, were most pronounced in DA_{spec} of Bennekom sludge, less pronounced in PA_{spec} of this sludge and in DA_{spec} of Zeist sludge, but absent in PA_{spec} of Zeist sludge. The pronounced seasonal fluctuations of enzymic activities in Bennekom sludge may be explained by the greater fluctuations of temperature in this sludge, due to the long retention time of the sewage in the oxidation ditch (about 2–3 days). During mild winters the temperature of the sludge falls to about 5 °C, during warm summers (as 1976) temperatures of at least 20 °C were reached. However, the temperature of Zeist sludge does not decrease much below 10 °C during mild winters.

The Q_{10} of amylolytic enzymes in activated sludge is about 2 (or slightly higher at temperatures between 0–10 °C, 3.5.2). Furthermore, sludge loading during winter probably does not differ from that during summer. It is therefore suggested that the reduced activity of amylolytic enzymes under the low-temperature conditions as prevailing in the outdoor sludge during winter, is compensated by the production of more enzymes. In the Bennekom sludge where the difference between winter and summer temperature was over 10 °C, DA_{spec} measured under standard conditions was more than twice as high in winter, compared to that in summer. In the Zeist plant with a difference in temperature less than 10 °C, DA_{spec} in winter was less than twice as high as in summer.

Although the Q_{10} of proteolytic enzymes in activated sludge was also 2, the reduction of activity during winter was not or only partly compensated by the production of additional amounts of enzymes. The synthesis of proteolytic enzymes in the activated sludge of outdoor plants probably depends to an important part on the presence of proteins derived from dead cell material (turnover of cell components). At low temperature the death rate of bacteria decreases, resulting in lower numbers of dead cells and consequently a decreased supply of bacterial protein. This might explain why the reduced activity of proteolytic enzymes in activated sludge under winter conditions was only partly compensated by the production of more enzyme in Bennekom sludge at low temperature and was not compensated at all in Zeist sludge at moderately low temperature.

4.8. SUMMARY

1. Colonies of several amylolytic bacteria isolated from activated sludge (SL = 0.3) showed no halo of amylolysis on starch agar medium. Total (viable), starch-, glucose- or casein-decomposing bacteria were counted with the dilution frequency method using liquid media and applying carefully defined growth criteria and cultural conditions resembling those in sludge.
2. Loading of sewage-fed Zeist activated sludge with starch resulted in a severalfold increase of numbers of amylolytic bacteria within 12 days. This was not observed when this sludge was loaded with maltose.
3. DA_{spec} of starch-grown sludge I was considerably higher than that of sludges fed with other substrates. In lactate-grown sludge DA was hardly detectable.
4. When the quotient SL/DA of starch activated sludge (SL = 0.3) was raised suddenly by a factor 4–5, e.g. by quantitative shock loading, the residual carbohydrate concentration in the sludge-free liquid remained approximately at the same low level as before the shock loading. It is concluded that the amylolytic activity of this sludge was far in excess of the activity required for adequate purification.
5. Laboratory-grown sludge I continuously loaded with starch and $(NH_4)_2SO_4$ (C/N = 5, pH 7.0) shows very poor settling characteristics due to the presence of cell-bound slime and usually of filamentous bacteria.
6. The response of maltose- or glucose-grown sludge I to a qualitative shock loading with starch (SL = 0.3) depended on the DA value of the preformed amylase system in the sludge. An adequate carbohydrate conversion, accompanied with an increase of biomass and DA, was found when the (initial) DA was higher than about 0.70; when it was lower than this (critical) value, inadequate carbohydrate removal by the sludge organisms was observed and the increase of biomass and DA was delayed. The accumulation of high-molecular dextrins in the sludge-free liquid indicated that the amylolytic activity was limiting the purification. In glucose sludge with a low DA this incomplete starch removal continued for one week in spite of the fact that starch is an easily degradable substrate.
7. The acclimatization of maltose- and glucose-grown activated sludges to starch was not due to an induction of enzyme synthesis in an existing population, but to a population shift. When the initial DA was higher than the critical DA, amylolytic bacteria increased from 6 % to about 100 % of the total viable number of bacteria within 4 days. When it was lower than the critical value, this increase was attained after 7–10 days. In the former case, i.e. under conditions of immediate complete starch removal, only amylolytic bacteria were able to grow, in the latter case also non-amylolytic bacteria were presumably able to grow (at the expense of accumulated dextrins). During the initial phase of the rapid acclimatization, generation times of amylolytic bacteria were calculated to be clearly lower (less than 20 h) than those of amylolytic bacteria under conditions of continuous operation (about 3 days).
8. Under apparently steady-state conditions the average amount of anthrone-

positive material in the liquid of starch-loaded sludges I and II fluctuated around 1 % of the substrate administered over the SL range 0.075–2.4. In addition to anthrone-positive substances greater amounts of other organic carbon compounds were present in the liquid of sludge I. Both, anthrone-negative substances and the major part of the anthrone-positive substances were probably produced by the sludge biomass and did not represent non-utilized substrate residues. The accumulation of resistant products is in fact the limiting factor in the purification process.

9. DA_{spec} of starch activated sludge, cultivated at pH 7.0, showed a pronounced rise with SL increasing from 0.075 to 2.4 g COD/g sludge.day. The fluctuations of the single DA_{spec} values were small at SL = 0.075 and extremely large and unpredictable at SL = 1.2 and 2.4.

10. DA_{spec} depended on the number of amylolytic bacteria, which increased sharply with SL, and on the apparent amylolytic activity calculated per amylolytic cell. The latter value, on the average, did not vary clearly with SL over the range 0.3 to 2.4, but at SL = 0.075 it was considerably higher than at SL = 2.4. The high activity at very low SL was thought to be due to amylolytic activity associated with dead cells and cell debris.

11. In continuously loaded starch activated sludge amylolytic bacteria accounted for about 80 to 90 % of the total viable cell number over the whole range from SL = 0.3 to even SL = 2.4. At SL = 0.075, amylolytic bacteria accounted for only about 60 % of the total viable count. Since at all loadings the percentages of glucose-consuming bacteria were only slightly higher than the percentages of amylolytic bacteria, it was concluded that (under conditions of complete starch removal) the starch-degrading bacteria hardly excreted degradation products of starch. At SL = 0.075 about 1/3 of the viable bacteria apparently utilized only substrates derived from decaying cell material (entirely cryptic growth).

12. The average amylolytic activity, calculated per (viable) amylolytic cell, was about equal in maltose and glucose activated sludge (containing 4–10 % amylolytic bacteria) and in starch sludge (80–90 % amylolytic bacteria) when submitted to equal conditions (SL = 0.3; 20°C, pH 7.0). In activated sludge from the outdoor plant at Zeist (10–13 % amylolytic bacteria) the amylolytic activity per cell seemed to be considerably higher than that of the sludge grown in the laboratory.

Cultivation of starch activated sludge at pH 8.8, a pH value adversely affecting amylolytic activity, resulted in the production of considerably greater amounts of amylolytic enzymes than required for compensation of the adverse pH effect (overcompensation). This higher activity was attributed to higher amylolytic activity per amylolytic cell. The adverse influence of winter conditions on amylolytic activity of activated sludge in outdoor plants resulted in the production of greater amounts of amylolytic enzymes, compensating for the low temperature effect. In the case of proteolytic activity of the sludge this compensation hardly occurred.

13. The amount of amylolytic enzymes in activated sludge (grown at pH 7.0) and estimated under standard conditions was assessed to be about 0.06 ‰ of the biomass of the viable amylolytic bacteria which produced the enzymes.

5. EXTRACELLULAR PROTEOLYTIC ENZYMES AND TURNOVER OF CELL PROTEIN, INCLUDING AMYLASES, IN SLUDGE

5.1. Introduction

5.1.1. *General introduction*

In section 4.7 the suggestion was made that proteolytic enzymes might play a role in the supposed turnover of nitrogenous cell constituents in activated sludge of outdoor plants. It was observed that a considerable proteolytic activity was present in laboratory-grown activated sludge that had been loaded continuously for 10 months or more with starch, maltose or glucose as the sole C source (SL = 0.3) and $(NH_4)_2SO_4$ as the sole N source (C/N = 5). As this substrate did not contain proteins and the cultivation of activated sludge with hardly detectable amylolytic activity was shown to be possible (4.3.2), the above-mentioned observation led to the hypothesis that degradation of cells and reutilization of released material might play an important part in the metabolism of activated sludge.

The study of turnover was largely limited to proteolysis, the degradative phase of protein turnover. Since we were interested in extracellular enzymes (cf. estimation of PA, 2.6.12) and since intracellular proteolysis presumably was slight compared to extracellular proteolysis (Chapter 7), we confined ourselves to proteolytic processes occurring outside living cells.

5.1.2. *Protein turnover*

Protein turnover can be defined as a dual process, viz. 1) degradation of protein to peptides and amino acids and 2) subsequent reincorporation of the degradation products into proteins. A description of protein turnover in a cell population should distinguish between three possibilities: a) intracellular turnover: degradation and resynthesis of protein occurs exclusively within the cell; b) intercellular turnover: excretion of low-molecular protein degradation products by living or lysed cells and utilization of these products for the protein synthesis of other cells which may or may not be of the same bacterial species; c) extracellular turnover: protein originating from living or dead cells is hydrolysed by extracellular proteolytic enzymes from other cells; the resulting degradation products are utilized for the growth of the latter (or other) cells which may or may not belong to the same bacterial species. A combination of these 3 processes may occur.

Impressive progress has been made in recent years in clarifying the regulation, selectivity and physiological significance of intracellular protein breakdown in bacterial and mammalian cells (PINE, 1972; GOLDBERG and DICE, 1974; GOLDBERG and ST. JOHN, 1976). LEVINE (1965) studied the intercellular turnover of protein between non-growing *E. coli* cells. This amounted to no more than

0.16–0.18 % per h and it was concluded that intracellular turnover accounts for almost all of the observed total protein turnover of 3 to 4 % per h in non-growing *E. coli* cells. The protein turnover experiments described in the above-mentioned literature were performed with pure cultures of growing or non-growing viable cells (usually non-proteolytic *E. coli*). In natural systems, with mixed cultures and dead cells, extracellular in addition to intracellular turnover may be an important process of the nitrogen cycle. In this respect it is remarked that, at least in some bacteria, cellular proteins under starvation conditions are only to a small extent degraded by intracellular proteolytic enzymes. Pine (1972)claimed that about 70 % of the proteins of *E. coli* are not subject to breakdown at all even during prolonged starvation as opposite to protein breakdown in mammalian tissues, where virtually all of the intracellular proteins are subject to continuous turnover (Goldberg and St. John, 1976). The apparent decrease of protein breakdown in *E. coli* during prolonged starvation (more than 6–8 h) may simply indicate that this process does not occur in dead cells (Goldberg and St. John, 1976). It is tempting to relate this apparent decrease of protein breakdown in (dead) cells to the known unexplained fact that intracellular protein degradation apparently requires metabolic energy. None of the known proteolytic enzymes of mammalian, bacterial or plant origin requires energy-rich cofactors.

5.1.3. *Effect of growth rate of continuously growing pure cultures and activated sludge on cell yield*

Generally the reduction of cell yield, with decreasing growth rate, on the substrate used as energy source is attributed to the need for maintenance energy of the micro-organism. Metabolic energy requirement is resolved in growth rate-dependent and growth rate-independent terms. The growth rate-independent terms, the maintenance functions, probably include turnover of macromolecules, osmotic work to maintain concentration gradients between the cell and its exterior, cell motility and cellular organization. Although there are two different conceptual models with correction factors for the growth rate-independent 'maintenance' functions, both can be expressed mathematically as identical linear functions of reciprocal yield versus mean cell residence time.

In the model of Herbert (1958) the maintenance energy requirement is represented as consumption of biomass through endogenous metabolism. By modification of the growth rate law, the biomass balance in differentiated form can be written as follows:

net growth rate = total growth rate – endogenous consumption rate of biomass

$$\left(\frac{\mathrm{d}X}{\mathrm{d}t}\right)_{\mathrm{net}} = \left(\frac{\mathrm{d}X}{\mathrm{d}t}\right)_{\mathrm{total}} - aX$$

$$\mu_{\mathrm{net}} = \mu_{\mathrm{tot}} - a = \frac{\mathrm{d}X_{\mathrm{net}}}{X.\mathrm{d}t} = \frac{1}{t_s} = D \text{ (equation of HERBERT)} \qquad (1)$$

where μ_{net} and μ_{tot} are the net (apparent) and total specific growth rates (day^{-1}), respectively, D = dilution rate (day^{-1}), a = specific endogenous consumption rate of biomass which may be regarded as a specific 'turnover' rate of biomass (g biomass utilized/g biomass.day = day^{-1}) and t_s = mean cell residence (or replacement) time in the reactor (or biological solids retention time, day).

In the model of PIRT (1965) the total energy requirement of growing cultures is represented in terms of substrate requirements for growth and for growth rate-independent processes (maintenance):

total rate of substrate consumption = rate of consumption for cell growth + rate of consumption for cellular maintenance

$$\left(\frac{dS}{dt}\right)_{total} = \left(\frac{dS}{dt}\right)_g + \left(\frac{dS}{dt}\right)_m$$

where g = growth and m = maintenance.
With the following substitutions

$$\left(\frac{dS}{dt}\right)_{tot} = -\frac{\mu X}{Y_{obs}};\ \left(\frac{dS}{dt}\right)_g = -\frac{\mu X}{Y_{max}} \text{ and } \left(\frac{dS}{dt}\right)_m = -mX,$$

the substrate balance can be written as:

$$-\left(\frac{dS}{dt}\right)_{tot} = \frac{\mu X}{Y_{obs}} = \frac{\mu X}{Y_{max}} + mX$$

and thus

$$-\frac{dS_{tot}}{X \cdot dt} = q = \frac{\mu}{Y_{obs}} = \frac{\mu}{Y_{max}} + m \qquad (2)$$

$$-\frac{dS_{tot}}{dX} = \frac{1}{Y_{obs}} = \frac{1}{Y_{max}} + \frac{m}{\mu} \text{ (equation of PIRT)} \qquad (3a)$$

Symbols: $Y_{obs} = dX_{net}/-dS_{tot} = dX_{net}/(-dS_g-dS_m)$ = observed growth yield coefficient (g biomass produced/g energy source consumed); $Y_{max} = dX_{net}/-dS_g$ = maximum ('true') growth yield coefficient, if the maintenance energy were zero; m = maintenance coefficient (g energy source consumed/g biomass. day or h; $q = -dS_{tot}/X.dt$ = specific rate of substrate uptake (g substrate transported into the cells/g biomass·day). In all these equations μ represents μ_{net} of equation (1). Upon introduction of the coefficient $a = m \cdot Y_{max}$ (SCHULZE and LIPE, 1964), the linear equation is:

$$\frac{1}{Y_{obs}} = \frac{1}{Y_{max}} + \frac{a}{Y_{max}} \cdot \frac{1}{\mu} \text{ (cf. MARR et al., 1963)} \qquad (3b)$$

In the latter equation (3b) a represents the consumption of the source of carbon and energy for purposes that are not a function of the rate of growth. The specific maintenance rate a = g biomass not synthesized due to utilization of substrate for maintenance per g biomass per day or h.

The model of MARR et al. differs from that of PIRT in the notation ($a = m \cdot Y_{max}$), but not in the concept used, whereas the model of MARR differs from that of HERBERT in the concept, but not in the notation used.

Both linear equations (3a and 3b) are the reciprocals of the orthogonal hyperbolae:

$$Y_{obs} = Y_{max} \frac{\mu}{\mu + m . Y_{max}} \text{ and} \tag{4a}$$

$$Y_{obs} = Y_{max} \frac{\mu}{\mu + a}, \text{ respectively,} \tag{4b}$$

The assumption that maintenance parameters which, as does Y_{max}, depend on the nature of the micro-organism, the substrate composition and the growth conditions, are independent of the growth rate is an approximation. PIRT (1965) and MARR et al. (1963) showed that the yield data for certain pure bacterial cultures with cell residence times of less than 1 day (with nearly 100% living bacteria) could approximately be described with the linear equations (3a) and (3b), respectively. However, some plots might be interpreted as curvilinear graphs but because of the small ratio of a to μ_{max} it is quantitatively of little importance whether a is assumed to be constant or to be a function of μ in the range mentioned.

The effect of either low viability or endogenous metabolism can be represented by the same linear yield formulae (3), under certain assumptions (e.g. non-viable organisms have the same mean size as viable organisms; the culture contains a fraction depending on μ, of living organisms measured by weight) (POWELL, 1967).

However, even if it is assumed that both the maintenance coefficient of viable organisms and the decay coefficient (equivalent of a) of non-viable cells are constant but different for the whole broad range of feasible cell residence times, the combined effect of these two constants likely will give a curvilinear graph of Y_{obs}^{-1} versus t_s. This conclusion is derived from a comparable effect of two or more different constants (viz. K_m values) on the reciprocal (L-B-plot) of an orthogonal hyperbola (saturation curve; 6.1) comparable to that of equation (3b) and from a model incorporating viability and different decay coefficients of viable and non-viable cells (GRADY and ROPER, 1974).

It should be realized that the equations (3) and (4) refer to populations and their 'maintenance'. The equations in themselves do not distinguish between resynthesis of proteins in existing cells and 'resynthesis' of completely new cells from the products of autolysis of dead cells.

It can be tried to use the equations for mixed cultures with retention of flocculated biomass. In this case the net specific growth rate is less than D and is equal to the reciprocal of the mean sludge residence time t_s (called sludge age): $\mu_{net} = \mathrm{d}X_{net}/X \cdot \mathrm{d}t = 1/t_s$. Therefore the equations (3a) and (3b) can be rewritten as:

$$Y_{obs}^{-1} = Y_{max}^{-1} + m \cdot t_s \text{ and} \tag{5a}$$

$$Y_{obs}^{-1} = Y_{max}^{-1} + a \cdot Y_{max}^{-1} \cdot t_s, \text{ respectively,} \tag{5b}$$

TABLE 5.1A. Micro-organism decay (turnover) coefficient (a^*) for aerobic mixed culture (activated sludge) systems.

Waste water composition	Experiment	Temperature (°C)	t_s (days)	Calculation on basis of	a^* (day^{-1})	Reference
Settled domestic waste	lab	19–22	normal range	VSS	0.055	Heukelekian et al. (1951)
Domestic waste				VSS	0.07	Benedek and Horvath (1967)
Domestic waste	lab	20–21	c. 7–20	VSS	0.048	Middlebrooks and Garland (1968)
Domestic waste	lab	20–21	c. 7–22	VSS	0.043	Middlebrooks and Garland (1968)
Domestic waste	field	different seasons	c. 19–66	VSS	c. 0.014	Middlebrooks and Garland (1968)
Skim milk				VSS	0.045	Gram (1956)
Peptone	lab	21–22	2–18	SS	0.093	Sherrard and Schroeder (1972)
Synthetic waste (largely soluble)	lab		including extended aeration	biodegradable VSS	0.18	McCarty and Broderson (1962)

TABLE 5.1B. Maintenance coefficient (m) for aerobically growing Gram-negative organisms.

Organism	Limiting substrate	Temperature (°C)	m (g substrate per g dry wt per h)	a[1] (day^{-1})	Reference
E. coli	glucose	30	0.055	0.67	Schulze and Lipe (1964)
Aer. aerogenes (*Klebsiella pneumoniae*)	glycerol	37	0.076	1.00	Herbert (1958), calculated by Pirt (1965)
Aer. cloacae	glucose	37	0.094	0.99	Pirt (1965)
Pseudomonas fluor.	glucose	30	0.25	4.01	Palumbo and Witter (1969)
		20	0.20	2.64	
		8	0.15	1.64	

[1] $a = m.Y_{max}$.

Although microbiologists mostly use the equation of PIRT (3a, 5a) because of its more direct concept for maintenance, in the present study the equation derived from the model of HERBERT and, as to the notation used, that of MARR et al. (3b, 5b) is preferred. The concept of negative growth or 'endogenous metabolism' seems to be applicable to systems with an important fraction of dead cells. The use of a turnover rate of biomass is also more convenient for comparing with degradation rates of biomass components like enzymes. Moreover, an equivalent of the constant a, in this thesis indicated by a^*, has been used already for a long time in the sanitary engineering literature, viz. in a linear equation describing net microbial growth and the amount of substrate utilized by activated sludge (HEUKELEKIAN et al., 1951; for a review see: LAWRENCE and MCCARTY, 1970). This equation was derived empirically from waste treatment studies and is here rewritten as:

$$\left(\frac{dX}{dt}\right)_{net} = -Y_{max}\left(\frac{dS}{dt}\right)_{tot} - a^*X \text{ or } \frac{1}{t_s} = Y_{max}\cdot q - a^* \qquad (6)$$

The symbol a^*, according to the literature, stands for the micro-organism, or biomass, decay coefficient. However, this symbol represents the overall decrease of cellular mass including besides the several 'maintenance' terms attributed to a growing pure culture, also decay by cell death and lysis, predator activities of protozoa, rotifers and other higher forms of life minus the increase of cellular mass, due to cryptic growth of bacteria. Therefore, the term 'biomass turnover coefficient' is preferred in the present study. Y_{max} is in this case the cell yield that would have been observed if no substrate were required for maintenance purposes and if no decay, predation and reutilization of sludge material occurred. To get an impression of the magnitude of micro-organism turnover coefficients and of maintenance coefficients Table 5.1 is included.

5.1.4. *Stability of α-amylases*

Purified and crystallized amylases (completely free from proteases) from human saliva, hog pancreas, *Bacillus subtilis* and *Aspergillus oryzae* contain per mole of enzyme at least 1 gram-atom of very firmly bound calcium, which is essential for their activity. Dialysis against a large molar excess of the sequestering agent ethylenediaminetetraacetate (EDTA) results in a decrease of the calcium content of amylase to less than 1 gram-atom per mole and a completely reversible inactivation of the amylases. This inactivation varies greatly with the biological origin of the enzyme. The amylase of *A. oryzae* is not inactivated under the conditions mentioned; it seems that this enzyme competes effectively with the chelating agent for Ca.

The addition of 1% of soluble starch protects the enzymes against inactivation by EDTA when the polysaccharide is not degraded too far. This protective action cannot be achieved with a protective colloid, e.g. 0.5% albumin, or with a different polysaccharide like dextran (VALLEE et al., 1955; FISCHER and STEIN, 1960).

Amylases of different biological origin show the same order of stability (viz. mould > bacteria > hog pancreas = human saliva) towards dialysis against EDTA and proteolytic attack (STEIN and FISCHER, 1958). α-Amylases from which the divalent metal ions have been removed by EDTA are highly susceptible to proteolytic attack except the amylase of *Aspergillus oryzae* from which the metal ions are not removed by EDTA.

Extensive proteolytic degradation of purified α-amylases can only occur through the joint action of a metal-binding agent and a protease. Only in the case of human saliva α-amylase, degradation by trypsin occurs unless Ca (10^{-2} M) is added. The degree of removal of metal ions determines the overall rate of the degradative process.

5.2. PROTEOLYTIC ACTIVITIES AND PROTEOLYTIC BACTERIA IN CONTINUOUSLY OPERATED SLUDGES

The hypothesis that turnover of cell constituents, particularly nitrogenous compounds, plays an important part in the metabolism of activated sludge (5.1.1), may be tested by estimating proteolytic activity and numbers of proteolytic bacteria in sludge. For this reason it was tested if and to which extent both parameters are affected by SL, using starch as the substrate; these results were also compared to those of activated sludges fed (partly) with proteins. Turnover was expected to be affected by the mean cell residence time, t_s (5.1.3), and by SL which is closely connected with t_s.

5.2.1. *Proteolytic activities of sludges I*

Proteolytic activities were estimated in differently loaded starch and glucose sludges I (Fig. 5.1 and Fig. 4.6B). Figs. 5.1A, C and D deal with the same sludges as presented in Fig. 4.6A.

It was observed that PA_{spec}, after a period of acclimatization of the activated sludge, was rather constant at SL = 0.075 (A), 0.3 (B) and 1.2 (C); it fluctuated clearly less than DA_{spec} (Fig. 4.6A). Qualitative differences of loading (glucose or starch, Figs. 5.1 B-1 and B-2) did not clearly affect PA_{spec}, provided that SL was kept constant. When SL was varied, fluctuations of PA_{spec} occurred; upon decrease of SL from 0.3 to 0.075 (B-2, A) these fluctuations were small, but upon increase from 0.3 to 1.2 (B-2, C) they were extremely large. Also loading of Zeist sludge with starch (SL = 0.3, B-3) initially led to a high PA_{spec} (the starvation experiment of B-3 will be discussed in 5.6).

The values of the successive maximum to minimum ratios of PA_{spec} at SL = 1.2 (C) were 23, 6.3, 2.7, 1.5 and 1.1; these fluctuations flattened after almost 3 months, i.e. 30–45 mean cell residence times. Nevertheless, continuing shifts of population were observed and DA_{spec} did not at all attain a steady-state value (4.6.1). Only during the first 19 days of loading fluctuations of DA_{spec} and PA_{spec} coincided (Fig. 5.1C). When SL was raised from 1.2 to 2.4 (Fig. 5.1D), PA_{spec} was even fluctuating during about 300 cell residence times without showing a

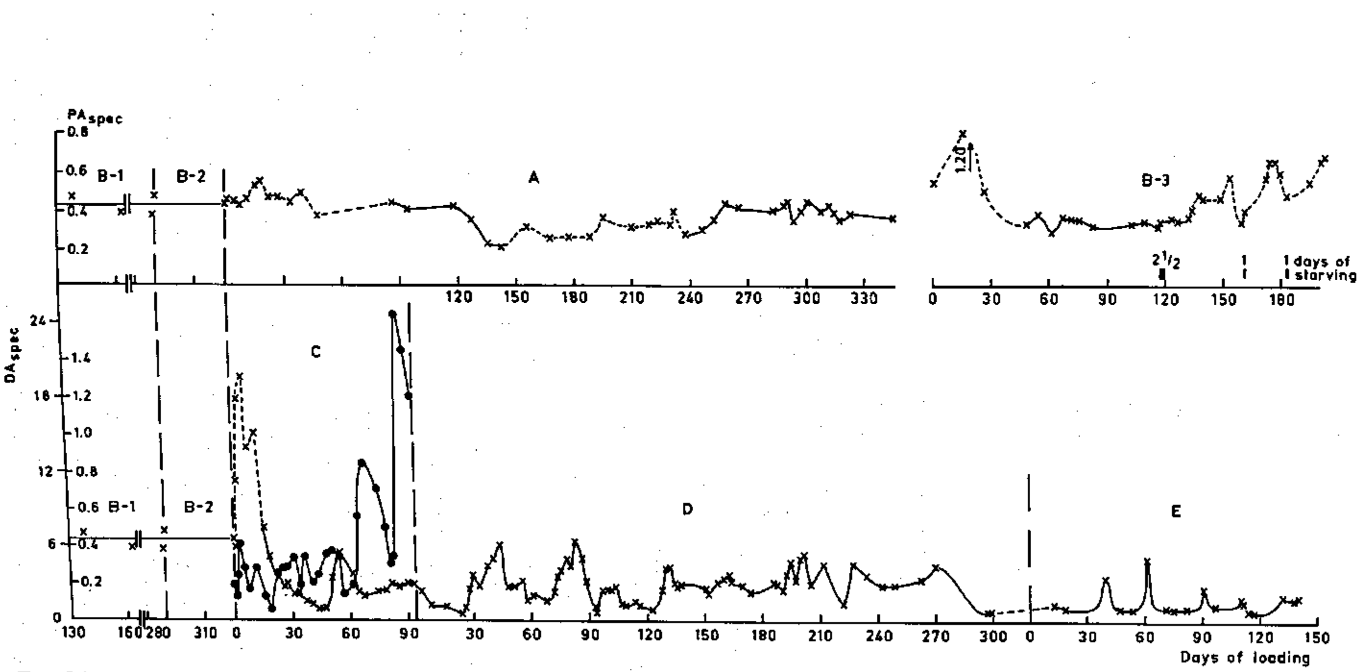

FIG. 5.1. PA$_{spec}$ values of sludge (with retention, I) during continuous loading with starch or glucose at pH 7.0 and 20°C. Quantitative alteration of loading is indicated by starting at zero time. A, B-2, B-3, C, D and E denote starch loadings of sludge with 0.075, 0.3, 0.3, 1.2, 2.4 and 2.12 g COD/g sludge.day, respectively. B-1: glucose, SL = 0.3. B-3: separate experiment started with a mixture of Zeist activated sludge, very high-loaded and very low-loaded laboratory-grown starch activated sludges in the ratio of 4:1:1; starch supply was interrupted by periods of starvation. E: sludge without retention of flocculated biomass (II). Broken lines include values which are not used for calculating averages. Between 150 and 240 days the dry wt of sludge A was less than half the planned 2.5 g/l. x, PA$_{spec}$; ●, DA$_{spec}$.

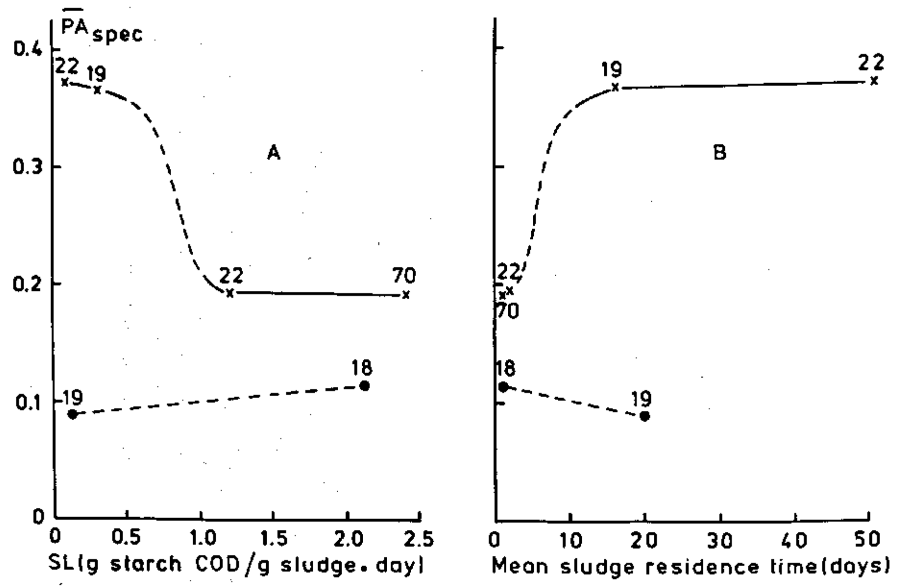

FIG. 5.2. Arithmetical means of PA_{spec} values of starch (or glucose) sludges (I and II) as affected by SL (Fig. 5.2A) and t_s (Fig. 5.2B). The t_s values were obtained from Table 5.7, the PA_{spec} values from Figs. 4.6B and 5.1 (values of periods indicated by broken lines were eliminated). Figures included in the graph of Figs. 5.2A and B indicate number of measurements. x and ●, sludges with and without retention of flocculated biomass, respectively.

tendency to attain a new steady-state value. However, in contrast to DA_{spec} (Fig. 4.6A), PA_{spec} was to some extent stationary.

Arithmetically averaged PA_{spec} values plotted against SL are given in Fig. 5.2A and plotted against t_s in Fig. 5.2B. At high and very high loadings of activated sludge (t_s about 1–3 days) PA_{spec} was on the average about 0.2. Over the range from very low to medium loadings (t_s more than about 12 days) PA_{spec} was relatively constant and approached 0.4. Although PA_{spec} was not measured at intermediate SL values, the observed data point to a negative correlation between PA_{spec} and SL with starch and to a positive correlation between PA_{spec} and t_s. This relationship is entirely different from the response of DA_{spec} to SL with starch (Fig. 4.8). DA_{spec} may be regarded as an extrinsic parameter, since it reflects a response of a sludge system to its external substrate. PA_{spec}, at least in non-protein-fed systems, may be regarded as an intrinsic parameter, as it apparently reflects a response to internal factors (turnover), which are independent of type of external substrate and which essentially characterize the system. The relative importance of extrinsic and intrinsic parameters in starch-fed sludge is shown in Table 5.2. The results of these experiments clearly show that at low loadings with starch the amylolytic activity of the sludge is relatively low compared to the proteolytic activity which represents turnover.

The average PA_{spec} of activated sludge from the outdoor plant at Bennekom, supplied with mainly domestic sewage (containing protein), was only 37%

TABLE 5.2. Average DA_{spec} and PA_{spec} at different SL values of starch activated sludge (data of Figs. 4.8 and 5.2).

SL	0.075–0.10	0.24–0.3	1.2	2.4
$\underline{D\tilde{A}}_{spec}$	0.635	1.80	4.72	7.02
PA_{spec}	0.372	0.366	0.193	0.191

higher than that of very low-loaded starch sludge, grown in the laboratory (Tables 5.3 and 5.2). This suggests that, at least in such an extensively aerated activated sludge as that of the Bennekom plant, the majority of proteolytic enzymes is synthesized for the turnover of proteinaceous cell material. The comparison of both sludge types confirms that the PA_{spec} values of starch activated sludges as recorded in Fig. 5.2 are unexpectedly high if one keeps in mind that these sludges were not supplied with proteins or peptides. Although PA_{spec} of the Zeist sludge was clearly higher than that of starch activated sludge with corresponding SL values (cf. Table 5.3 and Fig. 5.2A), also in the Zeist sludge an important part of the proteolytic enzymes apparently served turnover. If extrinsic protein sources of the relatively high-loaded Zeist sludge had largely been responsible for the development of proteolytic activity, the PA_{spec} of this sludge would have been higher than that of the Bennekom sludge, analogously to the observed DA_{spec} values of both sludges (Table 5.3). Additional evidence concerning this statement can be derived from the results of the following laboratory experiment in which activated sludges were loaded with casein or casamino acids (acid-hydrolysed casein, not free from peptides). Similar to DA_{spec} of starch-grown sludge with increased starch loading (Fig. 4.8), PA_{spec} of these sludges tended to increase with SL (Table 5.4).

5.2.2. *Proteolytic and non-proteolytic bacteria and their relation to proteolytic activities of sludges I*

The question arose whether the relation between the average PA_{spec} and SL with non-proteinaceous substrates (Fig. 5.2A) was due to differences in numbers of proteolytic bacteria or to other factors (cf. DA_{spec}, 4.6.2). Testing of the hypothesis of turnover also prompted us to estimate numbers of total (viable) and proteolytic bacteria and PA_{spec} at different SL values with starch (Table 5.5A).

Unlike PA_{spec}, numbers of total (viable) and proteolytic bacteria of starch

TABLE 5.3. Arithmetically averaged PA_{spec} and DA_{spec} of activated sludge from outdoor plants during summer periods (May–Oct./Nov.).

Activated sludge	SL	PA_{spec} ('75–'76)	DA_{spec} ('74–'76)
Bennekom	c. 0.063	0.51 ($n = 6$; 0.36–0.64)	0.54 ($n = 7$; 0.40–0.67)
Zeist	c. 1.5	0.54 ($n = 7$; 0.43–0.73)	1.08 ($n = 8$; 0.96–1.30)

TABLE 5.4. PA_{spec} of activated sludge grown for 46 days at SL = 0.83 and subsequently for 21 days at SL = 0.17 with casamino acids or casein as the sole carbon and nitrogen source. The proteolytic activities, fluctuating considerably during the experiment, are extremes observed after an acclimatization period of only 10 days.

SL	PA_{spec}[1] Sludge substrate	
	Casamino acids	Casein
0.83	0.6–1.6 ($n = 5$; $\overline{PA}_{spec} = 1.1$)	1.2–4.5 ($n = 5$; $\overline{PA}_{spec} = 2.3$)
0.17	0.4–0.7 ($n = 2$)	1.2–1.9 ($n = 2$)

[1] Proteolytic activities were not determined under standard conditions and therefore are hardly comparable to activities of other sludges.

sludge I markedly increased with SL. However, percentages of proteolytic bacteria decreased with SL (see also Fig. 5.3). It is remarkable that on the average about 50% of the viable population was proteolytic over the entire range from very low to medium loadings, in spite of the complete absence of proteinaceous material in the influent. Average percentages of less than 40 may be expected (Fig. 5.3B) only at mean sludge residence times (t_s) of less than about one week. Even in starch activated sludge with a t_s of 1 day on the average 20% of the viable bacteria was proteolytic; the lowest percentage of proteolytic bacteria in this very high-loaded sludge ever observed was 8. From these results it can be concluded that extracellular proteolysis and likely cell protein turnover never were completely absent in activated sludge with retention of biomass, even not at the highest SL values.

TABLE 5.5A. Proteolytic activities and proteolytic and total (viable) bacteria (estimated in the same samples) of starch sludges with retention of flocculated biomass (I), (cf. Fig. 5.1).

SL	Days of loading	PA_{spec}	Total (viable) bact. per 10^{-7} mg	Proteolytic bact. per 10^{-7} mg	Prot. act. per prot. cell[1]
0.075	135	0.223	4.69	2.02 (43)	110.4
	138		6.18	3.33 (54)	
	142	0.200	5.12	2.27 (44)	88.1
	146		5.94	3.17 (53)	
	287	0.420	12.35	6.24 (51)	67.3
	289	0.439	16.98	9.70 (57)	45.3
	296	0.390	15.54	6.55 (42)	59.5
	310	0.419	9.20	2.45 (27)	171.0
	313	0.385	9.85	8.07 (82)	47.7
	316	0.342	8.17	4.15 (51)	82.4
	321	0.374	4.79	2.57 (54)	145.5
	$n = 11$	($\overline{PA}_{spec} = 0.355$)	$\overline{\ln d} = 2.095$ $s = 0.465$ $\tilde{d} = 8.1$	$\overline{\ln d} = 1.383$ $s = 0.551$ $\tilde{d} = 4.0$ (49)	$\overline{\ln \text{rat.}} = 4.410$ $s = 0.468$ $\tilde{\text{rat}}. = 82$

TABLE 5.5A. continued.

SL	Days of loading	PA_{spec}	Total (viable) bact. per 10^{-7} mg	Proteolytic bact. per 10^{-7} mg	Prot. act. per prot. cell[1]
0.3[2]	109	0.340	38.9	20.3 (52)	16.7
	116	0.304	54.3	26.5 (49)	11.5
	123	0.353	28.0	22.7 (81)	15.6
	126	0.337	24.1	14.0 (58)	24.1
	133	0.397	21.7	21.7 (100)	18.3
	153	0.569	86.0	75.0 (87)	7.6
	173	0.647	50.0	16.3 (33)	39.7
	176	0.644	23.8	19.3 (81)	33.4
	179	0.592	22.0	17.7 (81)	33.4
	194	0.543	57.5	28.4 (50)	19.1
	200	0.644	16.6	9.5 (57)	67.8
	202	0.664	15.1	9.3 (62)	71.1
	$n = 12$	($\overline{PA_{spec}} = 0.503$)	$\overline{\ln d} = 3.454$ $s = 0.548$ $\tilde{d} = 31.6$	$\overline{\ln d} = 2.992$ $s = 0.547$ $\tilde{d} = 19.9$ (63)	$\overline{\ln \text{rat.}} = 3.188$ $s = 0.677$ $\tilde{\text{rat}}. = 24$
2.4	41	0.332	127.4	64.1 (50)	5.2
	44	0.401	154.6	96.1 (62)	4.2
	48	0.180	137.0	52.6 (39)	3.4
	52	0.185	146.2	27.6 (19)	6.7
	115	0.088	162.1	24.3 (15)	3.6
	122	0.059	174.2	14.1 (8.1)	4.2
	130	0.276	125.6	22.5 (18)	12.3
	138	0.190	118.5	14.8 (13)	12.8
	193	0.247	46.0	14.3 (31)	17.3
	195	0.316	169.6	25.4 (15)	12.4
	202	0.357	82.0	28.5 (35)	12.5
	222	0.093	78.8	16.0 (20)	5.8
	227	0.302	82.7	13.9 (17)	21.7
	$n = 13$	($\overline{PA_{spec}} = 0.233$)	$\overline{\ln d} = 4.754$ $s = 0.392$ $\tilde{d} = 116$	$\overline{\ln d} = 3.254$ $s = 0.628$ $\tilde{d} = 25.9$ (22)	$\overline{\ln \text{rat.}} = 2.055$ $s = 0.639$ $\tilde{\text{rat}}. = 7.8$

All MPN-based counts were made in combined-substrate tubes with casein yeast extract medium A_3 (10 tubes per dilution). They were computed at sludge dry wt. Figures in parentheses denote proteolytic bacteria as % of total numbers of bacteria. n = number of estimates; d = MPN-based estimate of density of bacteria; s = sample standard deviation (of ln); rat. = ratio; $\bar{}$: superscript denotes arithmetical mean; $\tilde{}$: superscript denotes logarithmic mean.

[1] Expressed as 10^{-10} mg casein/proteolytic bacterium·h and calculated from $PA_{spec} \cdot 10^{10}$/proteol. bact. mg^{-1}; [2] the activated sludge with SL = 0.3 was three times disturbed by periods of starvation (Fig. 5.1B-3).

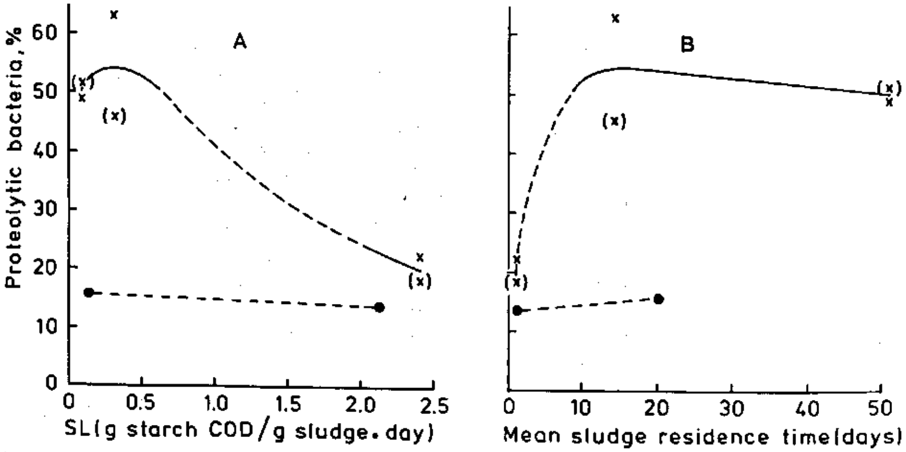

FIG. 5.3. Relation between proteolytic bacteria as average % of total (viable) count (Table 5.5) and sludge loading with starch (Fig. 5.3A) or mean sludge residence time (Fig. 5.3B). x, Sludge I; ●, sludge II. If the arithmetically averaged PA_{spec} values of the activated sludge measured at the times of counting deviated from those of Fig. 5.2, a correction was made for the estimated average percentages of proteolytic bacteria, indicated by (x).

From the average percentages of amylolytic and proteolytic bacteria in starch activated sludge at SL = 0.3, viz. about 90 and 50, respectively, it is derived that on the average about half of the amylolytic bacteria was also proteolytic and that the majority of the proteolytic bacteria was also amylolytic. Since the majority of the proteolytic bacteria apparently grew in situ also on the primary substrate (starch), this type of growth is called 'partly cryptic'. Loading with starch at SL = 0.3 did not or hardly lead to a secondary population; such a population, characterized by an entirely cryptic growth, was found only at SL = 0.075 (4.6.2).

As PA_{spec} decreased with SL (Fig. 5.2A) but the numbers of proteolytic bacteria markedly increased, the average proteolytic activity calculated per (viable) proteolytic bacterium sharply decreased with SL (Table 5.5A and Fig. 5.4A), and thus increased with t_s (Fig. 5.4B). At SL = 0.3, proteolytic activity per proteolytic cell was approximately 3 times and at SL = 0.075 – 0.1 about 10 times higher than that at SL = 2.4. The sharply increased proteolytic activity above the average 'basic' level of about $5 - 8 \times 10^{-10}$ mg casein per proteolytic bacterium per h (Fig. 5.4) can be explained by (a) proteolytic enzyme activity associated with non-viable cells or cell debris (similar to the increased amount of amylolytic enzymes above a distinct 'basic' level at a very low loading and (b) the release of relatively large amounts of proteinaceous cell constituents from dead bacteria resulting in an increased induction/stimulation of the synthesis of proteolytic enzymes. That assumption (a) accounted for at least a considerable part of the observed phenomenon is concluded from the fact that amylolytic enzymes, which presumably are less stabile in activated sludge than proteolytic

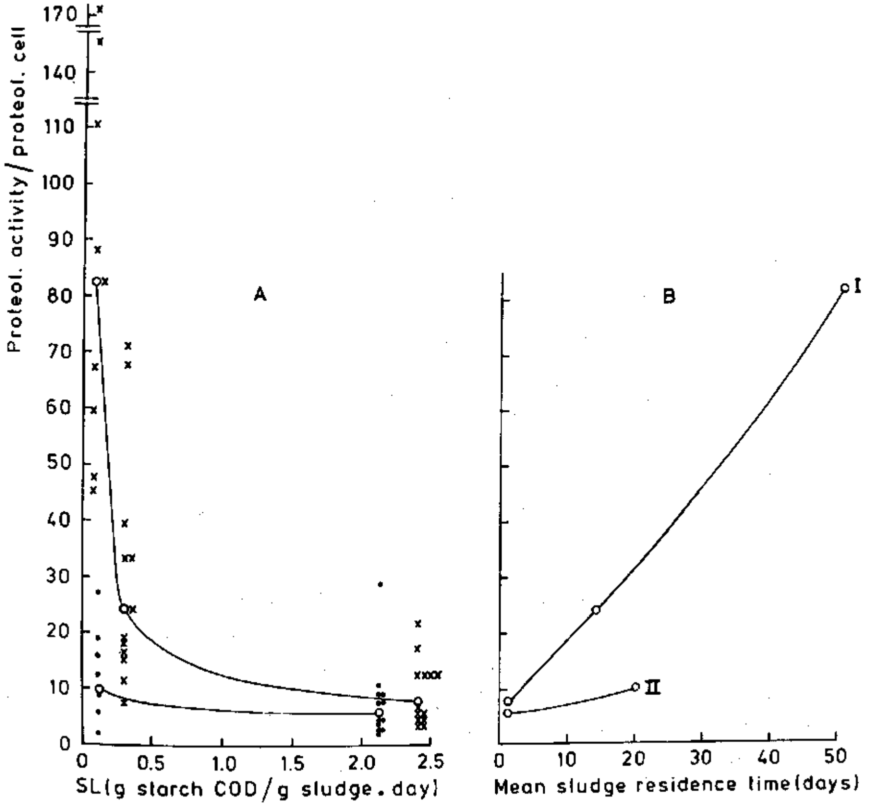

FIG. 5.4. Relationship between proteolytic activity calculated per (viable) proteolytic bacterium (MPN-estimates; Table 5.5) and sludge loading with starch (Fig. 5.4A) or mean sludge residence time (Fig. 5.4B). x, Estimates of proteolytic activity per (viable) proteolytic bacterium (10^{-10} mg casein/proteolytic bacterium.h) in sludge I; ●, estimates in sludge II; O, logarithmic averages of these estimates.

enzymes, behaved similarly at very low SL with starch (Fig. 4.9). Additional evidence in favour of (a) was derived from the fact that with increased supply of starch no increased amylolytic activity per amylolytic cell was found (Fig. 4.9). It is remarkable that the reduction of the viable count at SL = 0.075 as compared with that at SL = 2.4 with a factor 14–20 is accompanied with an increase of the percentage proteolytic bacteria with a factor 2.5 and with a ten times increased proteolytic activity calculated per (viable) proteolytic cell.

Whether the fluctuations of PA_{spec} of very high-loaded starch activated sludge, operated continuously (Fig. 5.1D), have to be attributed to variations of number of proteolytic bacteria and/or variations of amount of enzyme present per proteolytic cell is difficult to decide, due to the poor repeatability of the MPN-based counts. Fig. 5.5 suggests that both variations may have influence (cf. variations of DA_{spec}, Fig. 4.10). Nevertheless, variation of the number of proteolytic bacteria can be the only cause of variation during periods of large

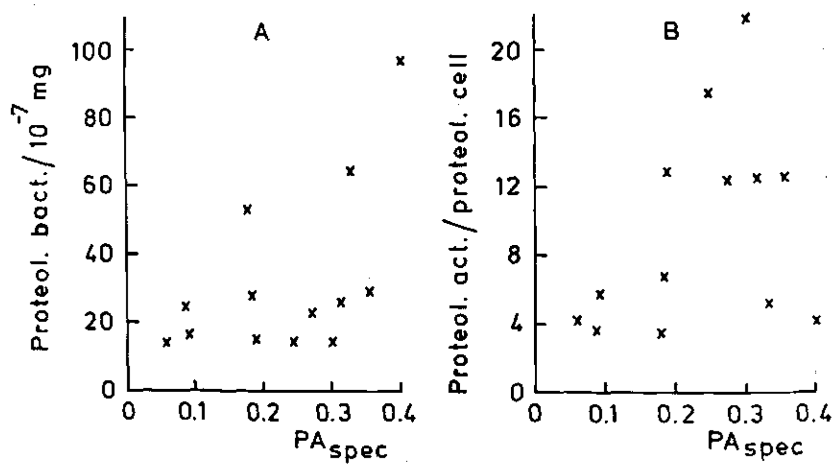

FIG. 5.5. Relation between PA_{spec} of very high-loaded starch activated sludge and number of proteolytic bacteria per 10^{-7} mg of sludge dry wt (A), or proteolytic activity per (viable) proteolytic bacterium (10^{-10} mg casein/proteolytic cell.h) (B). Data were obtained from Table 5.5A.

fluctuations of PA_{spec} (see first 4–6 data of this sludge in Table 5.5A).

The proteolytic parameters of activated sludge from the Zeist plant were estimated only once (17/10/1975): $PA_{spec} = 0.43$ (cf. Table 5.3); total (viable) bacteria: 21.4×10^7/mg; percentage of proteolytic bacteria: 46 and proteolytic activity expressed as 10^{-10} mg casein/proteolytic bacterium.h: 43. The percentage of proteolytic bacteria in this sludge was of the same order of magnitude as that of carbohydrate-grown sludges.

5.2.3. *Proteolytic activities and proteolytic bacteria in sludges II*

The high-loaded sludge system (SL = 2.12) without retention of flocculated biomass (II) (Fig. 5.1E) was generally characterized by a low PA_{spec} and by the predominance of few (usually 2) types of bacteria growing in suspension. Higher PA_{spec} values were sometimes observed, usually after some irregularity of the growth conditions, e.g. a temporary disturbance of the supply of substrate solution or of dilution water (Fig. 2.1). This was accompanied with an increase of usually yellow flocs in the system.

Fig. 4.6B shows that PA_{spec}, in spite of the low loading applied, dropped to a very low level within some weeks after cessation of the retention of biomass in contrast to the PA_{spec} of the same activated sludge submitted to prolonged loading with retention of flocculated biomass (Figs. 5.1B-1, B-2). The drop of PA_{spec} (Fig. 4.6B) was accompanied with the alteration of a flocculated mixed culture into a culture which was predominated by the suspended growth of a non-proteolytic *Nocardia*-like bacterium, which maintained its predominance for 500 days (see also 4.6.1). Nevertheless, the PA_{spec} increased after about 150 days. Since growth of algae was observed, the system was darkened, after which the algae disappeared and PA_{spec} decreased sharply. Another low-loaded sludge II which was predominated by the suspended growth of a different strain and

TABLE 5.5B. Proteolytic activities and proteolytic and total (viable) bacteria (estimated in the same samples) of starch sludges without retention of flocculated biomass (II) (cf. Figs. 4.6B and 5.1).

SL	Days of loading	PA_{spec}	Total (viable) bact. per 10^{-7} mg	Proteolytic bact. per 10^{-7} mg	Prot. act. per prot. cell
0.121	398	0.110	71.2	11.45 (16.1)	9.6
	402	0.128	22.8	6.78 (29.7)	18.9
	412	0.114	22.9	4.20 (18.3)	27.1
	441	0.067	24.0	4.21 (17.5)	15.9
	448	0.067	100.6	5.47 (5.4)	12.3
	454	0.060	218.9	32.3 (14.7)	1.9
	496	0.062	57.1	10.8 (18.9)	5.7
	$n = 7$	$(\overline{PA}_{spec} = 0.087)$	$\overline{\ln d} = 3.964$ $s = 0.872$ $\bar{d} = 52.7$	$\overline{\ln d} = 2.111$ $s = 0.726$ $\bar{d} = 8.3$ (16)	$\overline{\ln \text{rat.}} = 2.306$ $s = 0.894$ rāt. = 10.0
2.12	54	0.071	399	26.3 (6.6)	2.7
	61	0.332	68.2	11.6 (16.9)	28.6
	71	0.076	52.6	7.2 (13.6)	10.6
	75	0.067	119.1	15.6 (13.1)	4.3
	82	0.073	231.5	40.9 (17.7)	1.8
	91	0.178	304.6	24.0 (7.9)	7.4
	111	0.105	96.8	25.0 (25.9)	4.2
	114	0.056	54.2	7.4 (13.6)	7.6
	117	0.053	87.3	13.2 (15.2)	4.0
	132	0.136	136.8	15.4 (11.2)	8.8
	138	0.117	110.4	12.8 (11.6)	9.1
	140	0.133	196.5	46.1 (23.5)	2.9
	$n = 12$	$(\overline{PA}_{spec} = 0.116)$	$\overline{\ln d} = 4.838$ $s = 0.658$ $\bar{d} = 126$	$\overline{\ln d} = 2.853$ $s = 0.601$ $\bar{d} = 17.3$ (14)	$\overline{\ln \text{rat.}} = 1.753$ $s = 0.752$ rāt. = 5.8

Notes are equal to those of Table 5.5A.

also was characterized by a very low PA_{spec}, is shown in Fig. 5.9B.

The $\overline{PA}_{spec}$ of sludge II was low and seemed to be rather constant over a broad range of sludge ages (Fig. 5.2B). It indicates that $\overline{PA}_{spec}$ cannot be increased by increasing sludge age only, but that retention of flocculated biomass is an important condition. The high PA_{spec} in the systems with sludge retention may be correlated with the occurrence of mixed cultures growing in flocs.

The lower PA_{spec} of sludge II, as compared with sludge I, may be explained at least partly by lower percentages of (viable) proteolytic bacteria. Even at a cell residence time of about 20 days on the average only 16% of proteolytic bacteria were found in sludge II which did not exceed the percentage counted at a cell residence time of 1 day (Table 5.5B; Fig. 5.3B). Sludge I of corresponding high sludge age contained 3–4 times higher percentages of proteolytic bacteria (Fig. 5.3B). Fig. 5.4A shows that at very high loadings with starch no significant difference between the average proteolytic activity per proteolytic cell in systems with and without sludge retention was observed. This suggests that the 'basic' proteolytic level per proteolytic bacterium was more or less independent of the system (either mainly flocs or mainly suspended bacteria) if most of the bacteria were living. Both very high-loaded systems contained about equal numbers of total (viable) bacteria per mg of sludge (Table 5.5). However, at low loadings with starch the proteolytic activity per proteolytic cell in system II was lower than that in system I (Fig. 5.4A). It is conceivable that within the flocs of activated sludge, conditions are less favourable for growth resulting in an increased death rate and/or lysis. An increased release of proteins might stimulate the sludge fed production of proteolytic enzymes. However, the content of measurable Lowry-positive material of the sludge-free liquid was very low over the whole range of loadings. The number of total (viable) bacteria in low-loaded sludge II seemed to be higher than that in corresponding sludge I (Tables 5.5 and 5.6). This would mean that the apparent proteolytic activity per proteolytic cell in sludge II had increased less with increasing sludge age. In the sludge without retention of biomass the average reduction of viability with a factor 2.4 (going from very high to low loadings) resulted in an average increase of the proteolytic activity per proteolytic cell with a factor of about 1.7 (cf. data of sludge I).

5.3. MINIMUM SPECIFIC GROWTH RATE OF (VIABLE) CELLS IN STARCH-LIMITED SLUDGES I AND II

The growth rate of micro-organisms in a chemostat culture can be varied between wide limits. The maximum rate is well defined; direct evidence for the existence of a finite minimum growth rate was provided for *Aerobacter aerogenes* (*Klebsiella pneumoniae*) by POSTGATE and HUNTER (1962) and TEMPEST et al. (1967). In a 'steady-state' culture with a viability of less than 100%, the specific growth rate of the viable portion of the population must be greater than the dilution rate as the viable organisms are replenishing both the viable and non-viable cells of the population. The doubling time (t_d) of a fully viable culture under steady-state conditions is equal to $(\ln 2)/\mu = (\ln 2)/D$ (where D is the dilution rate); when only a fraction (f) of the bacterial population is viable the doubling time of the viable cells (t_d^*) must equal $(\ln 2 + \ln \alpha)/D$, where α is equal to $(f + 1)/2$. The fraction α, called the viability index, represents the probability that a newly formed cell will be viable. The doubling time of the whole culture is still equal to $(\ln 2)/D$ (TEMPEST et al., 1967).

For description of the sludge system with retention of flocculated biomass, the mean cell residence time t_s (5.1.3) must be substituted for $1/D$ so that:

$$t_d = \frac{\ln 2}{\mu} = t_s \ln 2 \text{ and}$$

$$t_d^* = \frac{\ln 2\alpha}{\mu} = t_s \ln 2\,\alpha = t_s \ln (f + 1).$$

The minimum growth rate of viable cells in a mixed culture like activated sludge has not been estimated earlier, mainly due to the difficulty of estimating viability. In the present study an attempt was made to estimate this growth rate using the collected data but making the following assumptions.

a) The viability of bacteria in very high-loaded sludge ($t_s = 1$ day) is about 90%; consequently, the average number of (viable + non-viable) bacteria per mg dry sludge solids is calculated to be 1.40×10^9 (Table 5.6). The assumption was based on the data of POSTGATE and HUNTER (1962) and TEMPEST et al. (1967) obtained with *Aerobacter aerogenes*; these authors found a viability of about 90% in a glycerol-limited continuous culture at 37°C and a mean cell residence time of 1 day. The maximum number of viable bacteria per mg VSS in activated sludge fed with primary sewage was found to be 1.0×10^9 (WEDDLE and JENKINS, 1971) at very high loadings of 10.7 g COD removed/g VSS.day (net specific growth rate: 4.2 day^{-1}). WALKER and DAVIES (1977) found the maximum number of 4.5×10^8 viable cells per mg SS already at a net specific growth rate of about 0.9 day^{-1} (laboratory activited sludge fed with settled sewage).

b) The average weight of one bacterium is equal in sludges with and without retention of biomass and independent of the mean cell residence time in the range 1 to 60 days.

TABLE 5.6. Influence of the mean cell residence time of starch-loaded sludges I and II on the 'steady-state' viability and the mean doubling time of (viable) cells. The mean cell residence time ($t_s = \mu^{-1}$) was derived from the data given in Table 5.7 and Fig. 5.7. Almost all MPN-based data of total (viable) bacteria were obtained from Tables 4.4 and 5.5. In all combined-substrate tubes yeast extract was present to allow 'total (viable) growth'. Viabilities were calculated taking into account the assumptions reported in the text (e.g. 1 mg dry sludge solids corresponds with 1.40×10^9 bacteria).

	Sludge I				Sludge II	
SL[1]	0.075	0.3	1.2	2.4	0.121	2.12
Number of estimates	24	28	5	26	9	12
Arithmetically averaged dry solids (g/l)	c. 2	2.62	c. 2.73	2.72	6.21	1.42
t_s (days)	c. 60	13.2	c. 2.4	1.01	20	1.0
$\overline{\ln}\,(d/10^{-7}\text{ mg})$	1.84	3.58	4.58	4.85	3.92	4.84
s	0.54	0.69	0.61	0.48	0.85	0.66
$\tilde{d}/10^{-7}$ mg	6.3	35.9	97.0	127	50.1	126
f (viable fraction)	0.045	0.26	0.69	0.91	0.36	0.90
α (index of viability)	0.52	0.63	0.85	0.96	0.68	0.95
t_d^* (mean doubling time of viable cells in days)	2.6	3.0	1.3	0.65	6.1	0.64
t_d	42	9.2	1.7	0.70	13.9	0.69

d = MPN-based estimate of density of total (viable) bacteria; s = sample standard deviation of ln ($d/10^{-7}$ mg).

[1] See Table 2.3.

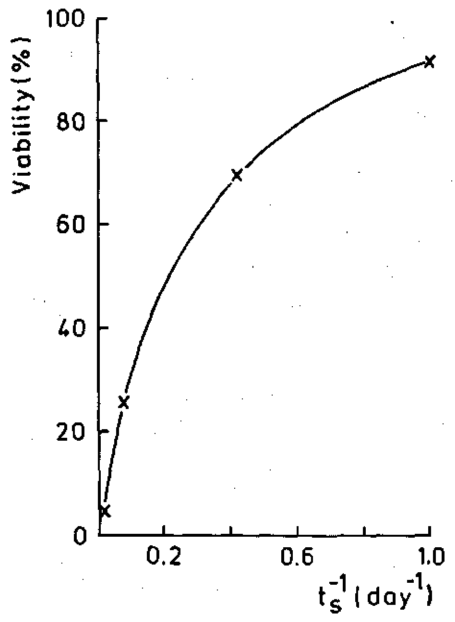

FIG. 5.6. Relation between calculated percentages of viable bacteria and net specific growth rate of starch activated sludge (cf. Table 5.6).

c) Flocculation of activated sludge does not result in an underestimate of the number of viable bacteria. The usefulness of our dispersion method was demonstrated by the fact that the average numbers of viable bacteria in very high-loaded sludges I (mainly bacteria occurring in flocs) and II (mainly suspended bacteria) were equal (Table 5.6).

The averaged results of the calculations are given in Table 5.6. Under apparent steady-state conditions as to viability the average number of total (viable) bacteria of sludge I showed a pronounced decrease with declining SL, resulting at SL = 0.075–0.1 in a viable count of about 5% compared to that at SL = 2.4. An approximately similar relationship is found when viable bacteria (calculated as % viability) are plotted against net specific growth rate (Fig. 5.6). This graph tends to go through the origin as contrasted with comparable graphs represented by WEDDLE and JENKINS (1971) and WALKER and DAVIES (1977), which intersected the ordinate above the origin. The former authors even did not find a significant increase of the viability (estimated to be 15–20%) with net specific growth rates rising from 0.026 to about 0.3 day^{-1}. In this respect it is remarked that also during the present study a low net specific growth rate of activated sludge was accompanied with a relatively high percentage of viable bacteria (17) when the counts were made shortly (viz. 34 days) after the reduction of SL from 0.3 to 0.075. Upon prolonged submission to SL = 0.075 (120 days in the present investigation), steady state was attained as to viability (Tables 4.4 and 5.5A).

Table 5.6 furthermore shows that the averaged doubling time of (viable) cells (t_d^*), in contrast to t_d, was not proportional to t_s and, as to sludge I, tended to attain a maximum of about 3 days above t_s values of about 10 days. This is equivalent with a minimum specific growth rate ($\mu^* = \ln 2/t_d^*$) of about 0.23 day^{-1}. This maximum t_d^* value is lower than that observed by TEMPEST et al. (1967) for a glycerol-limited *A. aerogenes* culture (3½ days, at 37 °C). Our results suggest that, in contrast to what is frequently thought and to what may be derived from the results of WEDDLE and JENKINS (1971) and WALKER and DAVIES (1977), very slowly growing or dormant bacteria do not and cannot emerge during very low loadings of activated sludge. The bacteria in activated sludge apparently are obliged either to multiply or to die and cannot maintain themselves indefinitely without division. Maintaining a resting population in a continuous culture would only be possible if the living organisms replenished the resting cells continuously.

The validity of the correction for non-viable bacteria may be questioned theoretically since dormant, i.e. non-growing, bacteria which appeared viable when incubated in fresh liquid media, could have been present in the sludge system. In this respect the number of growing bacteria and the maximum t_d^* could have been overestimated only. The growth of filamentous bacteria in activated sludge apparently did not interfere to a large extent with the countings because they were occasionally found in the highest diluted samples. A filamentous bacterium provisorily called *Flavobacterium* sometimes made up ¼ to ½ (or more) of the whole viable population.

The viable fraction of the low-loaded starch sludge II seemed to be higher than that of comparable systems I. The calculated high maximum t_d^* value of roughly 6 days (equivalent minimum $\mu^* = 0.11$ day^{-1}) might be attributed to the peculiar *Nocardia*-like bacterium (Plate 4.10) that predominated during 500 days (4.6.1 and 5.2.3). However, a relatively high viability may generally be expected in low-loaded sludges II since other characteristic parameters as a low PA_{spec} (5.2) and a high cell yield (5.4) were also observed in low-loaded sludges II predominated by other bacteria. These data indicate relatively low numbers of lysing cells and low death rates in sludges II where a suspended growth of bacteria predominated.

5.4. YIELD COEFFICIENT AND BIOMASS TURNOVER COEFFICIENT OF STARCH-GROWN SLUDGES IN RELATION TO MEAN CELL RESIDENCE TIME

The supposed (extracellular) turnover (5.1.1) in activated sludge is one of the factors (5.1.3) expected to minimize yield at increasing mean cell residence times. The yield coefficient is related to the specific growth rate by the equation:

$$Y_{obs}^{-1} = Y_{max}^{-1}\,(1 + a \cdot t_s) \qquad (5b)$$

This equation, or its equivalent, has been derived from experiments with pure continuous cultures (HERBERT, 1958; MARR et al., 1963) and with activated sludge (HEUKELEKIAN et al., 1951; LAWRENCE and MCCARTY, 1970) and has been discussed in 5.1.3.

The maintenance energy requirement of activated sludge cannot be estimated separately as in this system death, decay and lysis of cells, predating activities by protozoa and other higher organisms, and reutilization of (dead) cell material by bacteria can occur and result in an energy consumption. Possibly, non-viable cells consuming substrate also occur in activated sludge. Hence, the effect of these processes on the yield coefficient will be expressed as the biomass turnover (decay) coefficient (a^*), as is common practice in the sanitary engineering literature. Attempts will be made to express the degradation rate of sludge extracellular enzymes in the same unit (day^{-1}).

5.4.1. *Principles and experimental procedure*

The growth yields of sludge (Y_{obs}) on starch as the limiting substrate were estimated in systems I and II, most of which are represented in Figs. 4.6A, 4.6B and 5.1. Table 4.3 shows that the concentrations of the carbon compounds in the sludge-free liquid were low as compared to the amounts of starch administered. Therefore, growth yields were calculated on the basis of the latter amounts, expressed as glucose equivalents (as was done by MARR et al., 1963). As to sludge I at SL = 0.075 a long period was required, after the decrease of SL from 0.3 to 0.075, before a steady state was attained with respect to the yield observed (compare with DA_{spec}, 4.6.1 and viability, 5.3). The same was true of sludge II at SL = 0.121 (Fig. 4.6B).

The system without retention of biomass (II) is a completely mixed open continuous culture (chemostat). In this system the mean cell residence time and cell yield equal the mean hydraulic retention time and the amount of biomass in the reactor, respectively, provided that steady-state conditions were achieved.

The parameters Y_{obs}, μ_{net} and t_s of very high- and of lower-loaded sludge systems I were determined in two different ways. The very high-loaded activated sludge approximated a really continuously operated culture since the sludge concentration was kept about constant by automatic removal of waste sludge every 2 hours (2.4.2). This excess (waste, removed) sludge and the effluent were kept in ice water until the dry weight was determined. The biomass concentration in the reactor varied at most 18 % during the determination period of one day. The average concentration of reactor biomass ($\bar{X}_1$) is presented in Table 5.7. The t_s value was deduced from the biomass balance equation for a completely mixed reactor with retention of biomass: $V(dX/dt)_{net} = \mu_{net} X_1 V - QX_2$ or (with $D = Q/V$): $(dX/dt)_{net} = \mu_{net} X_1 - DX_2$, where X_1 is the averaged biomass concentration in the reactor, X_2 is the biomass concentration in the mixture of removed sludge and (sponge) effluent and Q is the liquid flow rate (l.day^{-1}). The condition of 'steady state' is defined by $(dX/dt)_{net} = 0$, thus:

$$\mu_{net} = t_s^{-1} = \frac{QX_2}{VX_1} = \frac{DX_2}{X_1}\ (\text{day}^{-1}) \qquad (7)$$

DX_2 is the productivity i.e. the daily production of biomass per unit volume of culture. It is corrected for the slight biomass variation in the reactor and is given in Table 5.7, for reasons of comparison with that at lower loadings, as corrected

ΔX_1/day (g/day.l), i.e. the imaginary increase of biomass in the reactor if the removed biomass would have been returned to the reactor.

At the other loadings (planned SL values 1.2, 0.3 and 0.075) no sludge was removed during the measurements, except for sludge withdrawn by sampling or wash-out (occasionally small amounts of cells escaped through the spongy filter element). The biomass increase in the reactor was corrected for these losses and for increased sludge volume in the reactor, resulting from an occasionally clogged filter (Table 5.7: corrected ΔX_1). The biomass in the reactor increased about proportionally with time when observed over limited periods. In this respect the unsteady-state system resembled a steady-state system, in which the biomass production is constant. This proportionality enabled the calculation of $\bar{\mu}_{\text{net}}$, averaged equally over the experimental period of unsteady state; this period was one day (SL = 1.2), several days (SL = 0.3) or several weeks (SL = 0.075). Half-way this period the biomass concentration approached $(X_{t=o} + X_{t=t})/2 = \bar{X}_1$ (Table 5.7), in which $X_{t=o}$ and $X_{t=t}$ represent the reactor biomass concentrations, not corrected for losses of sampling and wash-out, at the beginning and end of the experiment. Analogously to equation (7),

TABLE 5.7. Relationship between arithmetically averaged sludge loadings, mean cell residence times and yields (coefficients) of starch sludges I and II.

VL (g COD per l per day)[1]	D (day^{-1})	Viable[2] bacteria (%)	n[3]	$\bar{X}_1$[4] (g per l)	$\overline{\text{SL}}$[5] (g COD per g sludge per day)	Corrected ΔX_1 per day[6] (g per day per l)	$\bar{t}_s$[7] (day)	Y_{obs}[8] (g biomass per g GE)
sludge I								
6.0	3.0	91	6	2.70	2.23	2.68	1.01	0.475 ± 0.051
3.0	1.0	69	1	2.36	1.27	1.20	1.98	0.424
0.75	1.0	26	5	3.16	0.24	0.196	16.1	0.278 ± 0.027
0.188	1.0	4.5	4	1.80	0.104	0.0357	50.5	0.203 ± 0.013
sludge II				X_1	SL		t_s	
3.0	1.0	90	8	1.42	2.12		1	0.503 ± 0.031
0.75	0.05	36	28	6.21[9]	0.121		20	0.441 ± 0.042[9]

[1] 1.000 g COD is equivalent to 0.939 g glucose; [2] estimates obtained from sludges with slightly different SL values (Table 5.6); [3] data given are the averages of n averaged estimates ($\bar{X}_1$, $\overline{\text{SL}}$, $\bar{t}_s$) or averages of n single estimates (corrected ΔX_1/day, Y_{obs} and, as to sludge II: X_1, SL and t_s); [4] biomass concentrations in the reactor; [5] $\overline{\text{SL}} = \text{VL}/\bar{X}_1$; [6] productivity: (imaginary) daily increase of biomass per l reactor contents (biomass concentration corrected for all biomass losses); [7] sludge I: $\bar{t}_s = \bar{X}_1$/corrected ΔX_1 per day; sludge II: $t_s = D^{-1}$; [8] sludge I: Y_{obs} = corrected ΔX_1 per day/VL (VL expressed as g glucose equivalents/l. day); sludge II: $Y_{obs} = X_1/S_0$ (S_0 = g GE/l influent); the 95% confidence interval of the mean of n observations is given by the formula: mean $Y_{obs} \pm ts/\sqrt{n}$, where s = sample standard deviation and t = Student's t, as obtained from STEEL and TORRIE (1960; t tests); [9] biomass concentrations measured between 111 and 510 days of loading (Fig. 4.6B); because of the interdependency of these observations, the 95% confidence interval of mean Y_{obs} was calculated for $n = 7$ (intervals between observations more than 3 t_s days) instead of $n = 28$.

$$\bar{\mu}_{\text{net}} = t_s^{-1} = \frac{\text{corrected } \Delta X_1}{\text{day} \cdot \bar{X}_1} \ (\text{day}^{-1}) \tag{8}$$

This resembles the true steady-state situation in which the biomass concentration in the reactor is maintained at $\bar{X}_1$.

The batch formula:

$$\mu_{\text{net}} = t_s^{-1} = \frac{\ln X_{t=t} - \ln X_{t=o}}{t} \ (\text{day}^{-1}) \tag{9}$$

can also be used to describe this unsteady state. $X_{t=t}$ represents then the biomass concentration corrected for sludge losses. Formula (9) is derived from: $(dX/dt)_{\text{net}} = \mu_{\text{net}} X$. In our experiments $(dX/dt)_{\text{net}}$ appeared to be constant within certain biomass limits, so $\mu_{\text{net}} = \text{constant}/X$. Since X increased proportionally with time, μ_{net} decreased proportionally with time. When we calculate μ_{net} according to equation (9) over a whole growth period, $\bar{\mu}_{\text{net}}$ represents the (period) average of the decreasing μ_{net} values during the growth period. Since the productivity is constant and $\bar{X}_1$ represents the average X_1 in the reactor, it can be written: $(\Delta X/\Delta t)_{\text{net}} = \bar{\mu}_{\text{net}}\bar{X}_1$ or $\Delta X_{\text{net}} = \bar{\mu}_{\text{net}}\bar{X}_1 \, \Delta t$. It resembles a (continuous) exponential growth, in which the biomass concentration in the reactor is maintained not at $X_{t=o}$, but at $\bar{X}_1$.

5.4.2. *Results*

The averaged results obtained with both systems I and II are presented in Table 5.7, the individual estimates of Y_{obs} and t_s of sludge I are plotted against SL

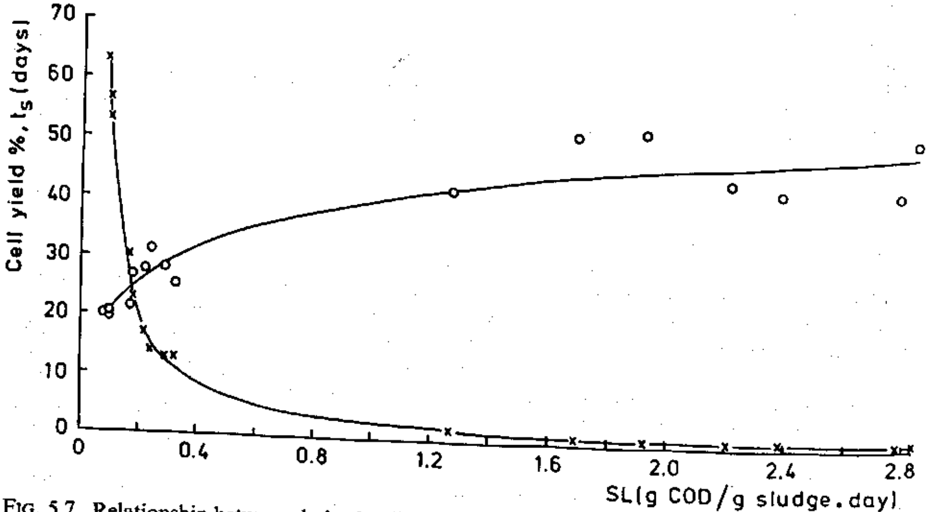

FIG. 5.7. Relationship between sludge loading, yield and mean cell residence time of starch-grown sludge I (cf. Table 5.7). O, Observed yield of biomass as percentage of added substrate (expressed as glucose); x, mean cell residence time.

in Fig. 5.7. The fluctuations of the yield coefficient obtained with sludge I increased with SL (Fig. 5.7). They may be attributed to the large variations of the predominating microbial species. The average Y_{obs} of this system decreased from about 50% at t_s values of 1 day to about 20% at t_s values of 50–60 days. This shows that at very low continuous loadings within feasible sludge retention times still 20% of the added substrate is converted to biomass. Consequently, biomass decay had proceeded only until about 40% (20% compared to 50%) of the maximally synthesized biomass (total suspended solids) had been left. According to KOUNTZ and FORNEY (1959) about 23% of the biological solids produced in activated sludge systems are relatively inert to biological oxidation within reasonable times and therefore accumulate in the system. In the systems II the decrease of Y_{obs} appeared to be much less pronounced (Table 5.7). The high Y_{obs} at a t_s value of 20 days was apparently due to the presence of a *Nocardia*-like bacterium which predominated for 500 days. This organism was growing in suspension, was non-proteolytic and was surrounded by a heavy slime layer (4.6.1 and 5.2.3). High yields were also observed during some shorter experimental periods with sludges having t_s values of 10 and 20 days. During such periods other types of bacteria, also growing in suspension or in loose aggregates, predominated. These results suggest that the high Y_{obs} in sludge II was due to the relatively favourable growth conditions prevailing in a suspended monoculture compared with cells occurring in flocs, rather than to the occurrence of one special type of organism. The characteristically low PA_{spec} (5.2.3) and possibly a high % viable bacteria (Table 5.6) also point to more favourable growth conditions in system II.

Plotting Y_{obs}^{-1} versus t_s values of sludge I does not show a linear relationship (Fig. 5.8). Although the validity of a constant value for the specific rate of biomass turnover (over the whole range of food-to-organism ratios) is doubtful, the concept has been found satisfactory when applied to laboratory scale

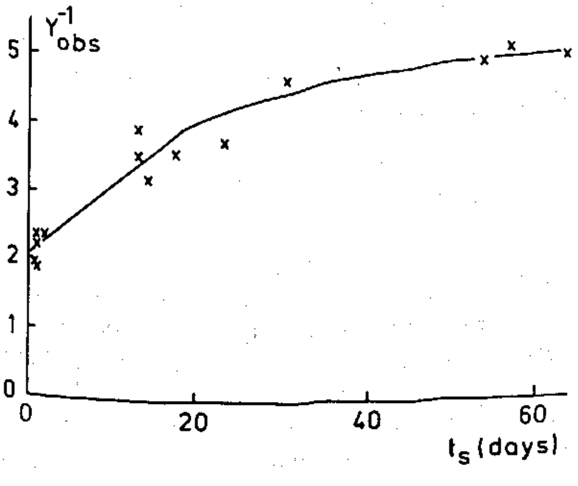

FIG. 5.8. The reciprocal of the observed yield coefficient versus cell residence time of starch-grown sludge I. Within t_s values of 1–18 days the yield may be approximately described as: $Y_{obs}^{-1} = Y_{max}^{-1} + a.Y_{max}^{-1}.t_s = 2.055 + 0.103\,t_s$ ($r. = 0.93$; least square fitting). Data are those plotted in Fig. 5.7.

activated sludge over a limited range of cellular residence times, viz. 2–18 days (SHERRARD and SCHROEDER, 1972) and to widely varying activated sludges and sewages (HEUKELEKIAN et al., 1951). If, therefore, a linear relationship is assumed to exist in the t_s range of 1–18 days, Y_{max} and a^* values of 0.49 g dry biomass per g glucose equivalent and 0.050 day^{-1}, respectively, can be calculated from the data available ($n = 11$, Fig. 5.8). The value of a^* is in agreement with the a^* value evaluated under a variety of environmental conditions in activated sludge (Table 5.1A). The 'apparent' maintenance coefficient, improperly derived from the turnover coefficient according to $m = a\ Y_{max}^{-1}$ and amounting to 0.103 g glucose per g sludge per day, i.e. 0.0043 g glucose per g sludge per h at 20 °C, is clearly lower than the m values of Gram-negative organisms grown aerobically in defined media (Table 5.1B), in spite of the presence of predators in starch activated sludge. However, such a comparison is not allowable as is concluded e.g. from the observation that the graph of Y_{obs}^{-1} of *Aerobacter aerogenes* (*Klebsiella pneumoniae*), for glycerol at 37 °C, against t_s departed sharply from linearity above a t_s value of less than 1 day (data of TEMPEST et al., 1967, presented by PIRT, 1972). At this stage the non-viable bacteria amounted to about 10%.

The decline of the turnover coefficient of activated sludge above t_s values of 20 days (Fig. 5.8; cf. the low, inaccurate, value of a^* of extensively aerated activated sludge, MIDDLEBROOKS and GARLAND, 1968, Table 5.1A) is likely to be attributed to an increased effect of two factors: cryptic growth and a decay coefficient of dead organisms being clearly smaller than that of viable organisms. Partly cryptic growth is likely to occur at all loadings (5.2), but completely cryptic growth was only clearly observed at SL = 0.075 (4.6.2). The organic material released from lysing cells apparently serves as a substrate for the bacteria and should therefore be corrected for in the yield formulae. In the present study no correction was made but the term biomass turnover coefficient was preferred to the term biomass decay coefficient. That the decay coefficient of dead organisms and cell debris is likely to be clearly lower than that of viable cells may be derived from the unexpectedly high Y_{obs} at t_s values of 50–60 days and the low % viable bacteria at these t_s values. This high Y_{obs} was only to a small extent due to a high ash content (Table 5.9) in spite of the presence of excessive amounts of mineral salts in the sludge liquid.

5.5. INACTIVATION OF AMYLASES

Although the data of 5.2, 5.3 and 5.4 strongly suggest that proteolytic activity is a measure of the intensity of extracellular turnover of cell material in sludge systems, no direct evidence of the degradation of any cell component by proteolytic enzymes was given. Preliminary experiments showed a rapid decrease of DA when starch-grown sludges (I, SL = 0.3) were starved in the reactor. In this section, experiments on the correlation between PA_{spec} and the inactivation rate of amylases are reported and discussed.

Two sludges, one of type I and one of type II, were low-loaded with starch during about 100 days, whereafter steady-state conditions were expected to occur, at least with regard to PA$_{spec}$ (5.2). After the supply of all nutrient components to the reactors had been ceased (SL = 0, VL = 0), the course of PA, DA and biomass concentration with time was followed (Fig. 5.9). The initial DA values of sludges I en II were about the same as contrasted to the proteolytic activity which in I was 10–20 times higher than in II. The initial decrease of DA in sludge I was 10–13 times greater than that in sludge II; the proteolytic activity remained rather constant in both systems. The amylases of the starving sludge systems I and II were inactivated for 50% in periods of slightly less than 1 day and 14 days, respectively. No DA was detected in the sludge-free liquid during starvation. When the continuously loaded sludge I with a constant DA$_{spec}$ (Fig. 4.11) was submitted to starvation, its amylolytic activity decreased immediately (Fig. 5.9A). This suggests that the ready inactivation of amylases occurring initially under starvation conditions, proceeds continuously under apparent

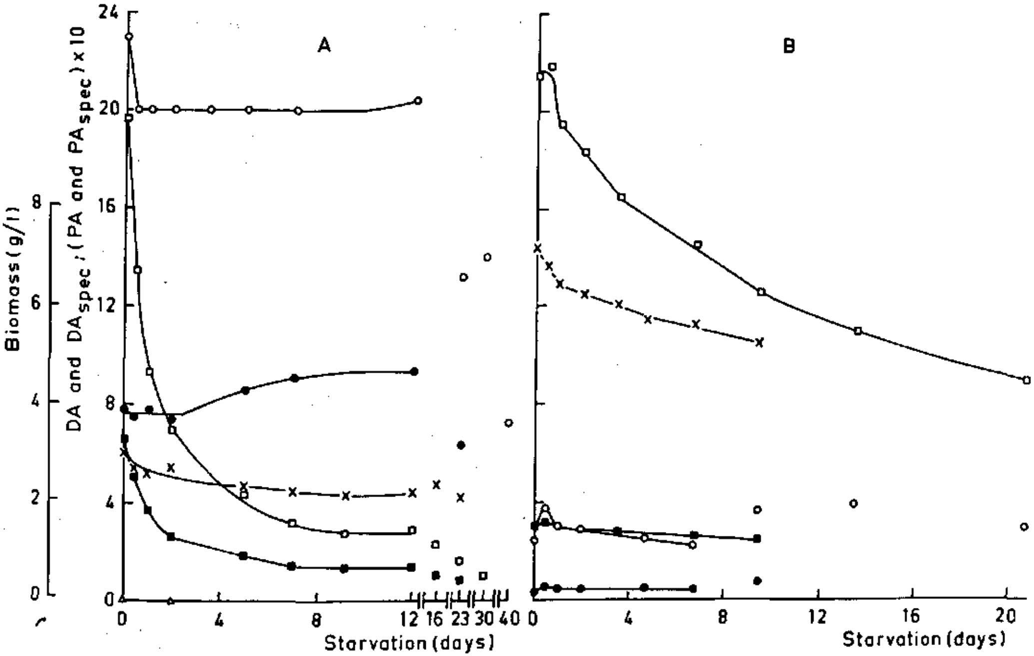

FIG. 5.9. Starvation of starch sludges I and II after previous loading during approximately 100 days with VL = 0.75 g COD/l.day and operating (A) with and (B) without retention of flocculated biomass. History during starch loading: (A) $D = 1$ day^{-1}, SL = c. 0.25, pH 8.8, 20°C (Fig. 4.11); (B) $D = 0.05$ day^{-1}, pH 7.0, c. 23°C, biomass increased from 1.4 g/l to c. 7 g/l (after about 9 weeks) which was maintained during the subsequent 6 weeks (SL = c. 0.1). During starvation (SL = 0, VL = 0) sludges were kept in the reactor under similar conditions as during starch loading. PA was measured under conditions somewhat different from standard conditions and some evaporation occurred during starvation of (A). ○ and ●, PA and PA$_{spec}$, respectively; □ and ■, DA and DA$_{spec}$, respectively; △, DA in sludge-free liquid; x, biomass.

steady-state conditions during continuous loading with starch. However, under these conditions it is compensated by a continuous enzyme synthesis so that $\mathrm{DA}_{\mathrm{spec}}$ remains constant (Fig. 4.11).

5.5.1. *Protection of amylases by excess casein or starch*

Although sludge I (mixed culture in flocs) differed considerably from sludge II (predominated by an unspecified bacterium growing in suspension outside the few flocs), the experimental data plotted in Fig. 5.9 suggested a correlation between proteolytic activity and inactivation rate of amylases. Such a correlation would be demonstrable if proteinase inhibitors, leaving DA unaltered, could be applied to the proteolytic enzymes of activated sludge. For lack of such inhibitors, the effect of an excess of a proteinase substrate (casein) on inactivation of amylases was studied. This excess of substrate was thought to prevent amylase degradation by saturating the proteolytic enzymes. Figs. 5.10A and B show that 15 g of casein added per litre of starving sludge completely protected the amylases present against inactivation, as long as casein was not degraded itself. The results of this experiment provide strong evidence that proteolytic enzymes are indeed fully responsible for the inactivation of amylases,

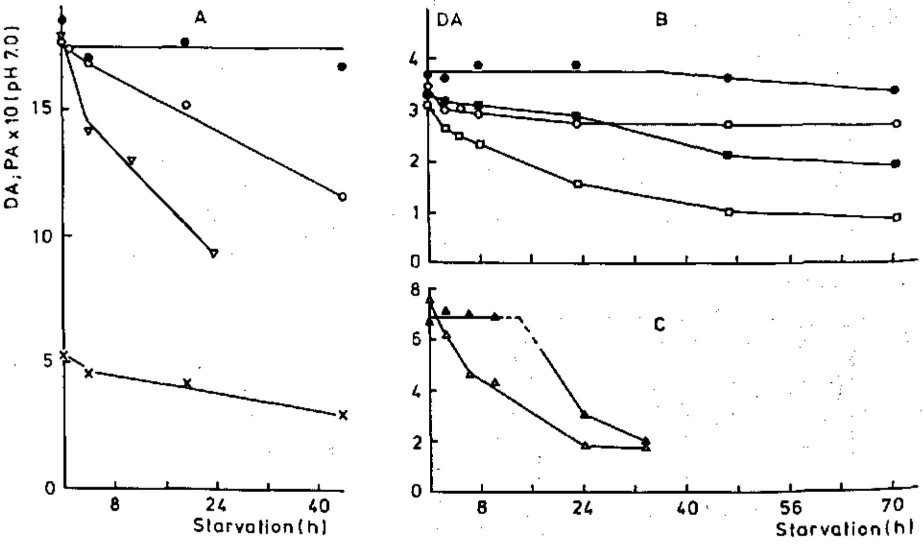

FIG. 5.10. Inactivation of amylases and proteinases in starving starch sludges I or cell-free ultrasonicates. The experiments were carried out in a water bath at 30°C under standard conditions as described in 2.6.13, with the exception of sludge C which was submitted to starvation in the presence of 1 mM $\mathrm{CaCl_2.2H_2O}$ and 5 mM potassium phosphate and whose DA was measured at pH 6.3. (A) SL was 1.2 during 83 days; $\mathrm{PA}_{\mathrm{spec}}^{\mathrm{pH}\,7.5}$ = c. 0.19; (B) SL was 0.3 during 82 days; $\mathrm{PA}_{\mathrm{spec}}^{\mathrm{pH}\,7.5}$ = 0.31; (C) SL was 0.3 during 325 days; $\mathrm{PA}_{\mathrm{spec}}^{\mathrm{pH}\,7.5}$ = roughly 0.87. ○ and ●, DA of sludge without and with 15 g of casein per litre of starvation mixture, respectively; □ and ■, DA of cell-free ultrasonicate of sludge (disintegration time 10 min; 2.6.8) without and with 15 g of casein/l, respectively; x, $\mathrm{PA}^{\mathrm{pH}\,7.0}$ of sludge; ∇, DA of sludge with 4% toluene in the starvation mixture; △ and ▲, DA of sludge without and with 5 g of starch per litre of starvation mixture, respectively.

at least under the conditions described (2.6.13). This applies to cell-surface-bound amylases as measured under standard conditions (3.5.1 and 7.2).

Under the conditions reported in 2.6.13 proteolytic enzymes were also inactivated (Fig. 5.10A). This may have been due to self-digestion or to instability. The fact that in the starving activated sludge in the reactor the rate of inactivation of amylases gradually decreased (deviated from a first order reaction), whereas the proteolytic activity remained constant (Fig. 5.9A), indicates the presence of part of the amylases in sites less accessible to proteolytic enzymes. Another explanation is the presence of different types of amylases being more or less resistant to proteolytic enzymes.

Two treatments were found to accelerate the inactivation of amylases in starving activated sludge, viz. addition of toluene (Fig. 5.10A) and ultrasonication of sludge (Fig. 5.10B). This enhanced inactivation probably was not completely abolished by adding casein, at least in ultrasonicate (Fig. 5.10B), which might suggest that in the absence of proteolytic activity sludge amylases are stable if bound to the cell, but unstable if released from the cell wall (Chapter 7).

In the experiments performed with starch activated sludge (shown in Fig. 5.1 B-3) under exactly the same conditions as prevailing during the experiments with other sludges, the protective effect of casein on amylase was not apparent. In the former experiments the primary effect of added casein was an immediate and sharp increase of DA. This suggests that in addition to protecting the cell-surface-bound amylases against proteolysis, the added casein released amylases from the cell (periplasmic space?). The latter enzymes apparently were not protected by the casein as can be seen from the drop of the initially increased amylase activity. This result resembles those obtained upon ultrasonic treatment of the sludge (Fig. 5.10B and Chapter 7).

Besides casein, relatively high concentrations of starch completely protected all of the sludge amylases, measured as standard DA, against proteolytic degradation (Fig. 5.10C). Mono- and oligosaccharides, derived from starch, and 1 mM $CaCl_2 \cdot 2H_2O$ gave no protection. The protection observed is probably due to a tightening of the conformation of the amylase molecule or to steric hindrance. The protection of amylase by starch and protein is very unlikely to occur under steady-state conditions of starch-grown activated sludge in the reactor. This is concluded from the very low concentrations of iodine-stainable, anthrone-positive (4.5) and Lowry-positive material in the sludge-free liquid under these conditions. Moreover, the substrate constants of the amylolytic and proteolytic enzymes are relatively high (Chapter 6). The rather constant amylolytic activity per (viable) amylolytic cell observed in the SL range from 2.4 (high viability) to 0.3 (low viability) (Table 4.4) is a further argument in favour of the absence of effective protection of amylases of (dead) cells. Effective protection may be expected to occur only after very high shock loadings, as occur under the conditions of the DA assay method (Fig. 3.4A). This adequate, prolonged protection is a fortunate condition, enabling the precise determination of DA.

5.5.2. *Quantification of the inactivation of amylases*

To quantify the relation between proteolytic activity of activated sludge and the inactivation rate of amylases in the reactor it was assumed that the inactivation of amylases in a distinct starving sludge follows first order kinetics, at least in the first phase. Furthermore, that this initial inactivation rate is equal to the rate of the (probably) continuously proceeding inactivation of amylases in sludge under steady-state conditions, i.e. in situ. The decrease of DA, following first order kinetics, is represented by:

$$-\frac{d(DA)}{dt} = k_i\,(DA) \text{ or, after integration: } (DA)_t = (DA)_o \cdot e^{-k_i \cdot t} \text{ or}$$

$$k_i = \frac{\ln (DA)_o - \ln (DA)_t}{t}$$ where $(DA)_o$ and $(DA)_t$ represent DA at o and t days, respectively, and k_i is the specific (amylolytic) enzyme inactivation rate (day^{-1}). This coefficient was calculated for the initial (maximum) rate of inactivation of amylases. When the log of the residual DA fraction of various starving starch-grown activated sludges was plotted against time (Fig. 5.13), an initially linear decrease of log DA was often observed. These experiments were performed with sludge samples taken from the reactors and incubated in a water bath under standard conditions (30 °C, 0.1 M buffer, 500 mg chloramphenicol/l; section 2.6.13). Fig. 5.11 shows that k_i at 30 °C was about twice that at 20 °C, which is in agreement with the observed Q_{10} value of about 2 for PA (3.6.2). Under reactor conditions at 20 °C, the decline of the inactivation rate with time was less pronounced than it was the case under standard conditions at 30 °C. This effect was probably due to the difference in applied buffer concentrations (see Fig. 5.12). However, the initial inactivation rates were independent of the buffer concentrations tested (Fig. 5.12). This was true even when the buffer was

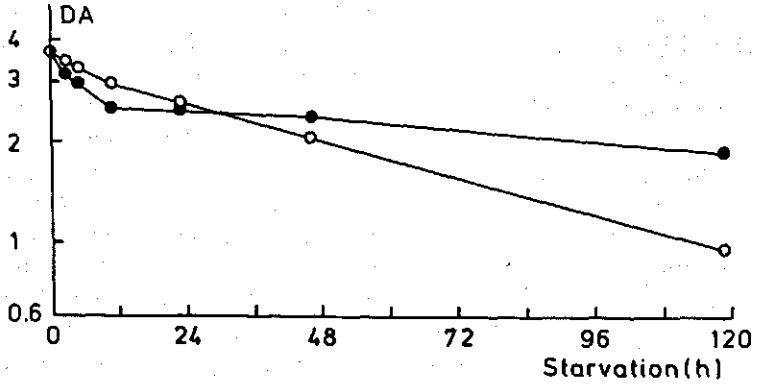

FIG. 5.11. Inactivation of amylases of starving starch activated sludge I under standard and reactor conditions. The sludge had been loaded previously during 202 days (SL = 0.3; Fig. 5.1B-3). DA values were measured at 30 °C and applied to the original sludge concentration: ○, sludge starved under reactor conditions i.e. in the reactor with continuous aeration, at pH 7.0 and 20 °C; ●, sludge starved in a water bath under standard conditions (30 °C; pH 7.0; 0.1 M potassium phosphate; 500 mg chloramphenicol/l and shaken at c. 100 strokes/min; see 2.6.13).

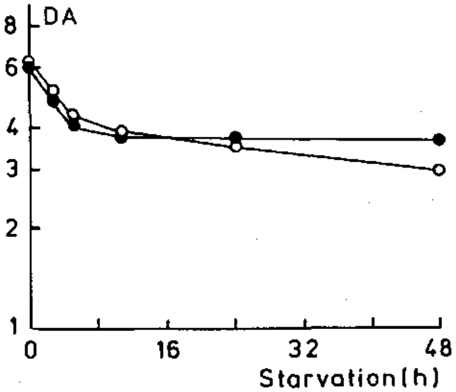

FIG. 5.12. Inactivation of amylases of starving starch sludge I at different buffer molarities. The sludge had been loaded previously during 26 days (SL = 0.3; Fig. 5.1B-3). Conditions: 2.6.13. The activities were applied to the original sludge concentration: ●, sludge starved at 30°C and 0.1 M potassium phosphate (k_i = 2.0); ○, sludge starved at 30°C and 0.005 M potassium phosphate (k_i = 1.8).

completely omitted (not shown). Since under standard conditions (100 mM buffer) surface-bound amylases in starving sludge were shown to be inactivated only by proteolytic enzymes (Figs. 5.10A and B), the same can be stated of the initial inactivation phase in sludge starving under reactor conditions and likely of the in situ inactivation in sludge under steady-state conditions. The greater decrease of amylase activity after prolonged sludge starvation at low as compared to high potassium phosphate concentrations might be attributed to adsorption of cell-released amylases by sludge. This suggestion originated from the results of experiments on adsorption of added fungal amylase by activated sludge. Such an adsorption occurred only at low potassium phosphate concentrations and resulted in immediate inactivation of the enzyme (7.4).

Some of the inactivation experiments performed under standard conditions with activated sludges grown at different starch loadings, are shown in Fig. 5.13. The initial DA and DA_{spec} values of the investigated sludges differed to a large extent; the ratios of the maximum to the minimum values were 78 and 31, respectively. Although these experiments were difficult to perform and consequently the k_i values, calculated over periods of 2½–46 h of inactivation, are rough estimates, the plot of the specific (i.e. relative) amylase inactivation rate (k_i; day^{-1}) against PA_{spec} suggests a linear relationship over the whole range of PA_{spec} values found in the SL range of 0.075 to 2.4 (Fig. 5.14A). Plotting the amylase inactivation rate ($k_i \cdot DA$; $DA \cdot day^{-1}$) against PA_{spec} fails to show any meaningful relationship (Fig. 5.14B). Fig. 5.14A shows only slightly differing k_i values at distinct PA_{spec} values in spite of widely different DA values. This leads to the conclusion that first order kinetics in amylase degradation by proteolytic enzymes is involved. This is in agreement with the fact that the reactions of amylase inactivation fall within the low substrate range (first order range) of the substrate saturation curve of proteolytic enzymes of activated sludge (6.6).

The average relation shown in Fig. 5.14A is defined as $k_i = 2.5\ PA_{spec}$ by application of the linear regression method to the data available. The corresponding value of k_i at 20°C under reactor conditions is calculated to be $1.25\ PA_{spec}$ (Fig. 5.11), which was approximately verified by 5 experiments

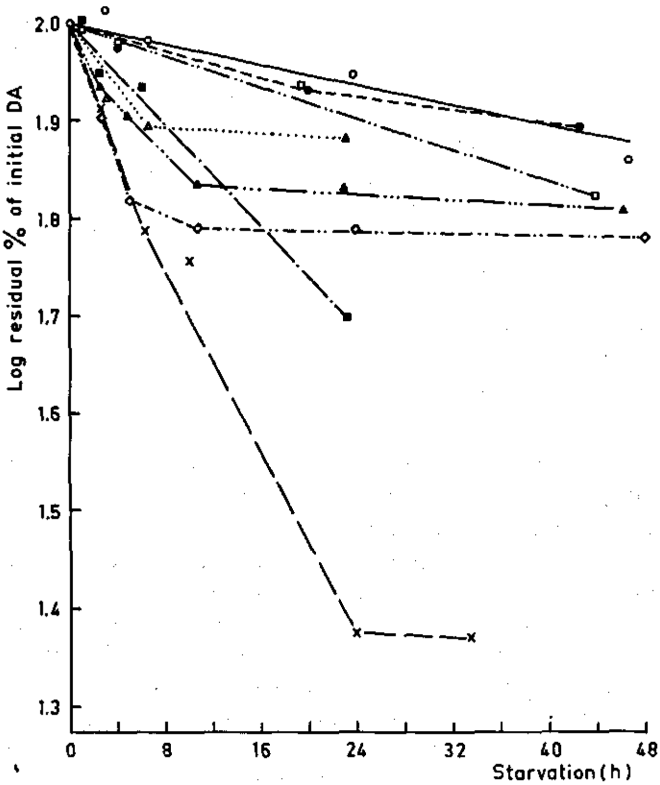

FIG. 5.13. Inactivation of amylases of differently loaded starch sludges I during starvation under standard conditions (2.6.13) in a water bath at 30°C. The conditions of one sludge (x) were as those reported in Fig. 5.10C. Sludge concentrations during starvation amounted to 40–100% of the original concentrations. The DA values of the original sludges varied between 0.90 and 71 and DA_{spec} between 0.71 and 21.8. All of the initial DA values were taken as 100% values. The sludges were arranged in order of increasing initial inactivation rates.

	SL	DA	PA_{spec}
○ (———)	2.4	14.9	0.038
● (---------)	1.2	71	0.20
□ (··—··—)	1.2	17.7	0.19
■ (·—·—)	1.2	5.9	0.32
△ (........)	0.075	0.90	0.39
▲ (···—···—)	0.3	3.7	0.66
x (— — —)	0.3	7.5	0.87 (rough estimate)
◇ (··—··—)	0.3	6.0	0.50

performed in the reactor. Although some variations of k_i values at a distinct PA_{spec} occur, presumably indicating different sensitivities of amylases to proteolytic attack, Fig. 5.14A permits to draw important conclusions and to transform the first order relation $-d(DA)/dt = k_i(DA)$ into the equation $-d(DA)/dt = 1.25\ (DA)\ (PA_{spec}^{30°C})$ (valid at 20°C when PA_{spec} measured at 30°C). The latter equation predicts roughly the initial amylase inactivation rate

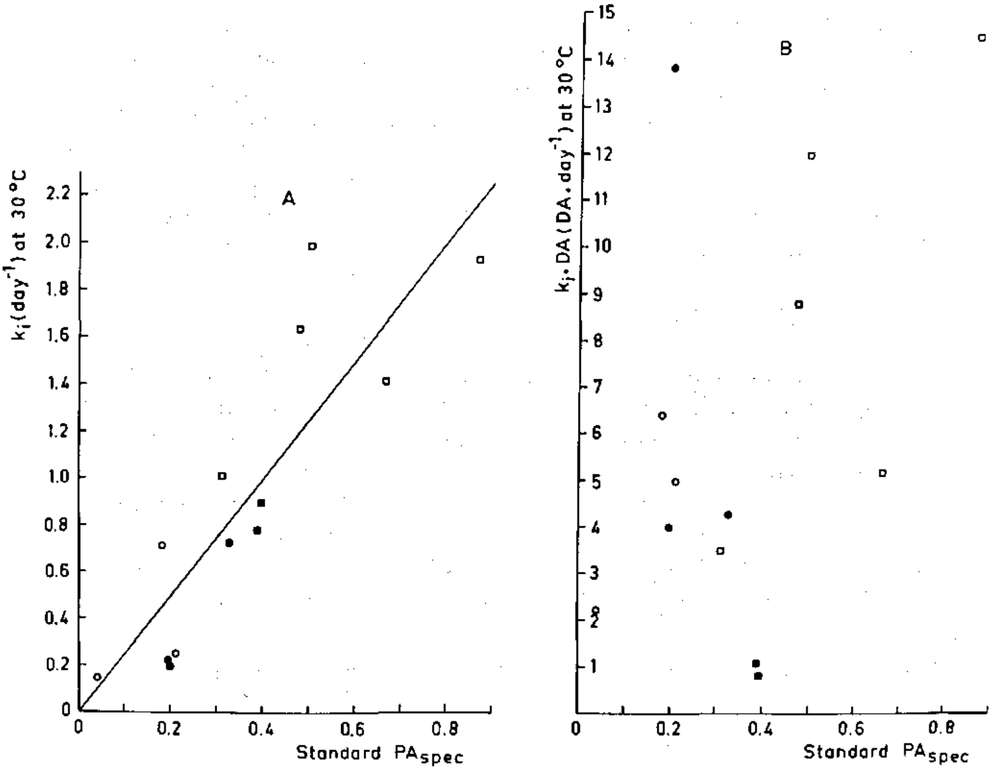

FIG. 5.14. Relation between PA$_{spec}$ of differently loaded starch sludges I and specific amylolytic enzyme inactivation rate (k_i; Fig. 5.14A) or amylolytic enzyme inactivation rate (k_i.DA; Fig. 5.14B). PA$_{spec}$ was estimated according to 2.6.12, initial inactivation rates in starving sludges according to 2.6.13. Part of the data were obtained from Fig. 5.13, where further information is given. The line was drawn by best linear fit of the experimental data ($n = 13$) giving equal weight to each data point: $k_i = 2.51$ PA$_{spec}$; $r = 0.87$. ○, ●, □and ■, sludge with SL = 2.4, 1.2, 0.3 and 0.075, respectively.

in various starving starch activated sludges with different DA and PA$_{spec}$ values. This equation confirms the evidence presented earlier (Fig. 5.10) that only proteolytic enzymes (and not e.g. adsorption by sludge) are responsible for the (initial) inactivation of amylases in laboratory-grown activated sludges. From the fact that k_i is determined only by PA$_{spec}$, it is derived that no other processes, if occurring at all, are rate-limiting the proteolytic amylase degradation. Such processes might include the release of amylases from the cell wall surface into the liquid, followed by a ready proteolytic inactivation, or they might include the removal of calcium ions from the amylase molecules (5.1.4). Amylases and proteases occur in cell wall-bound condition in continuously loaded activated sludge (7.2). Hydrolysis of amylases probably proceeds in cell wall-bound condition by cell wall-bound proteases. This is deduced from the equal initial inactivation rates at 0, 5 and 100 mM potassium phosphate (Fig. 5.12), the last concentration releasing amylases as well as proteases into the liquid (Table 7.2), and from the almost equal initial inactivation rates of amylases in whole sludge and cell-free ultrasonicate (Fig. 5.10B), the latter containing considerable amounts of 'free' amylases and proteases (Chapter 7). These results show that the rate of proteolytic degradation of amylases is hardly or not affected by the condition in which the enzymes occur in the sludge. As amylases (and probably also proteases) do not occur in the cell-free liquid of starving activated sludge (Fig. 5.9) it is concluded that proteolytic breakdown of amylases occurs in the cell wall-bound condition.

5.5.3. *Daily gross and net production of amylases in starch activated sludges in situ*

Table 5.8 gives a compilation of the data shown in Fig. 5.14A and those of other figures and tables recorded in Chapters 4 and 5. In addition, tentatively estimated average production rates of amylolytic enzymes in continuously operated starch activated sludges are given. Values of k_i in the lower loaded sludges were calculated to be on the average twice as high as those in the higher loaded sludges. The net production of amylolytic activity units (as g SE/h) per g sludge per day is $\mu_{net} \cdot \tilde{\mathrm{DA}}_{spec}$, i.e. the amylase wastage (removal) rate, since all of the DA is bound to the sludge. As was argued above, the rate of amylase

TABLE 5.8. Rough estimates of average daily net and gross production of amylases (expressed as amylolytic activity) per g starch sludge I over the whole range of applied loadings at 20°C.

SL	0.075–0.10	0.24–0.3	1.2	2.4
$\overline{\mathrm{PA}}_{spec}$[1]	0.372	0.366	0.193	0.191
Y_{obs} of biomass[2]	0.203	0.278	0.424	0.475
Viable bacteria %[3]	4.5	26	69	91
t_s (day)[2]	50.5	16.1	1.98	1.01
μ_{net} (day^{-1})[4]	0.020	0.062	0.51	0.99
k_i (day^{-1})[5]	0.47	0.46	0.24	0.24
$\tilde{\mathrm{DA}}_{spec}$[6]	0.635	1.80	4.72	7.02
Net production of amylol. act. per g sludge per day[7]	0.013	0.11	2.38	6.97
Gross production of amylol. act. per g sludge per day[8]	0.31	0.93	3.52	8.65
Imaginary $\tilde{\mathrm{DA}}_{spec}$, if $k_i = 0$[9]	15.56	15.06	6.96	8.71
Imaginary $\tilde{\mathrm{DA}}_{spec}$, if $k_i = 0$ and $a^* = 0$[10]	6.32	8.37	5.91	8.27

[1] Fig. 5.2A; [2] Table 5.7; [3] Table 5.6; [4] $\mu_{net} = t_s^{-1}$ = specific sludge wastage rate; [5] calculated with the formula: $k_i = 1.25\,(\overline{\mathrm{PA}}_{spec})$ (valid at 20°C); [6] Table 4.4; [7] $\mu_{net} \cdot \tilde{\mathrm{DA}}_{spec}$: amylolytic activity units (as g SE/h) removed daily from the reactor under steady-state conditions (calculated per g sludge); [8] $(\mu_{net} + k_i) \cdot \tilde{\mathrm{DA}}_{spec}$: net daily production + production compensating for daily degraded amylases (calc. per g sludge); [9] imaginary $\tilde{\mathrm{DA}}_{spec}$ = gross production of amyl. act./g sludge $\cdot$ day $\cdot\ \mu_{net}$, i.e. if no proteolytic degradation of amylases occurred but gross production of amylases remained equal to that reported in this Table; [10] imaginary $\tilde{\mathrm{DA}}_{spec}$ = gross production of amylolytic activity $\cdot\ Y_{obs}$/g sludge $\cdot$ day $\cdot\ \mu_{net} \cdot Y_{max}$, i.e. if $k_i = 0$ and no decrease of biomass, due to maintenance, decay etc. occurred (i.e. $a^* = 0$), but gross production of amylases remained equal to that reported in this Table; Y_{max} is supposed to be 0.5.

inactivation by proteases is $k_i \cdot \mathrm{D\tilde{A}_{spec}}$, whilst inactivation of excreted amylases by adsorption to sludge was negligible. Hence, the gross production of amylolytic activity per g sludge per day = $(\mu_{app} + k_i) \cdot \mathrm{D\tilde{A}_{spec}}$.

In the two low-loaded sludges the gross production of amylolytic activity per g sludge per day is about 50 % of $\mathrm{D\tilde{A}_{spec}}$, the net production is about 2 and 6 % of $\mathrm{D\tilde{A}_{spec}}$. The very high turnover rate of amylases is surprising since these sludges are characterized by a low viability, a low μ_{net} and a low net production of amylases. In the two high-loaded sludges the gross production of amylolytic activity per g sludge per day is about 75 and 125 % of $\mathrm{D\tilde{A}_{spec}}$, but in these sludges the net production accounts for the greatest part of gross production.

The net production rate of amylases at SL = 2.4 is roughly 550 times as high as that at SL = 0.075, as contrasted to the gross production rate which is roughly proportional to SL. This indicates that the gross yield of amylases (expressed as amylolytic activity) produced per g substrate, is roughly constant between SL = 0.075–2.4.

The constancy of the amylase yield coefficient can be illustrated by calculating the imaginary values which $\mathrm{D\tilde{A}_{spec}}$ would have if no turnover had occurred, i.e. if k_i were zero and Y always had the maximum value of 0.5 g biomass/g glucose equivalent. Table 5.8 (and Fig. 4.8A) show that these imaginary $\mathrm{D\tilde{A}_{spec}}$ values are about equal for all SL values applied, viz. on the average 7.2. This confirms that the proteolytic attack of amylases, estimated as k_i under the standard conditions of 2.6.13, is the only important factor responsible for inactivation of amylases in starch activated sludge under steady-state conditions. This is encouraging with respect to the reliability of the proposed model: gross rate of amylase synthesis = rate of proteolytic inactivation of amylase + rate of amylase wastage. The decrease of $\mathrm{D\tilde{A}_{spec}}$ with decreasing SL (Fig. 4.8) below this imaginary and maximum $\mathrm{D\tilde{A}_{spec}}$ value is explained only by large differences in decay rate between biomass and amylases of dead cells. From the data of Table 5.8 it is deduced that the $\mathrm{D\tilde{A}_{spec}}$ values at e.g. SL = 0.075 and 0.3 were reduced with factors 24.5 and 8.4, respectively, compared to the imaginary $\mathrm{D\tilde{A}_{spec}}$ (with $k_i = 0$) and the biomass only with factors 2.5 and 1.8, respectively. The different rates of decay resulted in an effective decay of $\mathrm{D\tilde{A}_{spec}}$ with factors 10 and 4.7, respectively.

The differences in decay rate can also be deduced from the high specific inactivation rate of extracellular amylase (k_i, assumed to represent amylase protein degradation) as compared to the specific turnover rate of biomass (a^*) including intracellular protein. For example, at SL = 0.3 (20 °C) k_i is 0.5 day^{-1} and a^* is 0.05 day^{-1} (5.4). Even if the observed decay of cellular mass in activated sludge was due only to real turnover (excluding processes as predation etc.), the degradation coefficient of biomass (without resynthesis) would maximally be twice as high as $a^* = 0.05\ \mathrm{day}^{-1}$.

The differences in decay rate between biomass and amylases discussed above can be derived directly from starvation experiments with sludge. Fig. 5.9A shows that $\mathrm{DA_{spec}}$ (i.e. the amylolytic activity per g sludge biomass) of sludge I decreased sharply during starvation. In contrast, $\mathrm{DA_{spec}}$ of sludge II hardly decreased (Fig. 5.9B), suggesting that the decay rates of biomass and amylases did not differ to a large extent in the latter sludge. Values of $k_i = 0.1$ (20°C) are not unusual in sludge II. This clearly demonstrates that sludge I is much more dynamic than sludge II, at least with respect to its amylolytic activity. Due to the high inactivation rate of amylases in sludge I, it is not surprising that dead cells have only small residual activities, as can be derived from the rather constant amylolytic activity calculated per (viable) amylolytic cell (Fig. 4.9, SL = 0.3 – 2.4).

5.6. SUPPOSED MECHANISM OF CELL PROTEIN TURNOVER

The results reported in 5.5 show that extracellular proteolytic enzymes only are responsible for the ready inactivation of the surface-bound extracellular amylases of starch-degrading bacteria in starch sludge I.

Extracellular proteolytic enzymes are probably also active in the degradation of intracellular protein of decaying sludge bacteria (extracellular turnover; 5.1.2). Hydrolysis of intracellular proteins may also be due to intracellular proteolytic enzymes (dying cells: autolysis and living cells: intracellular turnover). Some evidence is available in the literature that internal proteolysis in bacterial cells is much less important than that in mammalian cells (PINE, 1972; GOLDBERG and ST. JOHN, 1976). In continuous cultures of *Aerobacter aerogenes* (*Klebsiella pneumoniae*), TEMPEST et al. (1967) found a decrease of the RNA, DNA and carbohydrate content but no decrease of the protein content of the cells over the t_s range 1.25–250 h in spite of the low viability (less than 40%) of the culture at t_s = 250 h.

The fact that ultrasonic treatment of activated sludge gave no clear increase of proteolytic activity (7.2) was no evidence for an important role of intracellular proteolytic enzymes in the hydrolysis of intracellular proteins of sludge bacteria.

Access of extracellular proteolytic enzymes to the intracellular proteins of dying or dead bacteria requires preceding lysis of the cell wall of these cells, to be brought about either by autolytic enzymes or extracellular enzymes of bacteriolytic organisms acting on dead and, possibly, living cells.

During the work with laboratory-grown activated sludge, the following indications were obtained that lysis of the cell wall was the rate-limiting step in the process of degradation of cellular protein of dying or dead bacteria by extracellular proteases from proteolytic bacteria and likely in the whole process of extracellular turnover of intracellular protein.

1) Y_{obs} of sludge I with a t_s value of 50–60 days was unexpectedly high (Table 5.7). This high value was only partly due to the somewhat higher ash content of this sludge (Table 5.9). Although the percentage of viable cells of this sludge was no more than about 5 (Table 5.6), the protein content of the sludge was relatively high (Table 5.10); the concentration of Lowry-positive material in the sludge-free liquid was very low. An important part of this protein was apparently present in dead bacterial cells which presumably were poorly accessible to the

TABLE 5.9. Effect of sludge loading with soluble starch on the ash content of laboratory-grown sludge I.

Planned SL (g COD per g sludge per day)	Days of loading	Ash as % of dry wt of biomass
2.4	74	8.8
	129	8.7
1.2	93	6.7
0.3	123	8.3
	172	8.9
0.075	296	12.6
	307	12.1
	315	9.9
	343	12.5

extracellular proteolytic enzymes that were present in relatively large amounts (PA was high, Fig. 5.2). Hence lysis of dead cells was probably the rate-limiting step in reutilization (i.e. in turnover) of nitrogenous compounds.

2) When continuously operated starch activated sludge with a constant PA_{spec} was starved for 1–2½ days and subsequently continuously supplied with

TABLE 5.10. Effect of sludge loading with soluble starch on the protein content of laboratory-grown sludge. The protein was determined with the biuret method (2.6.10).

SL[1] (g COD per g sludge per day)	Days of loading	Protein as % of dry wt of biomass	Average %
Sludge I			
2.4	74	41.9	42.9
	129	46.4	
	269	42.0	
	278	44.8	
	306	39.2	
1.2	90	39.1	40.9
	93	42.7	
0.3	49	41.1	46.0
	77	46.9	
	154	48.3	
	169	47.6	
0.075	149	32.6	35.2
	152	32.1	
	315	43.9	
	343	32.1	
Sludge II			
0.12	464	32.8	
2.12	15	37.6	43.6
	92	44.1	
	107	49.1	

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[1] See Table 2.3.

starch, ammonium sulphate and other nutrients, a temporarily increased PA_{spec} was observed 10 or more days after resumed feeding (Fig. 5.1B-3). This peak is thought to have been due to the rise of (retarded) lysis of bacteria following increased death of cells during the starvation period. The postponed rise of PA_{spec} suggests that the retarded effective lysis had limited the supply of proteins from dead cells.

Aer. aerogenes (*Klebsiella pneumoniae*), harvested from continuous-flow culture, showed significant cryptic growth (increase of total count of population) after 4–6 days of starvation in 'saline-tris' buffer (POSTGATE and HUNTER, 1962).

To demonstrate the presence of extracellular bacteriolytic enzymes in activated sludge, use was made of a suspension of the Gram-positive *Sarcina lutea* which is sensitive to cell wall-dissolving enzymes like lysozyme and endopeptidases (2.6.14). Sludge-free liquid obtained by centrifuging starch activated sludge (SL = 0.3) was unable to clear up a suspension of *S. lutea*. That this negative effect was due to the absence of bacteriolytic enzymes in the sludge liquid and not to the presence of compounds inhibiting the functioning of these enzymes was shown by adding lysozyme (2 mg/l) to the mixture of sludge-free liquid and *Sarcina* suspension.

In a subsequent experiment, whole, unwashed starch sludge (SL = 0.3) was added to a suspension of *S. lutea* (+ buffer) and the mixture incubated aerobically at 25°C in a shaking water bath. A control culture had received no sludge. No lysis of *Sarcina* cells was observed in these cultures after $\frac{1}{2}$ and 2 days of incubation, as was checked by microscopic observation. The slight breakdown of several *Sarcina* cells observed after 5 days was about the same in both cultures. This means that the living bacteria of the activated sludge tested, which accounted for about 25% of all of the cells present, probably were unable to effect heterobacteriolysis. Degradation of the cell walls apparently depends on autolysis only, which was suggested before to be the limiting factor in the turnover of intracellular protein.

These results are in agreement with those of HANKIN and SANDS (1974) who attempted to estimate numbers of cannibalistic (i.e. lysozyme-producing) bacteria in activated sludge by using agar plates containing U.V.-treated (intact) *Micrococcus lysodeikticus* cells. In most sludge samples taken at different treatment stages from purification plants, less than 1% of the bacteria was found to be able to degrade intact cell walls.

5.7. SUMMARY

1. Laboratory-grown sludge I, fed continuously with starch, maltose or glucose and ammonium sulphate at C/N = 5 and pH 7.0, and activated sludges from sewage treatment plants show a considerable extracellular proteolytic activity. Attempts to grow activated sludge without detectable proteolytic activity were unsuccessful.

2. PA$_{spec}$ attained rather constant values after loading of sludge I with starch at SL = 0.075, 0.3 or 1.2. At SL = 2.4, PA$_{spec}$ continued to fluctuate even after 300 mean cell residence times, though fluctuations were less unpredictable and pronounced than those of DA$_{spec}$. The extreme fluctuations of PA$_{spec}$ at SL = 1.2 flattened after about 40 cell residence times. After this period DA$_{spec}$ and the population composition were still very variable.

3. The effect of SL of starch sludge I on the average PA$_{spec}$ was the reverse of that on DA$_{spec}$. At SL = 0.075 and 0.3 (t_s = 50–60 and about 14 days, respectively) PA$_{spec}$ approached 0.4. At SL = 1.2 and 2.4 (t_s = 2–3 days and 1 day, respectively) PA$_{spec}$ approached 0.2. Therefore PA$_{spec}$ and DA$_{spec}$ can be regarded as an intrinsic and an extrinsic parameter, respectively.

4. PA$_{spec}$ values of activated sludge from two outdoor plants treating (mainly) domestic sewage (SL = 0.06 and 1.5) were about equal, viz. 0.5, in contrast to the DA$_{spec}$ values (0.54 and 1.08, respectively, Chapter 4).

5. Although proteolytic cell counts per mg starch sludge I increased with SL, as was true of total (viable) cell counts, the average percentage of proteolytic bacteria decreased with SL. At SL = 0.075 (t_s = 50–60 days) and 0.3 (t_s = c. 14 days), proteolytic bacteria amounted on the average to about 50% of the total (viable) count. At SL = 2.4 (t_s = 1 day) the % proteolytic bacteria, like most other parameters tested, fluctuated considerably. On the average it amounted to about 20% of the total (viable) count whilst the lowest value observed was 8%. The average proteolytic activity calculated per (viable) proteolytic cell sharply decreased with rising SL, clearly more sharply than it was the case with the amylolytic activity per amylolytic cell. This different behaviour of PA and DA was probably caused by a greater stability of proteinases. Non-viable cells and cell debris constitute a large fraction of sludge mass at low SL.

6. At SL = 0.3 about half of the amylolytic bacteria was also proteolytic and the majority of the latter bacteria was also amylolytic (partly cryptic growth).

7. The adverse effect of winter conditions on proteolytic activity of activated sludge in outdoor plants did not or only to some extent result in the production of greater amounts of proteolytic enzymes. In the case of amylolytic activity compensation for the low temperature effect did occur (4.7).

8. From items 1, 3, 4, 5, 7 and 21 of this summary it was concluded that degradation of cells and reutilization of released cell material (turnover) play an important role in the metabolism of sludge I, especially at low SL. PA$_{spec}$ can be regarded as a measure of the intensity of extracellular turnover of cell protein.

9. PA$_{spec}$ of continuously loaded starch-grown sludge II, generally predominated by one or a few types of organisms growing in suspension, was low and was little affected by SL and t_s. This low activity was attributed to low percentages of proteolytic bacteria (about 15%) and probably also to a low proteolytic activity calculated per proteolytic cell at low loadings.

10. The viable bacterial cells in starch sludge I showed a pronounced increase with SL to a maximum of approximately 1.4×10^9 bacteria/mg dry wt. An average maximum t_d^* of roughly 3 days, corresponding with a minimum μ^* of 0.23 day^{-1}, was calculated from the relation between sludge t_s and % viable cell

mass. This maximum t_d^* applied to sludges I with a t_s of more than about 10 days.

11. The viable fraction of low-loaded starch sludge II seemed to be higher than that of comparable sludges I, indicating higher maximum t_d^* values (cf. item 21).

12. The average growth yield of starch sludge I increased with SL. It ranged from 0.20 g dry biomass (SL = 0.075 – 0.10, t_s = 50–60 days) to 0.48 g dry biomass (SL = 2.2, t_s = 1 day) per g glucose equivalent added. Growth yields of starch sludges II were higher, i.e. 0.44 (SL = 0.12, t_s = 20 days) and 0.50 (SL = 2.12, t_s = 1 day).

13. From the (assumed linear) plot of Y_{obs}^{-1} versus t_s at t_s values up to 18 days, an Y_{max} of 0.49 g biomass per g glucose equivalent and a biomass turnover coefficient (a^*) of 0.05 day^{-1} were calculated. At higher t_s values this coefficient decreased which was ascribed to an increased effect of cryptic growth and to increasing numbers of dead bacteria which are characterized by a clearly smaller decay coefficient as compared to that of viable organisms.

14. Standard DA of starch sludges I decreased rapidly during starvation of these sludges. The rate of (initial) inactivation of the amylases appeared to be correlated with proteolytic activity. The inactivation of all of the surface-bound amylases present was prevented by the presence of an excess of the proteinase substrate, casein, or by starch. It is highly improbable that protection of amylase by such substrates occurs in continuously fed starch activated sludges.

15. In some experiments, added casein immediately and sharply enhanced DA, possibly by releasing amylases from the cell periplasmic space. These amylases were unstable even in the presence of casein, like it was observed after ultrasonic treatment of sludge.

16. The (initial) inactivation rates of amylases in different starving starch sludges I obeyed first order kinetics according to the equation $-\mathrm{d}\,(\mathrm{DA})/\mathrm{d}t = k_i\,(\mathrm{DA})$. The specific amylase inactivation rate (k_i) was proportional to PA_{spec} (under reactor conditions, at 20°C, $k_i = 1.25\ \mathrm{PA}_{spec}^{30\,°C}$) over the whole range of PA_{spec} values (0.038 to 0.87) found in the SL range of 0.075 to 2.4. The values of k_i were much higher than those of a^*, i.e. the decay rates of amylase were much higher than those of biomass.

17. Proteolytic enzymes only are responsible for the (initial) inactivation of amylases in laboratory-grown starch activated sludges. During the degradation both amylases and proteinases probably remain cell wall-bound.

18. It is very probably that initial amylase inactivation rates as observed in starch sludge I under starvation conditions are also valid under steady-state conditions. Consequently rates of amylase production were calculated according to the equations: net production rate = $\mu_{net} \cdot \mathrm{D\tilde{A}}_{spec}$, gross production rate = $(\mu_{net} + k_i)\,\mathrm{D\tilde{A}}_{spec}$. In the two low-loaded sludges with SL = 0.075 and 0.3 the daily net production was about 2 and 6%, respectively, but the daily gross production amounted to about 50% of $\mathrm{D\tilde{A}}_{spec}$. This indicates a relatively high turnover rate of amylases in these sludges containing low numbers of viable organisms. In the two high-loaded sludges (SL = 1.2 and 2.4) the daily gross production of amylases was about 75 and 125% of $\mathrm{D\tilde{A}}_{spec}$, respectively; the daily net production was above $\frac{2}{3}$ of the gross production owing to the relatively less

important amylase turnover in high-loaded sludge. The gross production rate of amylases in starch activated sludge increased roughly proportionally to the starch loading; at SL = 2.4 it was almost 30 times higher than that at SL = 0.075. However, the net production rate at the latter loading was only roughly 1/550 of that at the former loading. Consequently, imaginary $D\tilde{A}_{spec}$ values (k_i = o, $Y = Y_{max}$) were rougly equal for all SL values applied. The decrease of $D\tilde{A}_{spec}$ actually observed with decreasing SL is explained only by the much higher decay rate of amylases of dead cells compared to the decay rate of biomass.

19. Amylase inactivation rates in low-loaded starch-grown sludge II were considerably lower than those in comparable sludges I. This is in agreement with the low PA_{spec} of sludges II. Obviously, sludges I are more dynamic with respect to amylolytic activity than sludges II.

20. Autolytic degradation of the cell wall of dead cells was apparently the rate-limiting step in the extracellular turnover of the protein of these dead cells in sludge I. Heterobacteriolysis was not demonstrated in sludge.

21. Starch-grown sludges I and II of comparable SL or t_s generally showed different properties. Sludges I grew mainly in flocs and consisted of many bacterial species; a large proportion of the sludge bacteria was proteolytic. These sludges showed a high PA_{spec} and concomitantly a high amylase inactivation rate, a relatively low biomass yield and a low viable count at low SL. Sludges II grew mainly in suspension, were predominated by one or a few bacterial species, showed a low PA_{spec} and a high biomass yield. Only within the few flocs a mixture of different bacterial types was observed. The retention of flocculated biomass in sludge I is responsible for these differences.

6. KINETIC ASPECTS OF STARCH AND CASEIN DEGRADATION BY ACTIVATED SLUDGE

6.1. INTRODUCTION

The potential amylolytic activity, as estimated under standard conditions (2.6.8), of starch-grown sludge I operated continuously (4.6.1) was much higher than it was expected on the basis of the starch removal rate in situ (in the reactor), i.e. the actual amylolytic activity. This excessive amylolytic activity might be associated with a relatively high Michaelis constant (K_m) of the sludge amylases, resulting in low reaction rates at the low starch concentrations present in activated sludge (4.5). To check this hypothesis, the relationship between starch concentration and amylolytic reaction rate was studied (6.3). Moreover, the role of starch adsorption to activated sludge was investigated in some detail as preliminary experiments indicated that this process might initiate starch degradation in sludge (6.2). In addition, the relationships between glucose and dextrin concentration and substrate respiration of sludge (6.4) and between casein concentration and proteolytic reaction rate (6.6) were investigated. The data obtained were used for a tentative assessment of residual carbohydrate concentrations in the liquid of starch activated sludge operated continuously (6.5).

Reactions catalysed by hydrolases are regarded as one-substrate reactions since water, the second participating reactant, is present in a large and constant concentration. Provided the reaction is performed under carefully controlled conditions, the relationship between the reaction rate (v) and the concentration of substrate (S) is described by the well-known Michaelis-Menten equation: $v = V_{max}/(1 + K_m/S)$ in which V_{max} is the maximum reaction rate obtained when the enzyme is saturated with substrate and K_m the Michaelis constant at which $v = \frac{1}{2} V_{max}$. The Michaelis-Menten equation predicts mixed first and zero order kinetics. Plotting v versus S gives a curve shaped as a section of a rectangular hyperbola. To determine the kinetic parameters K_m and V_{max}, the equation can be transformed into a linear form, e.g. in the double reciprocal plot of $1/v$ versus $1/S$ of LINEWEAVER AND BURK (1934):

$\frac{1}{v} = \frac{1}{V_{max}} + \frac{K_m}{V_{max}} \cdot \frac{1}{S}$ (L-B-plot) or in the single reciprocal form of S/v versus S of HANES (1932):

$$\frac{S}{v} = \frac{1}{V_{max}} \cdot S + \frac{K_m}{V_{max}}.$$

Starch hydrolysis by activated sludge for several reasons is not expected to obey Michaelis-Menten kinetics. (i) The substrate is a mixture of amylose and amylopectin which on their turn are not homogeneous with respect to molecular weight and degree of branching. (ii) The enzyme system is heterogeneous. It is the product of a variety of bacterial species and consists of α-amylases, debranching enzymes, and possibly other amylolytic enzymes. (iii) The binding of the enzymes to the sludge may affect their kinetic constants. It is clear that determination of a true Michaelis-Menten constant of sludge-bound amylolytic (and, analogously, of proteolytic enzymes) is impossible. If, however, plotting of v^{-1} versus S^{-1} will show a linear relationship, the maximum reaction rate and the half-maximum rate concentration (called $K_{1/2}$ of the system) can be calculated and used as overall kinetic parameters to predict the system's behaviour at very low starch and protein concentrations. V_{max} can be estimated more easily than $K_{1/2}$, viz. by increasing the starch concentration far above the $K_{1/2}$ value; the DA values estimated under standard conditions (2.6.8; 4.6.1) in fact represent V_{max} values.

The kinetic behaviour of a mixture of enzymes acting independently on the same substrate and heterogeneous with respect to V_{max} and K_m has been dealt with by DIXON and WEBB (1964). At any substrate concentration the total reaction rate will be the sum of the separate reaction rates (1, 2 etc.):

$$v_{total} = v_1 + v_2 + \ldots.. = \frac{V_1 \cdot S}{S + K_1} + \frac{V_2 \cdot S}{S + K_2} + \ldots..$$

As to 2 enzymes, the shape of the double reciprocal plot will depend on the relative values of K_1 and K_2. If $K_1 = K_2$, a straight line is obtained with intercepts $(V_1 + V_2)^{-1}$ and $-K_1^{-1} = -K_2^{-1}$. The greater the difference between the K_1 and K_2 value of the individual enzymes, the greater the deviation from linearity will be. This deviation is often difficult to detect. The shape and position of the curves depends on the relative values of K_1, K_2, V_1 and V_2. When the substrate concentration is sufficiently low, all of the curves approximate straight lines.

When L-B-plots of imaginary mixtures of 3 enzymes with K_m values (of 200, 50 en 10 mg starch/l) were fitted by linear regression by the method of least squares, we found the apparent K_m value to approach reasonably that of the enzyme showing the greatest substrate affinity. The approach was most adequate when this enzyme was present in reasonable amounts (as % of total V_{max}) and when the curve fitting was applied only to relatively low substrate concentrations. At these concentrations only the enzyme with the highest affinity will contribute appreciably to the observed rate. However, at higher substrate concentrations no linearity in the L-B-plot will be found and apparent K_m values calculated from relatively high substrate concentrations, considerably underestimate the K_m value of the enzyme showing the smallest substrate affinity.

Data of reaction rates at high substrate concentrations have more weight in the single reciprocal (least-square fitted) plot of Hanes than in the L-B-plot. The apparent K_m value calculated according to Hanes is more intermediate between the highest and lowest K_m values of the different enzymes and possible curvatures in the plots are better detectable than those in the L-B-plots.

TABLE 6.1. Survey of saturation constants of amylases.

Source	Compound measured	$K_{1/2}$ (mg starch/l)	Literature
K_m ($K_{1/2}$) values of amylases			
Germinated barley (α and β)	reducing sugars	770 and 790	HANES, 1932
Malt (α)		240	HOPKINS, 1946
Malt (β)		3000	HOPKINS, 1946
Pig pancreas (α)	reducing sugars	180^1	BERNFELD and STUDER-PECHA, 1947 and BERNFELD, 1951
Bac. subtilis (α)	reducing sugars	630^1	
Barley malt (α)	reducing sugars	likely $> 630^1$	
Ps. saccharophila (α)	reducing sugars	600	MARKOVITZ et al., 1956
Asp. oryzae EI 212 (α)		3850	KUNDU and DAS, 1970
Asp. oryzae (M-13) (α)	'blue value'	1300^2	YAKUBI et al., 1977
Lipomyces kononenkoae (α)	'blue value'	2700	SPENCER-MARTINS and VAN UDEN, 1979
Lipomyces kononenkoae (gluco-)	glucose	16200	
Apparent K_s values of α-amylase + pullulanase			
Klebsiella (Aerobacter) aerogenes NCIB 8017^3	starch	400	HERNANDEZ and PIRT, 1975
Klebsiella aer. NCIB 8017^4	starch	3600	HERNANDEZ and PIRT, 1975

^{1} mg amylopectin/l; ^{2} mg amylose/l; ^{3} starch-limited chemostat; ^{4} batch culture.

Nevertheless, L-B-plots are preferred as they reflect the low substrate concentrations observed in activated sludge more exactly than the Hanes plot.

K_m values of various α-amylases, some of which are of importance in this study, are reviewed in Table 6.1.

6.2. ADSORPTION OF STARCH TO ACTIVATED SLUDGE

The relationship between amylolytic reaction rate and starch concentration was studied in preliminary experiments with whole untreated starch sludge I. It was observed that the initial values of the extinction of the iodine starch complex, estimated by extrapolation of the initial linear part of the degradation curves, were considerably lower than it was expected from the amounts of starch administered (Fig. 6.1A). This phenomenon was hardly found in cell-free starch sludge ultrasonicate (Fig. 6.1B). Hence the apparent loss of starch cannot be explained by assuming interference of sludge-free liquid with the iodine starch reaction. Such an interference has never been observed with activated-sludge-liquid in contrast to cell-free ultrasonicate which sometimes decolourized a starch iodine solution (3.3.1). Neither can it be explained by enzymic starch degradation since the phenomenon was shown to occur also at

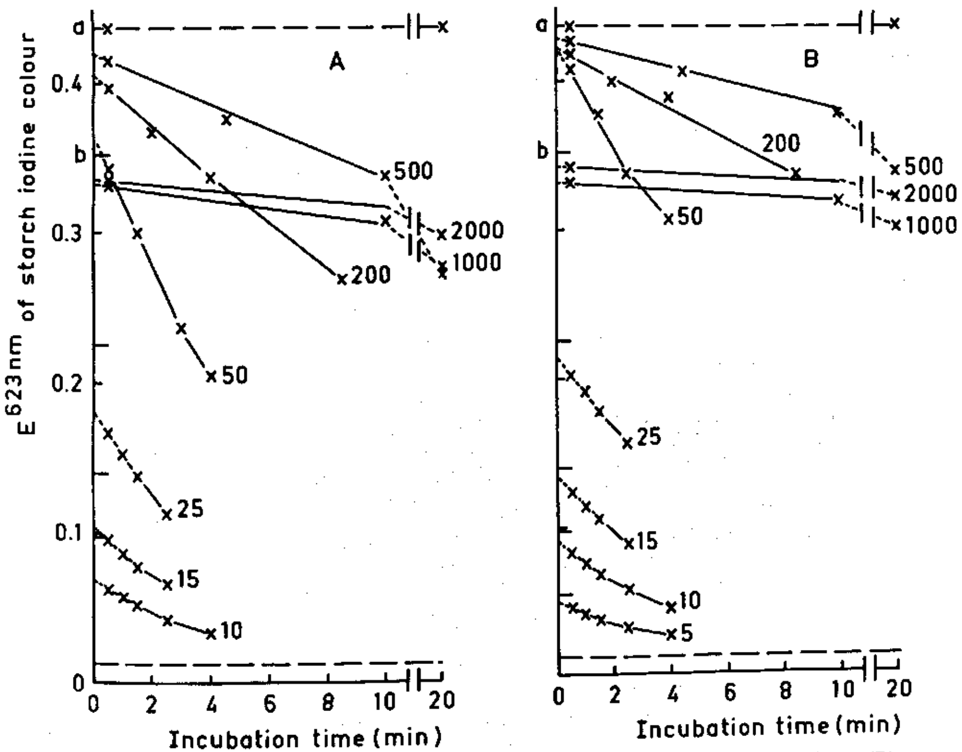

FIG. 6.1. Starch degradation by starch activated sludge (A) and cell-free ultrasonicate (B) at different starch concentrations. The sludge (I) had been loaded during 110 days (SL = 0.3). Tenfold diluted samples were incubated under the conditions of 2.6.9. The broken horizontal lines give the E^{623nm} of a blank incubation mixture containing 200 mg starch/l without enzyme sample (upper line) and of the reagent blank (lower line), both after iodine reaction. Samples of 5.0 ml (5–50 mg starch added per litre), 1.25 ml (200 mg/l), 0.5 ml (500 mg/l), 0.2 ml (1000 mg/l) and 0.1 ml (2000 mg/l) were analysed for starch (see 2.6.7). Numbers in the graph indicate the amounts of starch (mg/l) added to the incubation mixtures; the corresponding (initial) extinctions, calculated from the extinction of the blank incubation mixture, are indicated by short horizontal lines at the ordinate (a: initial extinction of samples with 50, 200 and 500 mg starch/l; b: of samples with 1000 and 2000 mg/l).

1 °C and even, although to a somewhat decreased extent, when 70% $HClO_4$ had been added to the sludge prior to the addition of starch. Further evidence that enzymic degradation was not involved was obtained from another experiment in which the apparent loss of starch was shown hardly to increase when $HClO_4$ was added 5 min instead of $\frac{1}{2}$ min after starch addition to starch sludge at 1 °C. It is therefore concluded that the above-mentioned loss of starch was due to an obviously very rapidly proceeding adsorption of the carbohydrate to the starch activated sludge (Fig. 6.2A). A saturation-type curve was obtained when the amounts of starch adsorbed to starch sludge were plotted against the residual concentrations of starch; double reciprocal plotting of these data gave a linear relationship (Fig. 6.2B). In this respect the curve resembles the adsorption isotherm of Langmuir. The isotherm of Freundlich predicts a linear relationship

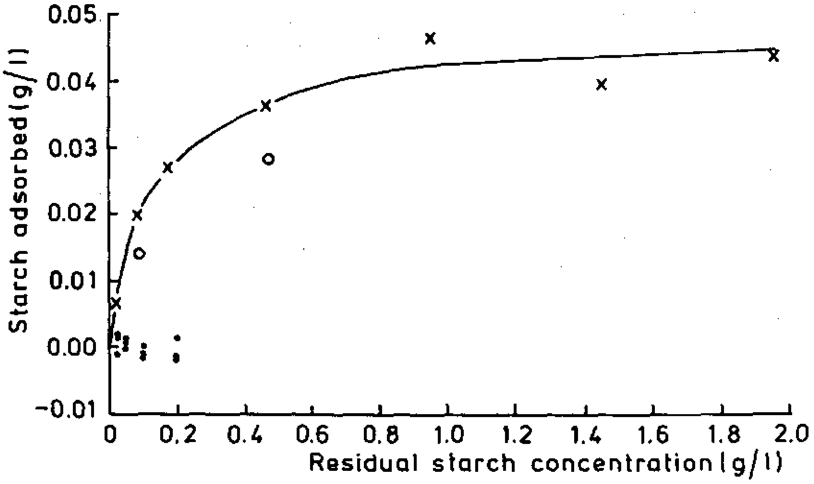

FIG. 6.2A. Adsorption of starch by some activated sludges at 1°C. Untreated sludge (5 ml) was mixed during $\frac{1}{2}$ minute at 1 °C with a starch solution (5 ml); 2 drops of 70 % $HClO_4$ were added and the mixture was centrifuged. Initial starch concentrations ranged from 25 to 2000 mg/l; supernatant samples for the determination of iodine-stainable material ranged from 5 to 0.2 ml. Adsorbed starch was calculated from the decrease of the starch iodine extinction. x and O: Very slimy starch sludge (176 days of loading with SL = 0.3; dry wt: 4.97 g/l; DA_{spec} = 1.23). The symbol O indicates that $HClO_4$ was added to the sludge prior to starch. The other sludges (●) were: glucose sludge (27 days of glucose loading preceded by 53 days of maltose loading with SL = 0.3; dry wt: 1.78 g/l; DA_{spec} = 0.19); Zeist sludge (7/8/1974; dry wt: 1.63 g/l; DA_{spec} = 1.30); Bennekom sludge (7/8/1974; dry wt: 2.70 g/l; DA_{spec} = 0.59).

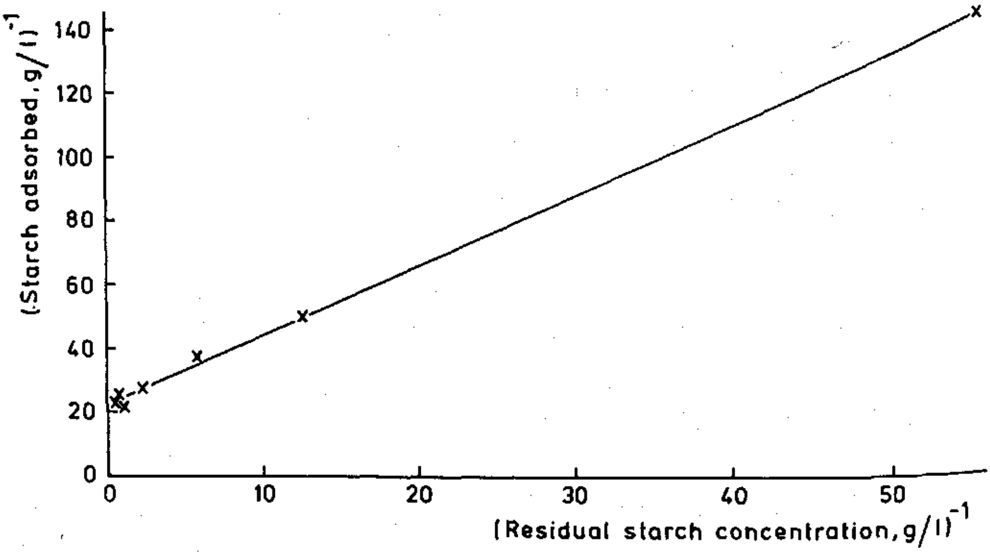

FIG. 6.2B. Double reciprocal plot of Fig. 6.2A. The data of the starch sludge (symbol x) were fitted by linear regression by the method of least squares (r = 0.999). The maximum amount of starch adsorbed, 44.1 mg/2.49 g sludge = 17.7 mg/g sludge, and the substrate concentration at half-maximum adsorption, 96 mg starch/litre, were derived from the plot.

between log amount of starch adsorbed and log residual amounts of starch/l; such a relationship was not found, in fact a curviform plot was observed.

Data of three experiments suggest that at relatively high starch concentrations the adsorptive surface of starch sludge was saturated with 10–50 mg of starch per g sludge, in the case of Fig. 6.2B 18 mg/g. Half-saturation took place at 50–100 mg of starch per litre. Although binding of substrate to enzymes according to Michaelis-Menten kinetics shows far-going similarity with adsorption as defined by Langmuir, the investigated adsorption of starch to starch activated sludge is not or hardly associated with sludge-bound amylolytic enzymes, but likely with slimy components of this sludge. If amylolytic enzymes were the receptors in the adsorption process, no adsorption would occur when $HClO_4$ had been added to the sludge before the addition of starch (Fig. 6.2A) whilst adsorbed starch molecules probably would be released by denaturation from these enzymes brought about by the addition of perchloric acid. Adsorption of starch was observed only with starch-grown activated sludge (SL = 0.3) which always was very slimy. None of the other activated sludges investigated, which were not slimy, showed this phenomenon (Fig. 6.2A).

The adsorption studied is unlikely to play a significant role under steady-state conditions in starch-grown activated sludge, because the starch concentrations under these conditions are very low (4.5) whilst the very small amounts of starch adsorbed are thought to prevent further starch adsorption.

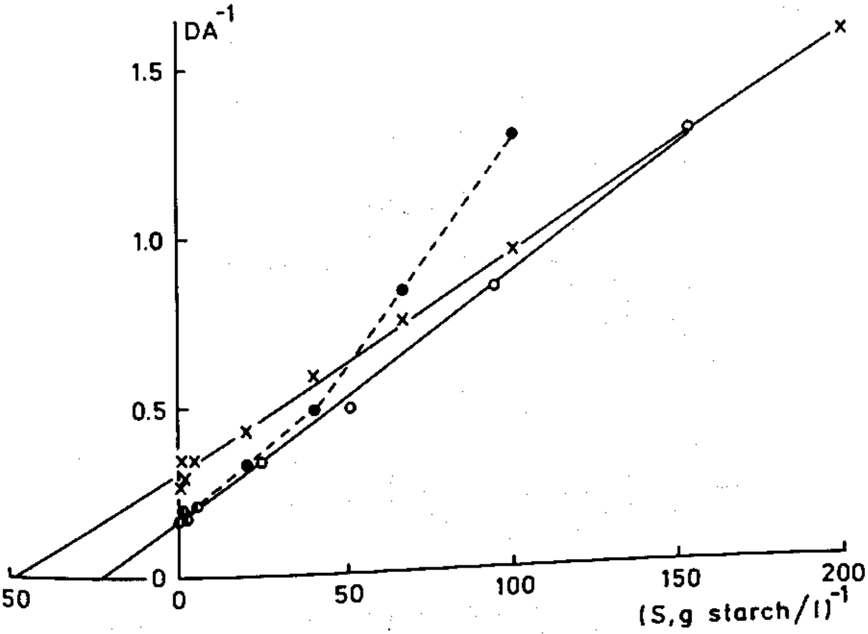

FIG. 6.3. Lineweaver-Burk plots of the initial DA values, derived from Fig. 6.1. x, 1/DA of cell-free ultrasonicate (least-squares fit line; $K_{1/2}$ = 0.021 g starch/l; sample correlation coefficient r = 0.998); •, 1/DA of sludge; o, 1/DA of sludge versus the reciprocals of the starch concentrations corrected for adsorption (least-squares fit line; $K_{1/2}$ = 0.044 g starch/l; r = 0.999).

6.3. HALF-RATE CONSTANTS OF AMYLASES OF STARCH ACTIVATED SLUDGES

The adsorption of starch to starch activated sludge hampers the correct estimation of the half-rate constant of amylases. The data of the experiment shown in Fig. 6.1 were used to calculate initial amylolytic reaction rates. Reciprocal plotting of $1/v$ versus $1/S$ yields a straight line only for the data obtained with cell-free ultrasonicate (Fig. 6.3). The data obtained with whole sludge show a curvilinear graph unless the initial starch concentrations were corrected for the amounts of starch adsorbed to the sludge. The V_{max} values of both systems are not comparable due to the effect of ultrasonic treatment upon the amount of enzymes estimated; see Chapter 7. The value of $K_{1/2}$ observed with cell-free ultrasonicate is lower than that observed with whole sludge corrected for adsorbed starch. However, in another experiment, using starch concentrations of 25–5000 mg/l, the $K_{1/2}$ values of amylases of cell-free ultrasonicate and of whole starch sludge (SL = 0.3), corrected for adsorbed

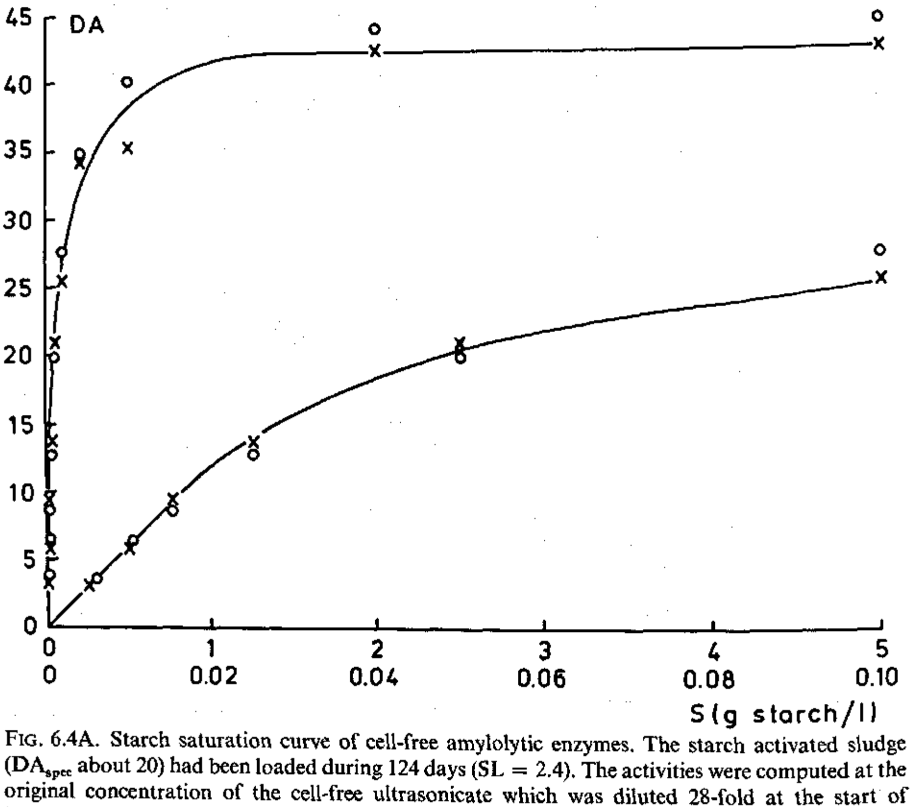

FIG. 6.4A. Starch saturation curve of cell-free amylolytic enzymes. The starch activated sludge (DA_{spec} about 20) had been loaded during 124 days (SL = 2.4). The activities were computed at the original concentration of the cell-free ultrasonicate which was diluted 28-fold at the start of incubating the assay mixtures. Conditions: see 2.6.9. x, (Upper curve) DA versus the whole range of starch concentrations; x, (lower curve) DA versus low starch concentrations; O, DA values calculated with the least-squares fit equation of Fig. 6.4B.

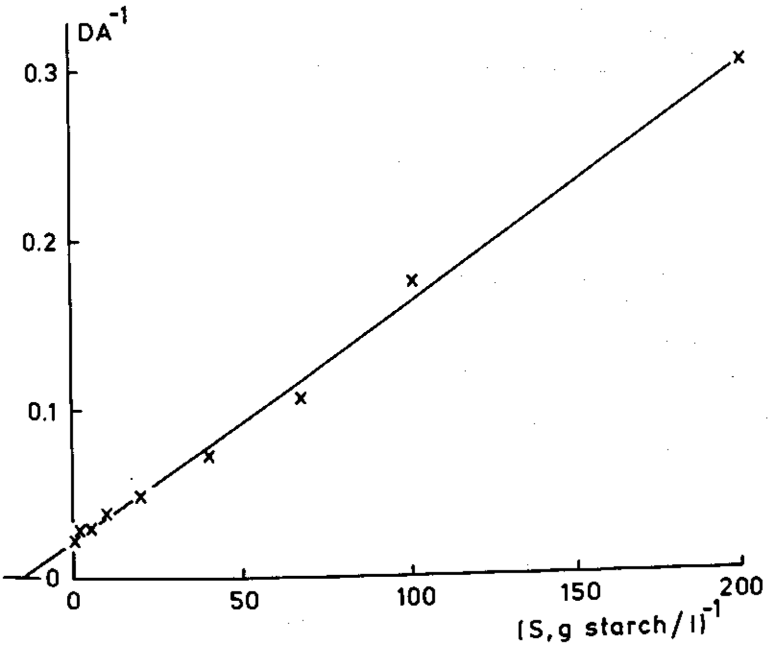

FIG. 6.4B. Least-squares fit of L-B-plot (data of Fig. 6.4A): $DA^{-1} = 0.00141\ S^{-1} + 0.0221$; $r = 0.998$; $K_{1/2} = 0.064$ g starch/l.

starch, were calculated to be 81 and 56 mg/l, respectively. If no correction was made for adsorption, the deviation from linearity in the L-B-plot was very pronounced. Plotting of the amounts of starch adsorbed (estimated by extrapolation to $t = 0$) versus residual starch concentrations gave a saturation-type curve, showing a linear double reciprocal plot (curve comparable to that of Fig. 6.2). These results indicate that the investigated adsorption of starch by starch sludge, observed during the estimation of the $K_{1/2}$ value, reduces the amount of starch instantly available for the amylases, resulting in decreased amylolytic rates (Fig. 6.1).

From the fact that the $K_{1/2}$ values estimated with cell-free amylases and sludge-bound amylases (the latter after correction for adsorption of starch to sludge) were of the same order of magnitude, it is concluded that the $K_{1/2}$ value of amylases is not appreciably affected by their binding to sludge. This is in contrast with some other bound enzyme systems, like for instance β-amylase and pullulanase immobilized by binding to an acrylic polymer (MÅRTENSSON, 1974).

Half-rate constants of amylases were estimated, according to 2.6.9, with cell-free ultrasonicates of starch activated sludges in order to rule out interference of starch adsorption by the sludge. Although ultrasonic treatment resulted in a considerable increase of amylolytic activity (7.2), this increase was assumed to be not due to the activity of intracellular enzymes, but presumably to extracellular

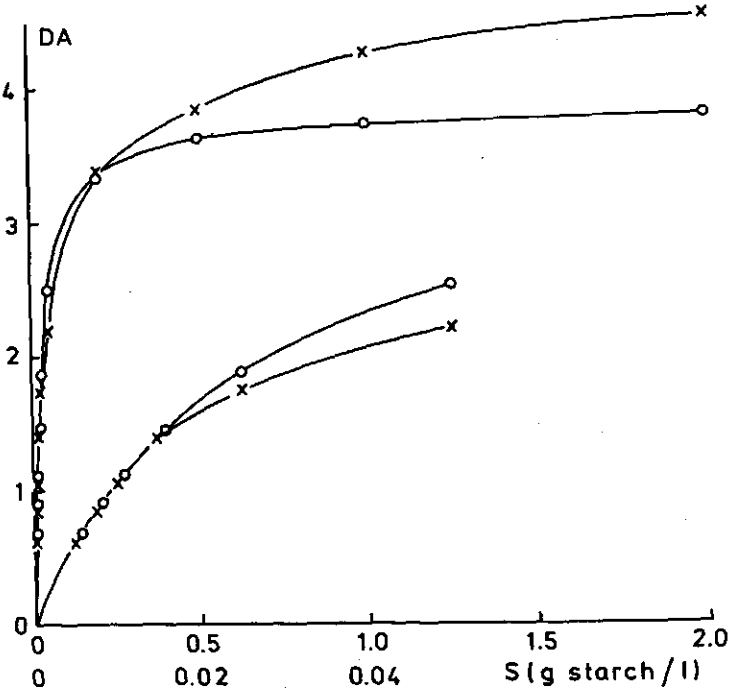

FIG. 6.5A. Starch saturation curve of cell-free amylolytic enzymes. The starch activated sludge (DA_{spec} about 3.7) had been loaded during 106 days (SL = 0.3). The activities were computed at the original concentration of the cell-free ultrasonicate which was diluted tenfold at the start of incubating the assay mixtures. Conditions: see 2.6.9. x, (Upper curve) DA versus the whole range of starch concentrations; x, (lower curve) DA versus low starch concentrations; ○, DA values calculated with the least-squares fit equation of Fig. 6.5B.

enzymes from the periplasmic space, since no effect of ultrasonic treatment on the DA/SA ratio was observed (3.2.3 and 7.2).

The $K_{1/2}$ value of cell-free amylases (SL = 2.4) at 30 °C did not deviate clearly from that at 20 °C; the $K_{1/2}$ values (SL = 0.3) estimated with the DA assay and the SA assay were of the same order of magnitude. The SA method is inaccurate so that the DA method was preferred (2.6.9).

The many L-B-plots made of starch concentration and initial amylolytic reaction velocity of cell-free ultrasonicates of starch activated sludges (SL = 0.075–2.4) rarely showed a real linear relationship. The same was true of whole starch sludges. The experiment shown in Figs. 6.4A and B is an example of the unusual situation of exact obeyance of the Michaelis-Menten relationship. A relatively high $K_{1/2}$ value (64 mg starch/l) and a sample correlation coefficient (r) of 0.998 were calculated by least-squares fitting of the linear relationship of S^{-1} and v^{-1}. Due to the high $K_{1/2}$ value, DA increased nearly proportionally with the starch concentration in the range of 0–15 mg/l (Fig. 6.4A), the latter value being far beyond the starch concentrations detectable in activated sludge liquid. Plotting the data calculated from the least-squares equation of Fig. 6.4B in Fig. 6.4A shows that these values correspond well with the original experimental

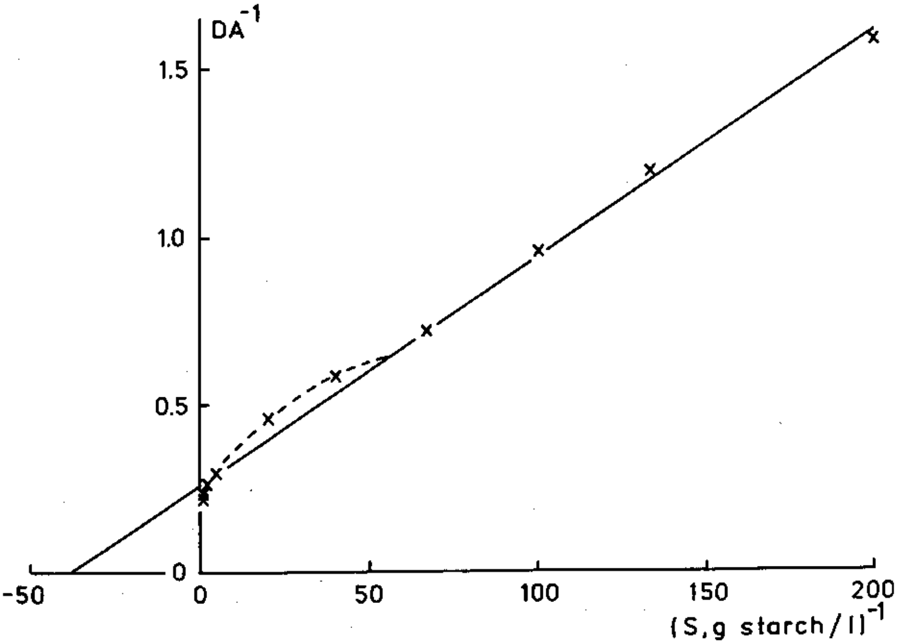

FIG. 6.5B. Least-squares fit of L-B-plot (data of Fig. 6.5A): DA^{-1} = 0.00682 S^{-1} + 0.263; r = 0.998; $K_{1/2}$ = 0.026 g starch/l.

data. In the experiment of Fig. 6.4 the $K_{1/2}$ values of the constituent amylases obviously did not differ considerably (6.1). Possibly, the straight L-B-plot and the high DA$_{spec}$ observed in this sludge at the time of sampling reflect the transient predominance of one type of amylase produced by one bacterial species.

L-B-plots of data obtained in almost all of the other experiments showed a deviation from linearity at higher substrate concentrations, indicating rather large differences between the $K_{1/2}$ values of the amylases present (6.1). Only two of such examples (SL = 0.3 and 2.4) are shown (Figs. 6.5 and 6.6). The $K_{1/2}$ values calculated from rectified substrate saturation curves amounted to 26 and 23 mg starch/l, respectively. The curvature in the L-B-plot of Fig. 6.6B was more pronounced than that of Fig. 6.5B, resulting in a lower sample correlation coefficient. It should be noticed that the sludge (SL = 2.4) of Fig. 6.6 had a very low DA$_{spec}$, in contrast to the sludge (SL = 2.4) of Fig. 6.4 which was characterized by a high DA$_{spec}$ and a straight L-B-plot. For reasons of simplicity, curvilinear L-B-graphs can be regarded as the result of two linear relationships, involving the data obtained at high ($\geqslant$ 50 mg/l) and at low starch concentrations ($\leqslant$ 15 mg/l). The calculated $K_{1/2}$ values were 50 (r = 0.995) and 22 mg starch/l (r = 0.999) (Fig. 6.5) and 76 (r = 0.993) and 15 mg starch/l (r = 1.000) (Fig. 6.6), respectively. These different values reflect to some extent the heterogeneity of the sludge amylases with respect to their $K_{1/2}$ values. The estimates of the lower $K_{1/2}$ values are likely to approach the lowest $K_{1/2}$ values of the amylases present,

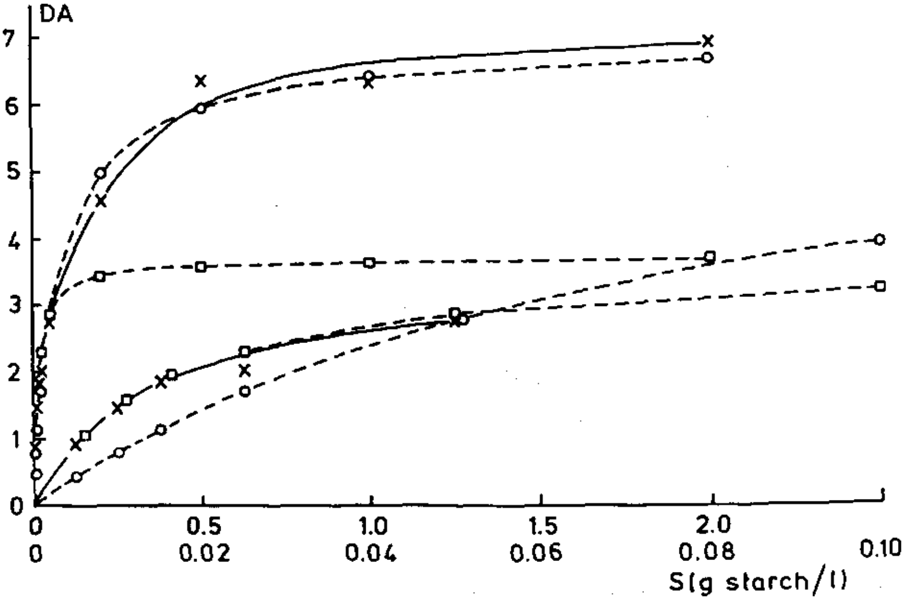

FIG. 6.6A. Starch saturation curve of cell-free amylolytic enzymes. The starch activated sludge (DA_{spec} about 2.5) had been loaded during 250 days (SL = 2.4). The activities were computed at the original concentration of the cell-free ultrasonicate which was diluted tenfold at the start of incubating the assay mixtures. Conditions: see 2.6.9. The upper 3 curves are related to the whole range of starch concentrations, the lower 3 to the low starch concentrations. x——x, DA as measured; □---- □, DA as predicted from a partial L-B-plot of Fig. 6.6B for $S = 0.005, 0.010$ and 0.015 g/l; $DA^{-1} = 0.00407\ S^{-1} + 0.271$; $r = 1.000$ and $K_{1/2} = 0.015$ g starch/l; ○----○, DA as predicted from a partial L-B-plot of Fig. 6.6B for $S = 0.05, 0.2, 0.5, 1.0$ and 2.0 g/l; $DA^{-1} = 0.0110\ S^{-1} + 0.146$; $r = 0.993$ and $K_{1/2} = 0.076$ g starch/l.

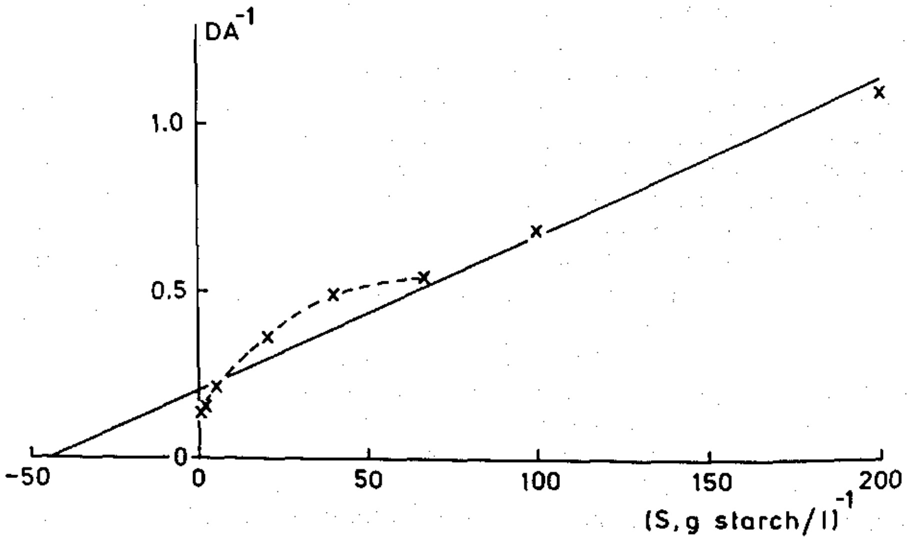

FIG. 6.6B. Least-squares fit of L-B-plot (data of Fig. 6.6A): $DA^{-1} = 0.00463\ S^{-1} + 0.203$; $r = 0.982$ and $K_{1/2} = 0.023$ g starch/l.

the estimates of the higher $K_{1/2}$ values considerably underestimate the highest $K_{1/2}$ values occurring (6.1). Actual values of e.g. 200 mg/l are imaginable. Plotting the data obtained from both extreme least-squares equations of the pronounced curvilinear L-B-plot of Fig. 6.6B in Fig. 6.6A shows that the values of both correspond with the original experimental data only in the respective substrate ranges. Generally the approach of the experimental data with the least-squares fit equation developed for the whole substrate range (Fig. 6.5A) is preferable. Due to the pronounced effect of the reaction rates at 5–15 mg starch/l on the L-B-plot, this equation is reliable at low substrate concentrations, such as is aimed at (6.1).

The two examples of Figs. 6.5 and 6.6 are representative of many ultrasonicates prepared from different starch sludges I (SL = 0.075–2.4). No evidence was obtained of a correlation between $K_{1/2}$ and SL or loading period of the sludge. In this respect it is remembered that very low starch concentrations were found over the whole range of loadings applied (4.5). No evidence was obtained of the selection of bacterial species producing amylases with the highest substrate affinity observed in the present investigation. The heterogeneity of sludge amylases with respect to their $K_{1/2}$ value (Figs. 6.5 and 6.6) might be associated with the different mean cell residence times of the suspended bacteria and of the retained flocculated bacteria occurring in activated sludge. The suspended bacteria characterized by short mean cell residence times might have

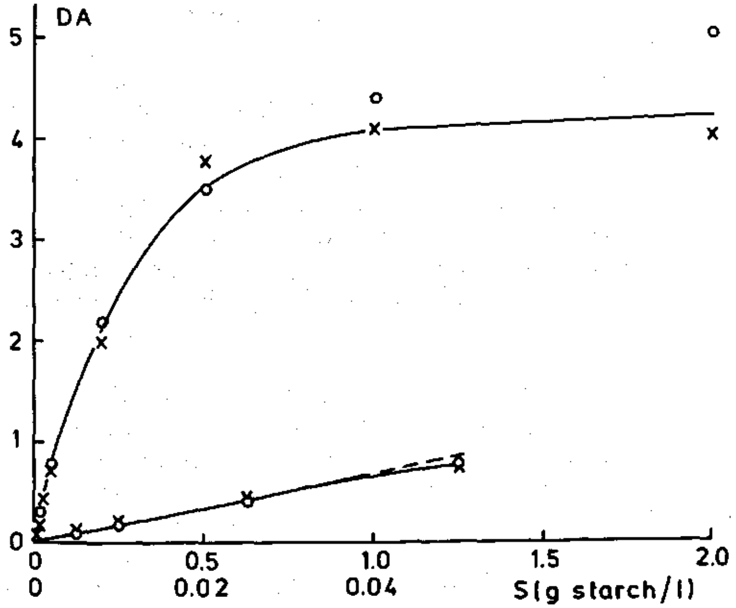

FIG. 6.7A. Starch saturation curve of Fungamyl 1600 (1 mg/l). Conditions: see 2.6.9. The upper curve is related to the whole range of starch concentrations, the lower curve to the low starch concentrations. x, DA as measured; o, DA values calculated with the least-squares fit equation of Fig. 6.7B.

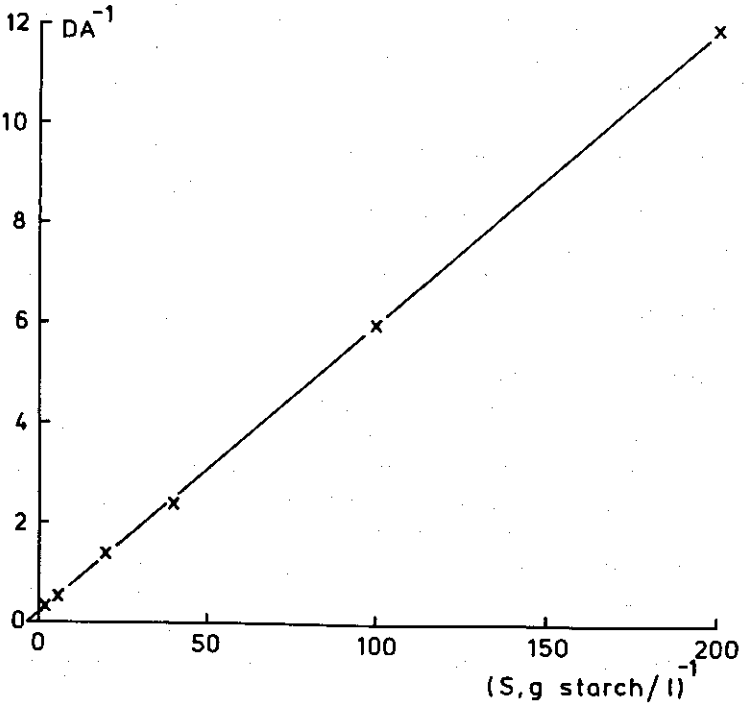

FIG. 6.7B. Least-squares fit of L-B-plot (data of Fig. 6.7A): DA^{-1} = 0.0578 S^{-1} + 0.170; r = 0.9999; $K_{1/2}$ = 0.341 g starch/l.

a lower $K_{1/2}$ for starch than the bacteria in the flocs. However, sludges which consisted almost only of flocs (at SL = 0.3) also gave curved L-B-plots, suggesting heterogeneity of floc-bound enzymes and likely of amylolytic bacteria. This might be explained by assuming starch concentrations to decrease from the surface to the interior parts of the floc, giving rise to different bacteria developing in different parts of the floc and producing amylases with different substate affinities. Moreover, competition of starch-degrading bacteria may also be affected by other growth-determining characteristics like for instance production of proteolytic enzymes.

Overall $K_{1/2}$ values of amylases of 20–25 mg starch/l were most frequently observed in starch activated sludge; such values include estimates of the lowest $K_{1/2}$ values amounting roughly to 15–20 mg starch/l. These values are very low as compared to those observed with the relatively pure α-amylase of *Aspergillus oryzae* (Fungamyl 1600), viz. $K_{1/2}$ values of 340 (Fig. 6.7B) and 295 mg starch/l (r = 0.999) were estimated in two experiments.

$K_{1/2}$ values of α-amylases of different bacterial and fungal species and from other sources reported in the literature are many times higher than the lowest $K_{1/2}$ values estimated for sludge amylases (Table 6.1). This may be one of the reasons for the absence of well-known amylolytic microbes like *Bacillus*, *Pseudomonas* and fungi in the microbiota of starch-fed activated sludge. Two weeks of cultivation of starch activated sludge at pH 5.0/5.5 (3.5.3) did not provoke the growth of fungi and yeasts. The relatively low $K_{1/2}$ values of sludge amylases and the large amounts of these enzymes synthesized explain the nearly complete removal of starch by activated sludge. BERNFELD (1951) observed that α-amylases with the highest affinity for a high-molecular substrate (see data of Table 6.1) convert the substrate to the shortest limit dextrins. It is tempting to suggest that the high conversion limit achieved by sludge amylolytic enzymes (Fig. 3.1) is associated with the unrivalled high affinity to these amylases for starch.

6.4. SUBSTRATE RESPIRATION OF ACTIVATED SLUDGE, INCLUDING ITS HALF-RATE CONSTANTS

$K_{1/2}$ values of sludge amylases are more meaningful if they are compared with $K_{1/2}$ values of processes succeeding starch degradation. For that reason experiments on substrate respiration rates were carried out with sludge I. These rates and their $K_{1/2}$ values may be assumed to be determined by the rate-limiting step in uptake and dissimilation of the substrate.

6.4.1. *Experimental procedure*

Sludge samples were washed with buffer, kept at 20°C, diluted, and transferred to the respirometer (2.6.4). After some minutes the oxygen uptake rate was (nearly) constant, provided that the sludge, withdrawn from a laboratory reactor, had previously been stored for some hours at 20°C. This rate was considered to represent endogenous respiration. Subsequent injection of a small amount of substrate solution with a syringe caused an almost immediate or sometimes a somewhat retarded increase of respiration rate (substrate respiration, v). After the oxygen uptake rate had fallen to the endogeneous level, another amount of substrate was injected into the same or another sludge sample. Rates at 1–2 mg COD/l were measured usually in triplicate. When the oxygen concentration had fallen to 3 mg/l, the sludge was reaerated to prevent oxygen limitation within the flocs.

6.4.2. *Preliminary experiments and principles*

Starch sludge I (SL = 0.3) always hardly responded to the addition of starch, DE-20, maltose, glucose or lactate. Hence, most respiration experiments were performed with glucose and maltose sludges I (SL = 0.3), which were shown to respire glucose, maltose, maltotriose, DE-20 and starch at a rate decreasing with the degree of polymerization of the substrate (Table 6.2). Respiration rates with glucose and maltose were considerably higher than those with the other substrates. Maltose sludge respired maltose slightly faster than glucose. Glucose sludge respired DE-20 and starch very slowly, a property attributed to its low amylolytic activity. With both sludges the dissimilation percentages decreased with increasing chain length of the carbohydrate. In all experiments performed

TABLE 6.2. Initial respiration rates of glucose and maltose activated sludge upon the addition of glucose-containing carbohydrates with different DP values. After washing, both sludges were pre-incubated aerobically (shaken at c. 100 strokes/min) for more than 4 h before substrates were added (2.6.4). The increased oxygen uptake until the rate had fallen approximately to the endogenous level is thought to represent substrate dissimilation. For the calculation of the dissimilation percentage the substrate was assumed to have been taken up completely.

Substrate[1]	Glucose sludge[2]			Maltose sludge[3]		
	Endo-genous resp. rate[4]	Sub-strate resp. rate[4]	% Dissimi-lation	Endo-genous resp. rate[4]	Sub-strate resp. rate[4]	% Dissimi-lation
Glucose	14.0	93.3	11.1	13.6	45.9	c. 20
Maltose	15.7	17.3	7.5	13.7	53.5	c. 14.3
Maltotriose[5]	17.4	7.7	5.0			
DE-20[6]	16.1	4.4	4.1	14.7	19.6	c. 10.1
Starch	17.4	1.9	–	12.9	9.6	6.0

[1] 20 mg COD/l; [2] loaded during 258 days at SL = 0.3 (DA_{spec} = c. 0.2 and biomass: 3.65 g/l); [3] loaded during 52 days at SL = 0.3 (DA_{spec} = c. 0.70–0.75 and biomass: c. 1.7 g/l); [4] mg O_2/h.l original sludge at 20°C; [5] the maltotriose preparation contained c. 0.5% isomaltotetraose and less than 0.1% maltose; [6] DP of DE-20 was about 7.

in this study the dissimilation percentages varied between 10 and 20 when glucose was added to glucose sludge and maltose to maltose sludge. These percentages are in agreement with the results of VAN GILS (1964; addition of glucose to glucose sludge and to sludge from the Zeist plant) and of HOUTMEYERS (1978; addition of glucose to glucose sludge). According to VAN GILS and other authors it indicates that only part of the utilized substrate was converted into protein and other nitrogen-containing cell constituents, while the remainder (almost 60% when no nitrogen was supplied along with glucose) was accumulated as intra- or extracellular polymerization products. Additional supply of $(NH_4)_2SO_4$ had no effect on the rate of oxygen consumption and on the dissimilation percentage (VAN GILS and present study). The markedly increased sludge respiration rate, associated with glucose uptake, dropped to the endogenous level, as soon as the substrate had been removed from the sludge liquid (HOUTMEYERS, 1978). Consequently, the uptake rate of a substrate is a factor 100/dissimilation % greater than the respiration rate.

To calculate substrate respiration rate and dissimilation percentage in the present study, a correction was made for the endogenous respiration rate by extrapolation. However, dependent on the organism tested, the endogenous respiration might be stimulated or reduced during substrate respiration (DIETRICH and BURRIS, 1967). The effect of a presumed constant, decreased or increased, endogenous respiration rate on the substrate dissimilation percentage is dealt with in the following calculations (data obtained from the experiment shown in Fig. 6.8A). If substrate respiration values observed at constant cell density and varying substrate concentration were corrected for endogenous respiration, dissimilation percentages of 19.4, 23.5, 20.1, 17.5 and 18.7 would be

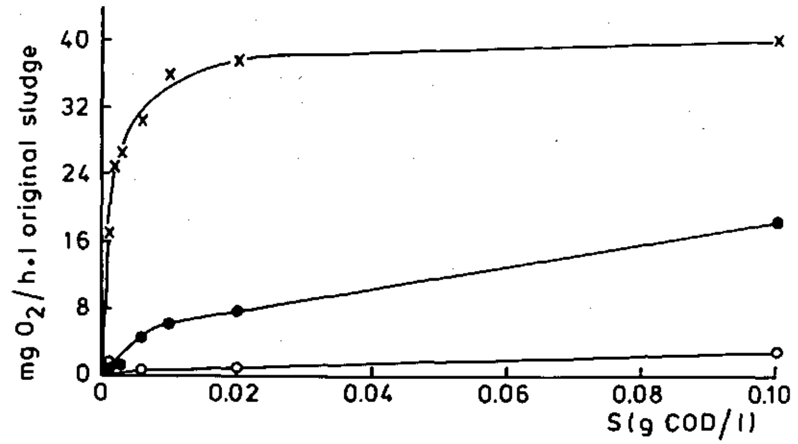

FIG. 6.8A. Substrate saturation curves of substrate respiration rates of glucose activated sludge. The sludge had been loaded for 48 days at SL = 0.3 and was pre-incubated, after washing, aerobically for 5 h before experiments were started (6.4.1). The endogenous respiration rates for which the substrate respiration rates always were corrected, varied between 19.7 and 14.1 mg O_2/l of original sludge.h. x, Glucose; ●, DE-20; ○, starch.

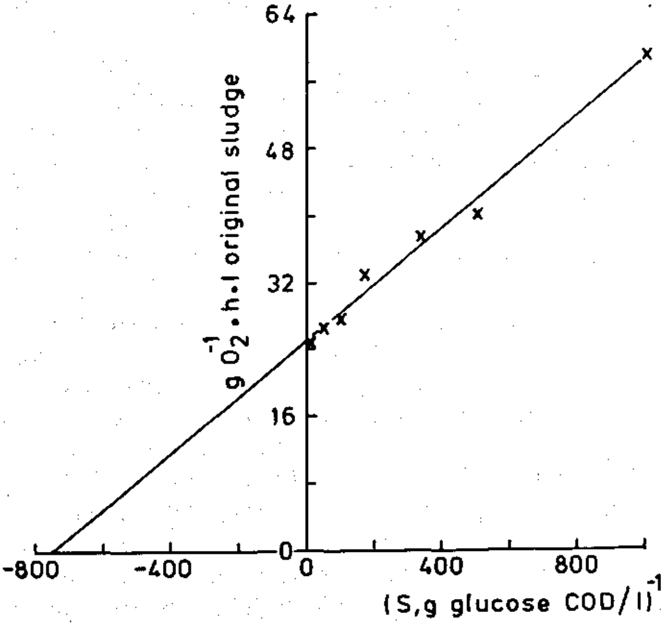

FIG. 6.8B. Least-squares fit of L-B-plot (data for glucose respiration rate, v, in g O_2/h. l original sluge, Fig. 6.8A): $v^{-1} = 0.0336\ S^{-1} + 25.21$; $r = 0.993$ and $K_{1/2} = 0.00133$ g glucose COD/l.

TABLE 6.3. $K_{1/2}$ values of substrate[1] respiration rates of glucose activated sludge (SL = 0.3).

Days of loading	Number of resp. rates[2]	$K_{1/2}$ values (mg COD/l) for glucose	maltose	maltotriose	Correlation coefficient
48	7	1.3			0.993
264	5		3.0		0.981
264	5			5.5	0.995
269	7	2.7			0.992
269	4		2.6		0.980
269	5			3.6	0.980

[1] Substrate concentration range: 1(2)–100 mg COD/l; [2] the rates at 1 and 2 mg COD/l were usually determined in triplicate or duplicate and averaged.

calculated for glucose concentrations of 1, 2, 3, 6 and 10 mg COD/l, respectively.

If complete suppression of the oxidation of endogeneous substrates by exogenous substrate was supposed, dissimilation percentages of 76.7, 72.6, 60.3, 34.1 and 34.7, respectively, would be calculated for glucose concentrations of 1, 2, 3, 6 and 10 mg COD/l, respectively. In this case no correction for endogenous respiration was made; the substrate respiration represented the oxygen consumed after substrate injection until the respiration rate became equal to the preceding endogenous respiration rate occurring before adding substrate.

If enhanced endogenous respiration during substrate respiration was supposed, even for only 20%, the calculated dissimilation percentages would be low and would increase with increasing substrate concentration. It seems fair to assume that the assimilated proportion of the added substrate is constant over varying substrate/cell density ratios (DAWES and RIBBONS, 1962), certainly at 1–10 mg glucose COD/l. In our experiments this was true only if the endogenous respiration rate was unaffected by glucose in concentrations up to at least 10 mg COD/l.

6.4.3. *Response of glucose activated sludge to added glucose, maltose or maltotriose*

Initial respiration rates of glucose sludge I (SL = 0.3) supplied with glucose apparently obeyed saturation kinetics over the substrate range applied (see e.g. Fig. 6.8). Least-squares fits of the double reciprocal plots yielded $K_{1/2}$ values listed in Table 6.3. The $K_{1/2}$ values for glucose, maltose and maltotriose, a few mg COD/l, were much lower than the lowest values of the initial amylolysis by starch sludge, viz. c. 15–20 mg starch/l, i.e. c. 18–24 mg starch COD/l. The values observed for glucose (c. 2 mg/l) agree with literature data on (glucose) utilization by suspended bacteria: 1.8 mg/l for *Zoogloea ramigera* (oxygen uptake method, KRUL, 1977a); about 5 mg/l for the filamentous bacterium *Haliscomenobacter hydrossis* (estimation of residual glucose concentrations, KRUL, 1977b); 2.0 mg glucose/l, 2.9 mg fructose/l and 2.6 mg mannitol/l for *Pseudomonas aeruginosa* (estimation of radioactive substrate uptake, EAGON and PHIBBS, 1971); 5.5–9 mg glucose/l for *Klebsiella aerogenes* (oxygen uptake method, NEYSSEL and TEMPEST, 1975); 5 and 8.5 mg glucose/l for *Beneckea natriegens* (oxygen uptake method, K_m, and chemostat, K_s, respectively; LINTON et al., 1977).

The oxygen uptake rates of sludge flocs, measured at different substrate concentrations, give an orientating rather than a precise description of the affinities of the cells towards substrate. The following critical remarks concerning the method and the interpretation of the measurements can be made.

1) The substrate respiration rate might not be proportional to the glucose uptake rate over the whole substrate range. However, DE BONT (1976) showed that the rate of methane uptake by whole-cell suspensions of a methane-oxidizing bacterium was directly proportional to the rate of oxygen uptake, after correcting for endogenous respiration. Both methods led to the same apparent K_m value.

That the substrate affinity values estimated with the aid of respiration data did not differ from those estimated with the aid of chemostat/growth data was shown by LINTON et al. (1977). A rather good agreement was found between the affinity of *Beneckea natriegens* for glucose measured in a chemostat culture (saturation constant $K_s = 8.5$ mg/l) and that measured in a closed oxygen electrode system using harvested bacteria ($K_m = 5$ mg/l). The K_m for glucose of harvested bacteria was measured in complete basal medium minus the carbon and nitrogen sources by adding known quantities of glucose. The K_m was independent of the specific growth rate (between 0.12 and 0.64 h^{-1}).

2) Diffusion may interfere with the uptake of substrate by cells occurring in flocs as was demonstrated by KRUL (1977a) with pure culture flocs of *Zoogloea ramigera*. However, in the case of glucose sludge diffusion was not expected to limit substrate uptake to a large extent since the flocs were relatively small and not markedly slimy whilst the percentage of living cells was clearly lower than in pure culture flocs (dead bacteria presumably predominate in the centre of the floc). That the uptake of substrate by cells in flocs is not affected to a large degree by diffusion resistance is derived from the observation that the $K_{1/2}$ value of amylolytic enzymes in cell-free ultrasonicate of sludge did not deviate to a large extent from the $K_{1/2}$ value of these enzymes in starch activated-sludge flocs (6.3).

6.4.4. *Response of glucose and starch sludge to added starch*

Since starch sludge I (SL = 0.3) always failed to respire clearly added carbohydrates, some experiments were performed with glucose sludge I (SL = 0.3), supplied with Fungamyl 1600, and with high-loaded starch sludge I (SL = 1.2) and starch sludge II (SL = 0.3–0.15). The first experiment (Fig. 6.9) revealed that glucose sludge not acclimatized to starch and dextrins (DA_{spec} = c. 0.2), but supplied with an α-amylase preparation, respired starch at a moderate rate (cf. Table 6.2), in spite of the fact that this α-amylase produces hardly glucose (3.2.2; NOVO enzyme information, Sept. 1971). The substrate respiration rate of this mixture with 100 and 200 mg starch COD/l, viz. 16.5 and 15.9 mg O_2/h.l of original sludge, respectively, equalled the rate at 20 mg maltose

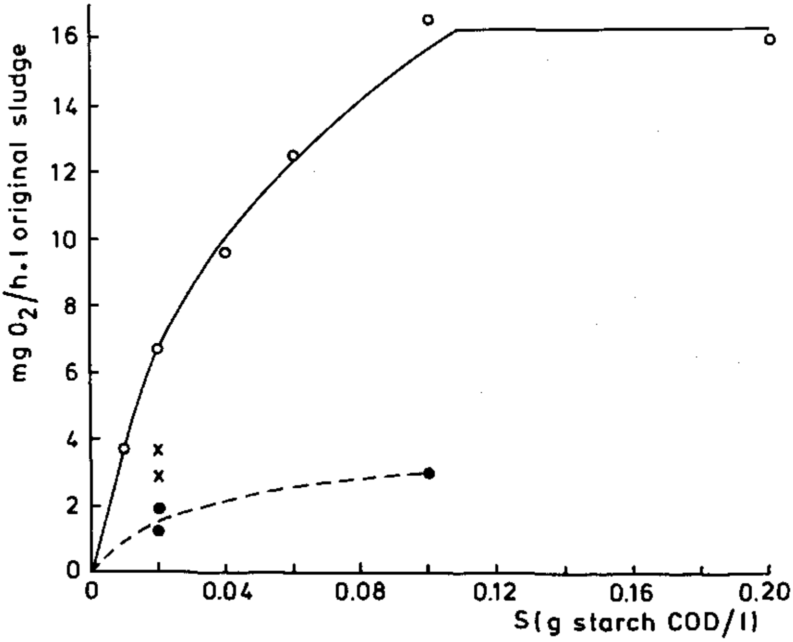

FIG. 6.9. Substrate saturation curves of starch respiration rates of glucose sludge in the absence and presence of Fungamyl 1600. Some characteristics of this glucose sludge are given in Table 6.2. After washing, the sludge was pre-incubated aerobically for 10 h before the experiments were started (6.4.1). Starch was added some minutes after the addition of Fungamyl. In the presence of 25 mg Fungamyl/l (DA about 75 at pH 7.0 and 30°C), the enhanced 'endogenous' respiration rate varied from 17.5 to 20.7 mg O_2/h. l original sludge. ○, Starch respiration in the presence of 25 mg Fungamyl/l (corrected for endogenous respiration with Fungamyl); from the least-squares fitted L-B-plot ($S = 0.01$–0.1 g COD/l) it was derived: $r = 0.999$, $V_{max} = 24.1$ mg O_2/h.l sludge and apparent $K_{1/2} = 54.5$ mg starch COD/l $= 46.0$ mg starch/l; x, starch respiration with 5 mg Fungamyl/l (corrected for respiration with Fungamyl); ●, starch respiration without added Fungamyl 1600.

COD/l, viz. 17.3 mg O_2/h.l of original sludge (Table 6.2). This suggests that enough starch was degraded to maltose by the Fungamyl 1600 preparation almost immediately after the addition of the polymer to allow maximum (i.e. maltose) respiration. The lower respiration rates at lower starch concentrations (Fig. 6.9) are therefore ascribed to a decreased and therefore limiting amylolytic activity, due to the high $K_{1/2}$ value (c. 300 mg starch/l; 6.3) of the Fungamyl. This limitation was more pronounced when only 5 mg Fungamyl/l had been supplied instead of 25 mg/l (Fig. 6.9).

The second conclusion drawn from the experiment of Fig. 6.9 is that the apparent $K_{1/2}$ value, derived from starch respiration measurements after correction for endogenous respiration inclusive Fungamyl, deviated from the $K_{1/2}$ value of respiration rates of glucose sludge on glucose or oligosaccharides (1–5 mg COD/l) and from the $K_{1/2}$ value of amylolysis by Fungamyl (c. 300 mg starch/l). This is not surprising since Fungamyl produces a mixture of dextrins which are respired by glucose sludge at widely different rates (Table

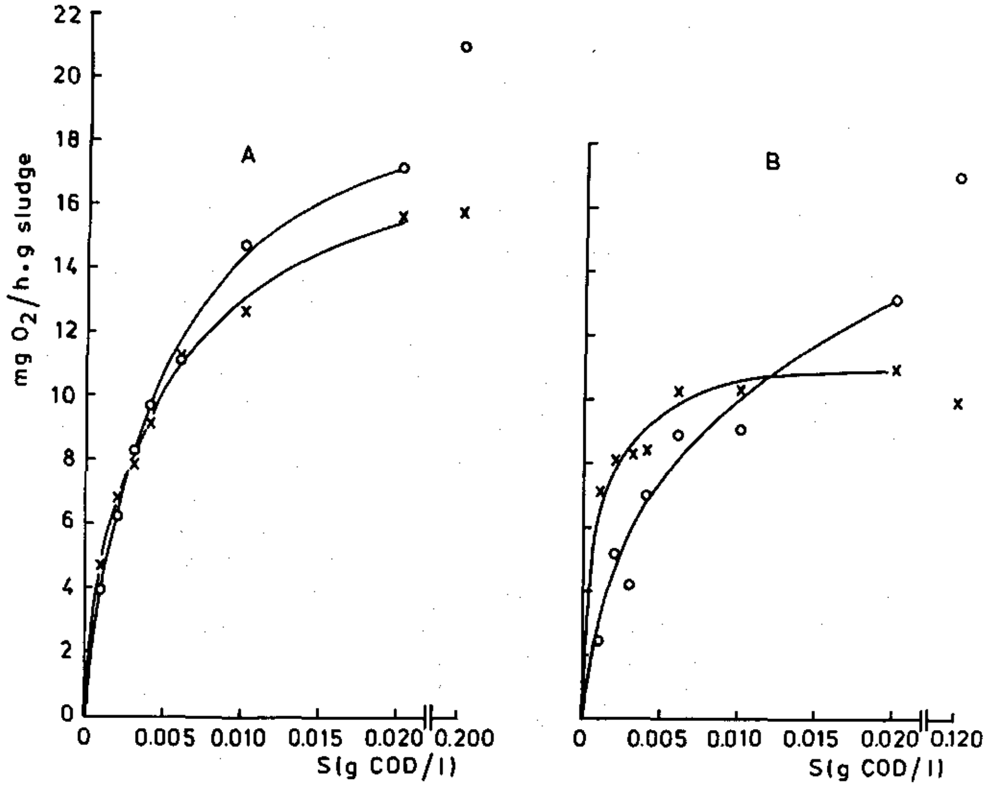

FIG. 6.10. Substrate saturation curves of substrate respiration rates of starch sludges II (Fig. A), and I (Fig. B). Both sludges were pre-incubated, after washing, for more than 4 h before experiments were started (6.4.1). Respiration rates were measured alternately on glucose and on starch. Sludge II (A) had been loaded for 58 days with 0.75 g COD/l.day (Fig. 4.6B); $D = 0.05$ day^{-1}; DA$_{spec}$ was about 65; amylolytic enzymes were completely bound to the sludge; dry wt of biomass was about 4.7 g/l; SL = 0.3–0.15. The endogenous respiration rate varied between 1.36 and 2.02 mg O$_2$/h.g sludge and the averaged dissimilation percentages for glucose and starch were 29.5 and 18.0, respectively. Sludge I (B) had been loaded for 23 days with 1.2 g COD/g sludge.day (Fig. 4.6A); DA$_{spec}$ = 3.74; dry wt of biomass: 3.52 g/l. The endogenous respiration rate varied between 11.7 and 17.5 mg O$_2$/h.g sludge and the averaged dissimilation percentages for glucose and starch were 13.4 and 8.1, respectively. x, Glucose; ○, starch.

6.2). In fact, the curve of Fig. 6.9 resembles the respiration curve of glucose sludge with DE-20, a mixture of dextrins (Fig. 6.8A with high apparent $K_{1/2}$ value).

Fig. 6.10 shows the results of two experiments performed with starch sludges. The starch respiration curve of sludge II (Fig. 6.10A) coincided reasonably well with the glucose respiration curve, except at higher substrate concentrations. The $K_{1/2}$ values calculated from the slightly curvilinear L-B-plots amounted to 4.1 mg starch COD/l ($r = 0.998$) as compared to 2.3 mg COD/l ($r = 0.991$) for glucose. This coincidence was not observed with sludge I (Fig. 6.10B). The extremely high DA$_{spec}$ of sludge II and the relatively low activity of sludge I suggest that, under the conditions reported, amylolytic activity was limiting only the respiration of sludge I and hardly or not that of sludge II. Although the data do not allow calculation of $K_{1/2}$ values of sludge I, that with glucose was low and comparable to that of glucose sludge (Table 6.3).

Over the substrate range applied, the starch respiration rates of sludge II amounted to 200–1000%, those of sludge I to 15–145% of their respective endogenous respiration rates. The glucose dissimilation percentages of sludge II were about 30 and those of sludge I 10–20, as was always found for glucose and maltose sludge I (SL = 0.3). The starch dissimilation percentages with both sludges were only 60% of those with glucose (cf. decreasing dissimilation percentages of glucose and maltose sludge with increasing chain length of the carbohydrate; Table 6.2). This suggests that substrates with a higher degree of polymerization are more completely converted to reserve polymers; in the case of starch, the amylolytic bacteria of sludge I even accumulated 90% or more of the added substrate in their cells. The different dissimilation percentages furthermore suggest that oligomers and polymers are not (completely) degraded by amylolytic enzymes to glucose outside the cytoplasm. The starch removal rates, calculated as 100 × substrate respiration rate/dissimilation percentage, in both sludges (I and II) appeared to be even clearly higher than the calculated glucose removal rates. Only at very low starch concentrations (around and below 1 mg/l) the starch removal rate in sludge I might be slightly lower under the described conditions. It is therefore concluded that dextrins are more readily taken up by starch sludge than glucose. In the same way maltose was taken up preferentially as compared to glucose by maltose sludge (Table 6.2).

6.5. KINETICS OF HYDROLYSIS AND REMOVAL OF STARCH AT CONTINUOUS OPERATION OF ACTIVATED SLUDGE

The most frequently occurring lowest $K_{1/2}$ values of amylases of starch sludges I were estimated at about 15–20 mg starch/l (6.3), as contrasted with a $K_{1/2}$ of about 2.5 mg glucose or maltose COD per litre for the respiration of glucose activated sludge (SL = 0.3) provided with glucose or oligosaccharides (6.4). The latter value is probably also found with starch activated sludge (SL = 1.2; Fig. 6.10B). The $K_{1/2}$ value of amylases appeared to be independent of the SL value at which the starch sludge had been grown.

Besides these $K_{1/2}$ values, V_{max} (potential) values were estimated, viz. standard amylolytic activities at different SL values (4.6.1), and some (maximum) substrate respiration rates at SL = 0.3 (6.4 and 6.5). The actual (symbol v) amylolytic activities and substrate removal rates in situ were both equal to SL since the substrate was removed almost completely at continuous sludge operation (4.5). On the basis of these data an attempt has been made to describe quantitatively processes of amylolysis and of substrate removal in the different starch-grown sludges I at continuous operation (Table 6.4).

$D\tilde{A}_{spec}$ values (Table 4.4) were recalculated as $S\tilde{A}_{spec}$ values, which were

TABLE 6.4. Tentative estimates of average potential, critical potential and actual amylolytic activities (at 20 °C) and saccharide concentrations in continuously operated, differently loaded starch sludges I.

SL (g COD per g sludge per day)		0.075	0.24	1.2	2.4
Actual amylolytic activity[1]	mg GE per g sludge per h	2.93	9.39	47.0	93.9
Potential $S\tilde{A}_{spec}$[2]	mg GE per g sludge per h	31.8	90.0	236	351
Critical potential SA_{spec}[3]	mg GE per g sludge per h	3.44	11.0	55.0	110
Potential $S\tilde{A}_{spec}$ ($\overline{DP} = 5$)[4]	mg GE per g sludge per h	159	450	1180	1755
$\frac{\text{Potential } S\tilde{A}_{spec}}{\text{Critical potential } SA_{spec}}$[5]	–	9.2	8.2	4.3	3.2
$\frac{\text{Potential } S\tilde{A}_{spec} (DP = 5)}{\text{Actual amylolytic activity}}$[6]	–	54	48	25	19
Logarithmically averaged concentrations of dextrins with DP ≥ 6[7]	mg glucose per l	0.40–0.53	0.45–0.59	0.88–1.17	1.18–1.56
Mean starch degradation or residence time[8]	min	4.3	1.5	0.6	0.4

[1] The actual amylolytic activity or carbohydrate removal rate (v) is equal to the sludge loading; 1.000 g COD equals 0.939 g glucose; [2] derived from Table 4.4 using $Q_{10} = 2$ and DA/SA = 10; [3] calculated from the critical DA_{spec} of 275 mg starch per g sludge per h at 30 °C at SL = 0.3 by multiplication with SL/0.3 × 2 × 10; [4] the $S\tilde{A}_{spec}$ (DP = 5) is 5 times as great as the $S\tilde{A}_{spec}$; [5] factor denoting average excess of purification capacity as to amylolytic activity; [6] estimate of $\tilde{V}_{max}/v$; [7] S calculated from $0.8\ \tilde{V}_{max}/v = (S + K_{1/2})/S$, with $K_{1/2} = 16.7$ and 22.2 mg glucose/l, respectively, (i.e. 15 and 20 mg starch/l, respectively); [8] $t = S.60/2.5v$, where S represents the (higher; see [7]) concentration of starch and dextrins with DP ≥ 6, t is the mean time of degradation of starch to dextrins with a $\overline{DP} = 5$ (i.e. the average residence time of dextrins with a DP of 6 and higher in the liquid assuming that they are taken up at $\overline{DP} = 5$) and 2.5 v represents the carbohydrate removal rate per litre containing 2.5 g biomass/litre.

expressed in the same units as the actual amylolytic activity, i.e. mg glucose per g sludge per h at 20°C. $S\tilde{A}_{spec}$ represents the potential $S\tilde{A}_{spec}$ i.e. the activity of starch-saturated amylolytic enzymes in the sludge at 20°C. Q_{10} and the DA/SA ratio of the amylases were shown to be about 2 (3.5.2) and 10 (3.2.3), respectively.

The critical potential SA represents the minimum potential amylolytic activity required for adequate starch removal, such as became apparent in the experiments with glucose and maltose sludge acclimatizing to starch (Fig. 4.3). The experiment of Fig. 4.3E at an SL value of about 0.3 (VL = 0.75; biomass concentration 2.63 g/l) allowed the assessment of the critical potential DA at 690 mg starch equivalents hydrolysed per litre per h at 30°C under standard conditions. At a biomass concentration of 2.5 g/l (with an actual amylolytic activity of 11.7 mg GE per g sludge per h) this corresponds with a critical potential DA_{spec} of 275 mg starch per g sludge per h at 30°C, i.e. a critical potential SA_{spec} of 13.8 mg glucose (i.e. equivalents) per h per g sludge at 20°C. The critical potential DA_{spec} and SA_{spec} were assumed to be proportional to SL (Table 6.4; Figs. 4.7 and 4.8). In other words, the ratio of the critical potential SA_{spec} to the actual amylolytic activity (1.17) is assumed to be independent of SL. This value is very low, particularly when it is kept in mind that the amylolytic production rate of glucose molecules corresponding with a critical potential SA_{spec} of 13.8 mg glucose equivalents per g per h at 20°C is much lower than 13.8 mg per g per h at 20°C, due to the decreasing amylolytic activity with decreasing $\overline{DP}$ of the substrate (3.2.2 and 3.2.4). The low ratio would suggest that starch and dextrin concentrations in the liquid of activated sludge with the reported critical activity would be much higher than the $K_{1/2}$ value for DA, i.e. higher than 15–25 mg starch per litre. In fact, however, the anthrone-positive material, including bacterial products, amounted to 10 mg glucose equivalents per litre whilst the concentration of iodine-stainable compounds was negligible (Fig. 4.3E after 6.5 days of loading with starch). This controversy demonstrates that starch degradation products are not taken up as glucose, but as dextrins. NAKAE and NIKAIDO (1975) showed that oligosaccharides like stachyose (DP = 4, mol.wt 666) penetrated through the cell wall of *Escherichia coli* and *Salmonella typhimurium*, whereas saccharides with a mol.wt of 900–1000 or higher were excluded. Other authors obtained similar results with *Proteus mirabilis* and *Proteus morganii*. DECAD and NIKAIDO (1976) and HANCOCK and NIKAIDO (1978) suggested that the exclusion limit for *Pseudomonas aeruginosa* and *Alcaligenes faecalis* was even larger. The molecular sieve effect of the cell wall of the Gram-negative bacteria was due to the outer membrane layer rather than to the peptidoglycan layer (NAKAE and NIKAIDO, 1975). A penetration limit of oligosaccharides consisting of 5–6 sugar residues is qualitatively in agreement with the results shown in Fig. 4.3E in which an almost complete elimination of carbohydrates (anthrone method) coincided with that of iodine-stainable compounds. According to SWANSON (1948) chains of 4 to 6 glucose units (1,4-α-linked) do not stain with iodine, chains of 8 to 12 units give a red stain and chains of 30 or more units a blue stain (see also MOULD and SYNGE, 1954). Assuming that dextrins consisting on the average of 5 glucose residues are passing the outer membrane of the cells, the true potential $\tilde{SA}_{spec}$ and the true critical potential SA_{spec} will be 5 times higher (designated as SA_{spec} ($\overline{DP} = 5$) in Table 6.4). This SA is defined as the (initial) increase of cell-available dextrins with a $\overline{DP}$ of 5 (cf. 2.6.8). The ratio of the critical potential SA_{spec} ($\overline{DP} = 5$) to the actual amylolytic activity will amount to about 6, indicating that starch degradation by continuously operated starch sludge will be clearly inadequate only if more than about 17% of the amylases is occupied with substrate[1].

The logarithmically averaged standard (i.e. potential) amylolytic activities of starch-grown sludges I (SL = 0.075 to 2.4) were 9 to 3 times as high as the critical amylolytic activities of these sludges (Table 6.4). Due to the relatively small fluctuations of the amylolytic activity of the lower-loaded sludges, all amylolytic activities estimated in these sludges far exceeded the critical amylolytic activities (Fig. 4.7). Only at SL = 2.4 the lowest amylolytic activities incidentally resulted in a somewhat decreased purification (Fig. 4.6A; Fig. 4.7). Fig. 4.7 predicts more serious and more frequently occurring limitations of purification by inadequate amylolytic activities only at SL values above 2.4.

From the overall ratios of the potential $\tilde{SA}_{spec}$ ($\overline{DP} = 5$) to the actual amylolytic activity, ranging from 54 to 19 at SL = 0.075 and 2.4, respectively (Table 6.4), it can be deduced that under apparently steady-state conditions in starch activated sludge on the average probably only 2 to 5.5% of the surface-bound amylases is occupied with substrate.[1] At all SL values the average amounts of surface-bound amylases in starch sludge apparently are very excessive.

The average concentrations of dextrins with a DP of 6 or higher in sludge-free liquids of continuously operated starch activated sludges can be approximated, assuming that the $K_{1/2}$ values estimated for initial starch ($\overline{DP} \geqslant 170$; Fig. 3.3) degradation rates (6.3) are also valid for these dextrins. Since at very low substrate concentrations amylases with the highest substrate affinities contribute most appreciably to the degradation rate (6.1), the $K_{1/2}$ values of these amylases, viz. 15–20 mg starch/l (6.3), are used in this calculation. To fit, at very low substrate concentrations, the substrate saturation curve derived from the V_{max} and $K_{1/2}$ value, calculated by the least squares method, and the curve for actually observed enzyme activities, V_{max} (potential $\tilde{SA}_{spec}$ ($\overline{DP} = 5$), estimated at 5 g starch/l), had to be corrected by multiplication with a factor 0.8 (cf. Fig. 6.5). Table 6.4 shows that the estimates of the average concentration of higher dextrins (DP of 6 and higher) increased from 0.4 to 1.6 mg/l (about 3-fold), when the SL values increased from 0.075 to 2.4, i.e. with a factor of about 30.

The available data allow the calculation of the average time needed for starch activated sludge in situ to degrade starch to dextrins with a $\overline{DP}$ of 5. This degradation time equals the average residence time of starch and its degradation products (dextrins with a DP of 6 and higher) in the sludge-free liquid assuming

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[1] Calculation based on the assumption that K_m approaches the dissociation constant of the enzyme-substrate complex.

that dextrins with a $\overline{\text{DP}}$ of 5 permeate through the cell wall. Table 6.4 shows that these residence times are only about 4 min at very low and 0.4 min at very high loadings.

Some data observed concerning glucose and maltose-grown activated sludge (SL = 0.3) allow the comparison of actual and potential substrate removal rates. The actual substrate removal rate at SL = 0.3 was 12.5 mg COD per g sludge per h. The potential glucose and maltose removal rates were calculated from the potential substrate respiration rates of glucose and maltose sludge, respectively, by multiplication of the latter rates with a factor 100/dissimilation % (6.4), and turned out to be in the range of roughly 80 to 230 mg COD per g sludge per h at 20 °C. Thus the ratio of potential (V_{max}) to actual (v) removal rates ranged from 6.4 to 18.4, values considerably lower than the corresponding ratio for amylolytic activities (Table 6.4). Substitution of these ratios and of the $K_{1/2}$ value of about 2.5 mg COD per litre (Table 6.3) in the equation $V_{max}/v = (S + K_{1/2})/S$ revealed that 'steady-state' substrate concentrations in sludge-free liquids ranged from 0.14 to 0.46 mg COD per litre. If it is justified to apply these data to starch-grown sludge I (SL = 0.3), the concentration of dextrins with an average DP of about 5 (i.e. permeable through the cell wall) would be somewhat lower than the average concentration of higher dextrins (Table 6.4).

The kinetic data of substrate removal and of amylolysis predict that the average substrate concentrations in the effluent of laboratory-grown sludge I (SL = 0.3) will be 0.1 to 0.5 mg glucose per litre (glucose sludge) and 0.5 to 1.0 mg higher and lower dextrins per litre (starch sludge). In accordance with these results hardly or no difference was found between the concentration of anthrone-positive material in both sludge-free liquids (4.5). In Table 4.3 it was shown that the effluent of starch sludge I (SL = 0.3) on the average contained 6.9 mg glucose equivalents per litre (anthrone method), hardly any iodine-stainable material, whereas the COD was much higher than the anthrone-positive material. A comparison of the calculated substrate concentrations and the concentrations detected suggests that residual substrate contributed to 5–10 % or less of the soluble COD in the effluent over the whole range of loadings applied. These percentages are even lower when related to the total COD of the effluent inclusive suspended solids. The major part of the soluble COD probably consisted of refractory soluble organic matter, derived from the sludge micro-organisms and limiting the purification process. This view is in agreement with the concept of product formation (by activated sludge), mathematically modelled by DAIGGER and GRADY (1977).

6.6. KINETIC ASPECTS OF EXTRACELLULAR PROTEOLYSIS IN ACTIVATED SLUDGE

Attempts have been made to estimate the $K_{1/2}$ values of extracellular sludge proteinases for casein. The structure of proteins is considerably more complicated than that of starch. Hence, proteolysis by sludge is less likely to obey Michaelis-Menten kinetics than it is true of amylolysis.

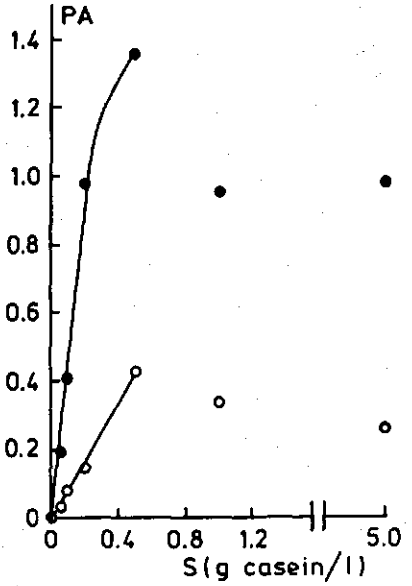

FIG. 6.11. Plots of initial PA values of glucose activated sludge (I, SL = 0.3) and of its cell-free extract (prepared by treatment with chloroform water) versus casein concentration. The sludge had been loaded during 285 days and was incubated at its original concentration (3.83 g/l) in the presence of 1% toluene (added at t = 0). The cell-free extract was prepared by adding 40 ml of demineralized water and 20 ml of chloroform to the centrifugate of c. 200 ml of glucose sludge, washed with demineralized water. The mixture (67 ml) was shaken for 3½ h at 25°C and centrifuged for 20 min at 38,000 × g. The resulting 56 ml supernatant was dialysed twice for 12½ h at 5°C against 4 l of 1 mM potassium phosphate pH 7.5 and the water layer (43 ml) centrifuged for 6 min at 2500 × g. The reactions were initiated by addition of the enzyme samples. Lowry-positive material was determined according to HERBERT et al. (1971; 2.6.11). ●, Sludge; ○, cell-free water extract.

One experiment was carried out with whole glucose sludge I (SL = 0.3) since it was found that Lowry-positive material in the perchloric acid-soluble fraction of cell-free ultrasonicates increased with time in the absence of added casein. This rise was probably the result of degradation of soluble cell protein by extracellular proteinases. Since the increase was not observed in the water phase of dialysed cell-free chloroform-water extract of glucose sludge, an additional experiment was performed with such an extract (Fig. 6.11). In the experiment with whole sludge the possible uptake of proteolytic reaction products by the sludge was prevented by the addition of toluene (1% v/v). In a preliminary experiment, glucose sludge, incubated with 100 mg casein/l in 0.1 M potassium phosphate, pH 7.5, at 1°C, seemed to adsorb at most a few mg casein per g sludge. Hence, the effect of substrate adsorption on reaction rates was not taken into account.

Initial proteolytic reaction rates were plotted against the casein concentration (Fig. 6.11). A linear relationship was observed at substrate concentrations up to 200 mg/l (whole sludge) and 500 mg/l (extract). Reaction rates at higher casein concentrations were relatively low. This may point to an inhibition of the enzymes by excess of substrate.

The data observed do not allow the exact estimation of the $K_{1/2}$ value which, however, amounts to at least a few hundreds of mg of casein per litre, a value much higher than that observed for sludge amylolytic enzymes. From the experimental data of SRIDHAR and PILLAI (1973) a $K_{1/2}$ value of 570 mg casein/l was calculated for proteolytic enzymes extracted from laboratory-grown sewage-fed activated sludge. The $K_{1/2}$ values of extracellular proteinase produced by *Streptococcus faecalis* var. *liquefaciens* (SHUGART and BECK, 1964) or a pathogenic pseudomonad (LI and JORDAN, 1968) were found to be 1.3–6.5 g protein/l (casein, haemoglobin, rainbow trout muscle albumin).

Total Lowry-positive material estimated once in cell-free liquid of sludge loaded with starch at SL = 0.075 and 2.4, amounted to about 2.5 and 10 mg casein equivalents per litre, respectively, values corresponding with the detection limit. The actual proteolytic activity and particularly the actual degradation rate of cell surface-bound amylases in sludge (5.5) are much lower than the potential proteolytic activity observed under standard conditions. This is concluded from the low protein and amylase concentrations in sludge-free liquid and sludge, respectively, and from the high $K_{1/2}$ value of PA. It is assumed that the protein of whole, non-lysed cells is inaccessible to proteolytic enzymes. Fig. 5.14 shows that no relationship exists between amylase inactivation rate and PA_{spec}, but that the specific amylase inactivation rate (k_i) is proportional to PA_{spec}. The high $K_{1/2}$ value of sludge PA (Fig. 6.11) and the small amounts of amylase protein present in starch-grown activated sludge (4.6.3) explain the first order kinetics observed in amylase degradation (Fig. 5.14A).

For estimation of the order of magnitude of the ratio potential to actual amylase degradation rate, the $K_{1/2}$ value of PA for amylase protein was assumed to be 570 mg/l (data derived from SRIDHAR and PILLAI, 1973); the amounts of surface-bound amylase protein present per litre of sludge containing 2.5 g of dry biomass were estimated to be 0.012, 0.034, 0.088 and 0.13 mg at SL = 0.075, 0.24, 1.2 and 2.4, respectively (4.6.3). Based on these assumptions, ratios of 48,000, 17,000, 6,500 and 4,400 were calculated at the respective SL values. Hence, the actual degradation rate of surface-bound amylases in situ might approximate 1/10,000 of the potential rate.

6.7. SUMMARY

1. Starch was adsorbed by whole starch-grown activated sludge but not by glucose-grown sludge, Zeist sludge and Bennekom sludge under the conditions used. Adsorption was observed at 1 and 30°C; it was prevented only to some extent by perchloric acid. The starch adsorption isotherm of starch activated sludge resembled the adsorption isotherm of Langmuir. The maximum adsorption capacity was 10–50 mg of starch per g of sludge; half-maximum saturation occurred at starch concentrations of 50–100 mg/l (data of 3 experiments).
   Starch adsorption likely took place by slimy components rather than by sludge-bound amylases. The adsorption studied is unlikely to play a significant role in continuously operated starch activated sludge.
2. Estimation of the $K_{1/2}$ value of starch hydrolysis by starch activated sludge was successful only if the initial starch concentrations were corrected for the amounts of starch adsorbed by the sludge. The $K_{1/2}$ value of amylases, calculated after such a correction, was not appreciably affected by the binding of the enzymes to sludge.

3. Plotting of DA^{-1} versus S^{-1} values of starch activated sludges (SL = 0.075–2.4) (after correction for adsorption) and of cell-free ultrasonicates almost always yielded deviations from linearity at higher substrate concentrations. This should be attributed to rather large differences between the $K_{1/2}$ values of the amylases present and suggests heterogeneity of amylases and likely of amylolytic bacteria in activated sludge.
4. No evidence was obtained of a correlation between $K_{1/2}$ of amylases and SL (0.075–2.4) or loading period of starch activated sludge. The most frequently occurring overall $K_{1/2}$ values (derived from L-B-plots) were 20–25 mg starch/l, including estimates of the lowest $K_{1/2}$ values of roughly 15–20 mg starch/l. These values are very low as compared with those observed with the α-amylase Fungamyl 1600, viz. about 300 mg starch/l and those of bacterial and fungal α-amylases reported in literature. However, the observed $K_{1/2}$ values are high as compared with the starch concentration in the liquid of starch sludge (see item 19).
5. The oxygen uptake by moderate-loaded starch sludge I was always hardly stimulated by added starch, DE-20, maltose, glucose or lactate, in contrast to the response of moderate-loaded glucose or maltose sludge I.
6. The endogenous respiration rate of glucose sludge was unaffected by glucose in concentrations up to at least 10 mg COD/l.
7. The respiration rate of glucose sludge (DA_{spec} = c. 0.2) and of maltose sludge (DA_{spec} = c. 0.73) with glucose-containing carbohydrates (1,4-α-linked) strongly and moderately, respectively, decreased with DP. In all experiments the dissimilation percentages varied between 10 and 20 when glucose was added to glucose sludge and maltose to maltose sludge. With both sludges the dissimilation percentages decreased with DP. The remaining parts of the substrates were propably mainly converted to polysaccharides. The maltose removal rate, calculated as 100 × substrate respiration rate/dissimilation %, in maltose sludge was clearly higher than the glucose removal rate.
8. $K_{1/2}$ values of glucose, maltose or maltotriose respiration rates of glucose activated sludge (SL = 0.3) amounted to 1–5 mg COD/l and agree with literature data on glucose utilization by suspended bacteria.
9. Glucose activated sludge (SL = 0.3, DA_{spec} = c. 0.2) was able to respire starch in the presence of a large amount of dissolved Fungamyl 1600 at a moderate rate; the maximum rate (at high starch concentration) equalled the respiration rate with maltose, the final product of α-amylolysis.
10. Apparent $K_{1/2}$ values of starch and glucose respiration of a starch sludge II with a very high DA_{spec} were 4.1 and 2.3 mg COD/l, respectively. At high substrate concentrations the starch respiration rate was higher than the glucose respiration rate. The glucose dissimilation percentage was high, viz. about 30%. The starch dissimilation percentage was 18%.
11. A high-loaded starch sludge I, SL = 1.2, respired starch at low substrate concentrations slower, but at high substrate concentrations faster than glucose. This was probably due to the relatively low DA_{spec}. The $K_{1/2}$ value

with glucose was low and comparable to that of glucose sludge.

12. Starch dissimilation percentages of starch sludges I and II (see items 10 and 11) amounted to about 60% of the glucose dissimilation percentages. This suggests that starch is for a greater part converted to reserve polymers than glucose and that it is not (completely) degraded by amylolytic enzymes to glucose outside the cytoplasm.
13. Starch removal rates, calculated as 100 × substrate respiration rate/dissimilation percentage of both sludges I and II were considerably higher than the calculated glucose removal rates, except possibly those at very low starch concentrations (about 1 mg/l and less) in sludge I. This demonstrates that dextrins were more readily taken up by starch sludge than glucose and that dextrins rather than glucose or maltose are to be considered as the end products of amylolysis at the surface of bacterial cell walls.
14. $D\tilde{A}_{spec}$ values measured under standard conditions (30°C), were recalculated as $S\tilde{A}_{spec}$ values, which were expressed as mg glucose equivalents per g sludge per h at 20°C. Both parameters represent potential activities, i.e. activities of starch-saturated amylolytic enzymes. Actual amylolytic activities and starch removal rates (i.e. in situ) of starch sludges I were expressed in the same unit and were both equal to SL.
15. From an experiment with glucose sludge acclimatizing to starch the critical potential SA_{spec} was deduced, i.e. the minimum potential amylolytic activity required for adequate starch removal (4.4). This activity was assumed to be proportional to SL.
16. From item 15 it follows that the ratio of the critical potential SA_{spec} to the actual amylolytic activity is independent of SL. If glucose is assumed to be the end product of cell-surface amylolysis and if the amylolytic activity did not decrease with decreasing $\overline{DP}$ of the substrate, this ratio would amount to only 1.17. If this was true, starch and dextrin concentrations in the sludge-free liquid would be much higher than the $K_{1/2}$ value of amylases. Since these concentrations were almost negligible, it is concluded that dextrins are passing the outer membrane of the cells (see also items 12 and 13). The assumption that these dextrins consist on the average of 5 glucose residues, results in true SA_{spec} values of 5 times higher than SA_{spec} values valid if glucose would be the end product of cell-surface amylolysis in sludge. As a consequence, the ratio of the critical potential SA_{spec} ($\overline{DP} = 5$) to the actual amylolytic activity is about 6. Hence, inadequate starch removal by continuously operated starch sludge I is expected only if more than about 17% of the amylases is occupied with substrate.[1]
17. Logarithmically averaged potential amylolytic activities of starch-grown sludges I (SL = 0.075 to 2.4) were 9 to 3 times as high as the critical amylolytic activities. At SL = 0.075 and 0.3 all amylolytic activities observed far exceeded the critical ones (Fig. 4.7); at SL = 2.4 the amylolytic activity fluctuated extremely and incidentally was lower than the critical

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[1] Assumption: K_m approaches K_{diss}.

activity, such as was observed once at SL = 1.2.

18. The ratio of the potential $\tilde{SA}_{spec}$ (DP = 5) to the actual amylolytic activity ranged from 54 to 19 with SL increasing from 0.075 to 2.4. Thus, under apparently steady-state conditions on the average about 2 to 5.5% of the surface-bound amylases were occupied with substrate[1].
19. Under certain assumptions average concentrations of dextrins that were unable to permeate into the cells (DP = 6 and higher) were estimated to range from 0.4 to 1.6 mg glucose equivalents, their average residence times ranging from about 4 to 0.4 min at SL values ranging from 0.075 to 2.4.
20. On the basis of some respiration experiments the ratio of potential to actual removal rates in glucose and maltose activated sludge (SL = 0.3) was calculated to range from about 6.4 to 18. With a $K_{1/2}$ value of about 2.5 mg COD/l, steady-state substrate concentrations in sludge-free liquid could be assessed at 0.1–0.5 mg COD/l. If application of these data to starch activated sludge (SL = 0.3) is justified, average concentrations of 0.5–1.0 mg lower and higher dextrins per litre can be calculated. These residual substrate concentrations likely amount to 5–10% or less of the soluble COD (and thus even less of the total COD) in the effluent over the whole range of loadings applied. The rest probably consists of refractory bacterial products and limits the process of purification.
21. The $K_{1/2}$ value of proteinases in glucose sludge was at least some hundreds of mg casein/l. From this apparently high $K_{1/2}$ value and the low concentration of Lowry-positive material in sludge-free liquid it is deduced that actual proteolytic reaction rates are very low as compared to the potential ones. The actual degradation rate of surface-bound amylases in situ might even approximate 1/10,000 of the potential rate; this very low rate explains the first order kinetics observed in amylase degradation (5.5).

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[1] Assumption: K_m approaches K_{diss}.

7. LOCATION, ACTIVITY AND ADSORPTION OF EXTRACELLULAR ENZYMES

7.1. Introduction

Extracellular enzymes of bacteria can be bound to the cell wall or excreted into the culture fluid (Chapter 1). The present Chapter deals with experiments on the distribution over liquid and sludge of amylolytic and proteolytic activities of different activated sludges (7.2; see also 3.5.1 and 3.6.1), and on the relative efficiency of these enzyme activities in both fractions (7.2). In addition some attention was paid to the location of the amylolytic and proteolytic enzymes in or on the cell wall of the bacteria (7.2). In section 7.3 enzyme activities of flocculated and suspended bacteria in activated sludge are dealt with. The binding of added enzymes to activated sludge is reported in 7.4.

The starch removal rate of cell-free liquid of starch-acclimatized activated sludge was found by Banerji et al. (1968a) to be 0.2–9 % of the initial whole-cell starch removal rate. However, the presence of amylolytic enzymes in the fluid might be attributed to the preceding homogenization of the sludge in a mixer (compare the effect of ultrasonication, e.g. Fig. 7.1). Verstraete et al. (1975) found 40 to 90 % of the total sludge amylase activity and also an important part of the total proteolytic activity in the sludge fluid. However, their 'sludge' was not a real activated sludge. Watanabe (1941) reported that hydrolytic enzymes such as amylase, protease and lipase were present in bacteria-free liquid of activated sludge. However, Ljubimov and Kagan (1958) found that only the solid phase of activated sludge from three aeration plants showed urease and protease activity. Sridhar and Pillai (1973) also found no protease activity in the effluent of activated sludge. Ingols (1939) found 'pepsin', lipase and diastase only on the surface of the activated-sludge flocs whereas 'trypsin' was found also in the liquor surrounding the flocs.

7.2. Location and activity of amylases and proteinases of activated sludge

Amylolytic and proteolytic enzyme activities were always found to be bound to the sludge i.e. they were completely precipitated with the biomass when sludge 1 from outdoor plants or from low-loaded laboratory units was centrifuged (Table 7.1). The same was true of low-loaded starch sludge II, even when DA_{spec} attained its highest value, viz. 69 (Fig. 4.6B, 64 days), observed during the entire period of investigation. Only in the higher-loaded starch sludges I (SL = 1.2 and 2.4) a small fraction of both enzyme activities was detected in the cell-free supernatants (1–3 % at SL = 2.4).

The sludge-bound condition of amylolytic and proteolytic enzymes is a salient

TABLE 7.1. Location of amylolytic and proteolytic activities in different sludges. Sludge samples were centrifuged for 30 min at 38,000 × g at 4°C and subsequently membrane-filtered (0.45 μm), if necessary. Occasionally the cell-free liquid was concentrated by vacuum rotation evaporation at 30°C.

Sludge	Days of loading	DA_{spec}	Excreted DA as % of total DA	PA_{spec}	Excreted PA as % of total PA
Sludge I					
Bennekom '74–'76		0.54[1]	0	0.51[1]	0
Zeist '74–'76		1.08[1]	0	0.54[1]	0
Starch SL = 0.075–0.10	0–351	0.635[2]	0	0.372[2]	0
Starch SL = 0.24–0.30	0–477	1.80[2]	0	0.366[2]	0
Starch SL = 1.2	41	3.03	0.5	0.086	1.8
	44			0.056	2.2
	93	18.2	0.9	0.194	0
Starch SL = 2.4	58	11.4	2.6	0.105	2.9
	100	5.62	2.2	0.163	1.6
	164	12.1	1.1	0.204	2.5
	191	3.96	1.7	0.159	2.7
	200			0.332	3.4
	234	12.1	2.6	0.243	c. 8
	298	18.1	1.7	0.045	1.9
Sludge II					
Starch SL = c. 0.121 $D = 0.05\ \text{day}^{-1}$	0–559	–[3]	0	–[3]	0

[1] $\overline{DA}_{spec}$, $\overline{PA}_{spec}$, Table 5.3; [2] $D\tilde{A}_{spec}$, $\overline{PA}_{spec}$, Table 5.2; [3] Fig. 4.6B.

property which prevents the continuous wash-out of these enzymes from the hydraulically high-loaded activated-sludge systems. It means that these enzymes, as compared with free extracellular enzymes, are distributed heterogeneously over the whole system which contains only about 0.25% (w/v) dry biomass. It suggests that an intimate contact between sludge(-bound) enzymes and suspended solids containing starch-like and protein-like components is necessary for degradation. These suspended solids can be sludge flocs, containing e.g. degradable amylases (5.5), or can already be present in the sewage.

Addition of salt solutions to activated sludge resulted in partial solubilization of proteolytic and amylolytic enzymes. In a preliminary experiment only rather low percentages of these enzymes were released during ½ h of magnetic stirring of laboratory-grown starch sludge (SL = 0.3) in 0.86 M sodium chloride, 0.42 M magnesium sulphate, or 0.027 M pentasodium tripolyphosphate. Potassium phosphate (0.1 M) released about 5–20% of the original activities of sludges from outdoor and laboratory plants (Table 7.2). Hardly any or no enzymes were extracted by the original sludge liquids after ½ h of shaking. These results show that only a small part of the amylolytic and proteolytic enzymes is not firmly bound to activated sludge.

Ultrasonic treatment (2.6.8) of sludge samples released more enzymes from

TABLE 7.2. Extraction of amylolytic and proteolytic enzymes (measured as DA and PA, respectively) from 3 activated sludges. Sludge samples were shaken for 30 min at 30°C in 0.1 M potassium phosphate, pH 7.0 (DA), in the same buffer at pH 7.5 (PA) and without buffer (control). The samples were centrifuged and the activities determined in the different supernatants and in the original sludges.

Activated sludge	Extracted extracellular enzymes[1]	
	DA (pH 7.0)	PA (pH 7.5)
Bennekom (26/2/'76)	20.7	18.0
Zeist (26/2/'76)	21.3	13.2
Starch, after 173 days SL = 2.4	c. 5.3	c. 9.2

[1] Activity as % of the activity of untreated sludge. The activities in the supernatant of the control were negligible.

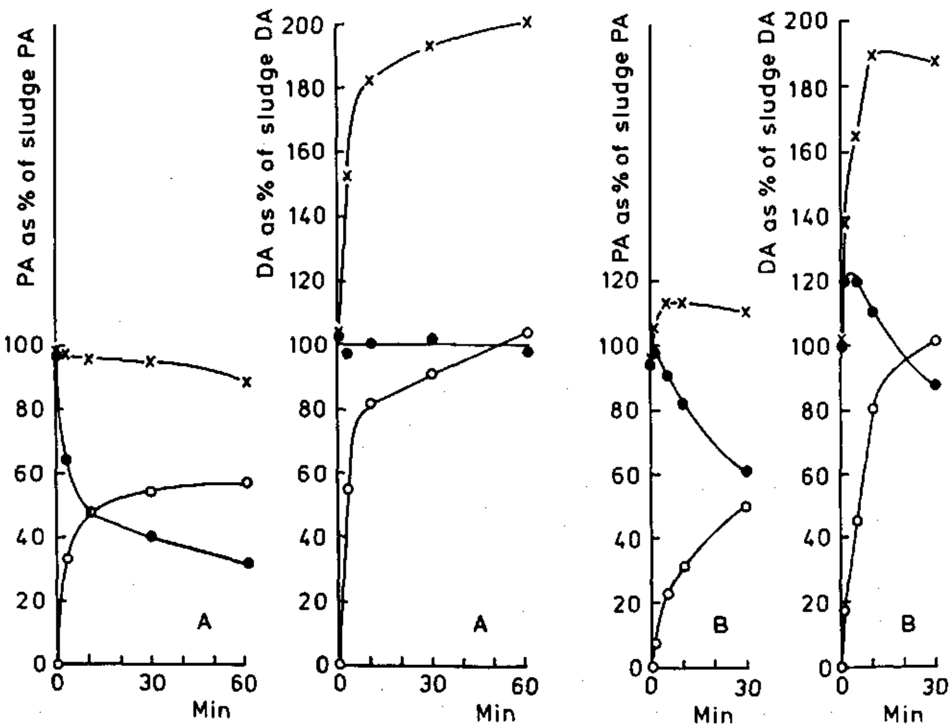

FIG. 7.1. Proteolytic and amylolytic activities of ultrasonically disintegrated sludges 1 in relation to the period of ultrasonication. The sludge DA and PA values were those of the whole untreated sludges (standard activities), viz. A: Zeist sludge (30/5/'75), DA = 2.56, PA = 1.51, biomass: 2.58 g/l and B: slimy starch sludge (loaded during 470 days with SL = 0.3), DA = 3.75, PA = 1.89, biomass: 4.97 g/l. Sludge samples were ultrasonically treated and centrifuged during 35 (A) and 90 min (B) according to 2.6.8. ●, DA or PA of resuspended pellet; ○, DA or PA of supernatant; x, total DA or PA as sum of the activities of pellet and supernatant.

activated sludge (Fig. 7.1) than extraction with potassium phosphate. PA and DA of the activated sludges tested (from the laboratory, Zeist and Bennekom) responded differently to such a treatment. DA increased, whereas PA did not, neither in the presence of 3 mM 2-mercaptoethanol during ultrasonic treatment (data not shown) nor in the absence of this protecting reagent.

The pattern of release of DA and to a less extent that of PA from different sludges showed a clear variation (cf. e.g. Figs. 7.1 and 7.2) which amongst others may have been due to differences in stability of the amylases. These enzymes appeared to be least stable during ultrasonic treatment in the case of very high-loaded starch activated sludge. The proteinases were rather stable during treatment of all of the sludges investigated. This stability was deduced from the observation that the sum of the gradually increasing activity of the liquid phase and the concomitantly decreasing activity of the residual sludge particles remained rather constant during ultrasonic treatment. In the case of starch activated sludge, SL = 2.4, the PA of the supernatant amounted even to 80–90% of the total activity of supernatant and pellet after 30–60 min of ultrasonic treatment (data not shown).

From the fact that the total PA did not increase during ultrasonic treatment, it is concluded that intracellular (5.6) and non-surface-bound (i.e. non-casein-accessible) extracellular proteolytic enzymes probably do not occur in significant amounts as compared to the surface-bound proteolytic enzymes. From the observation that free, soluble proteinases were released by ultrasonic treatment without concomitant increase of total PA (Table 7.4), it is furthermore concluded that sludge-bound extracellular enzymes are not limited by steric restrictions imposed by the carrier (i.e. bacterium or floc).

In contrast to PA, the standard activity of DA of Zeist, Bennekom and laboratory-grown sludges increased to at most twice the original activity during

TABLE 7.3. Amylolytic activities of activated sludges and of their ultrasonicates (sonication time 20 min) using starch and DE-9 as substrates. The data in brackets are the amylolytic activities expressed as percentages of the corresponding activities of whole untreated sludges.

Source	Amylolytic system[1]	Substrate[2]	DA	SA	DA/SA
Zeist[3] (15/12/'76)	activated sludge	starch	3.41	0.261	13.1
	whole ultrasonicate	starch	5.35 (157)	0.396 (152)	13.5
	activated sludge	DE-9		0.245	
	whole ultrasonicate	DE-9		0.382 (156)	
Starch sludge[4] after 237 days: SL = 0.3	activated sludge	starch		0.608	
	whole ultrasonicate	starch		1.086 (179)	
	activated sludge	DE-9		0.543	
	whole ultrasonicate	DE-9		0.935 (172)	

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[1] Reactions were initiated by the addition of enzyme samples (1:1 diluted); [2] starch solution contained 0.6% reducing sugars (related to total carbohydrate concentration), DE-9 solution contained 9.2% reducing sugars; [3] standard conditions, except 2.0 g substrate/l and 0.5% (v/v) of toluene; [4] standard conditions, except 1.5 g substrate/l, 0.5% (v/v) of toluene, 0.5% (v/v) of chloroform, 50 mg nystatin/l and 1% (v/v) of ethanol (see Fig. 3.3).

ultrasonic treatment (Fig. 7.1). In one case no decrease of pellet DA was observed during treatment (Fig. 7.1A). It is not certain that this constant pellet activity was only due to firmly surface-bound enzymes.

The different behaviour of DA and PA during ultrasonic treatment was frequently seen during three other treatments: toluene treatment (3.3.6 and 3.4.5), freezing and thawing (3.3.5 and 3.4.4) and addition of excess of casein (5.5).

The increase of standard amylolytic activity may be explained in several ways.

1) The release of amylolytic enzymes might promote the maximum reaction rate (V_{max}) and/or decrease the $K_{1/2}$ value of these enzymes due to mitigation of steric hindrance exerted by binding to slimy compounds or to cell wall constituents. This is improbable since the sludge-bound proteolytic enzymes did not show increased activity after solubilization. $K_{1/2}$ values of DA in cell-free ultrasonicates and in whole sludge did not differ appreciably (6.3).

2) The rate of diffusion of starch into the sludge flocs might limit DA under standard conditions. If this were true, such a limitation would also have been observed in PA, which is relatively abundant in sludge flocs (Table 7.5). The results of the following experiments exclude the possibility of an appreciable restriction of the overall reaction rate of starch and protein hydrolysis by intrafloc diffusion of the substrate under standard conditions.

a) Upon ultrasonic treatment of starch and Zeist sludge the per cent increase of DA or SA with starch as the substrate was equal to that of SA with the low-molecular substrate DE-9 (Table 7.3). This result is not in agreement with a

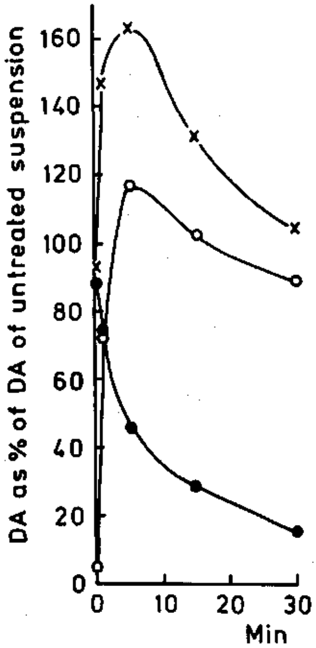

FIG. 7.2. Amylolytic activities of ultrasonically treated suspended cells of sludge I in relation to the period of ultrasonication. Starch activated sludge, loaded at SL = 2.4 for 234 days (Table 7.5) was passed through Whatman N° 1 filter paper and samples of the filtrate were ultrasonically treated according to 2.6.8. The suspension (DA = 11.48) was very rich in separate *Nocardia*-like bacteria, each cell being surrounded by a heavy slime layer. ●, DA of resuspended pellet; ○, DA of supernatant; x, total DA as sum of the activities of pellet and supernatant.

supposed restriction of the reaction rate due to diffusion (dextrins would move faster in sludge flocs than starch). The SA values with DE-9 were almost equal to those with starch, viz. 94 and 97% (amylolytic enzymes of Zeist sludge) and 89 and 86% (enzymes of starch sludge).

b) Ultrasonic treatment of suspended bacteria resulted in a sharp increase of DA within one minute (Fig. 7.2). Mass transfer resistance in floc material was obviously not involved.

c) Starch concentrations (from 2–20 g/l) and different agitation rates in the water bath did not affect DA of very slimy starch activated sludge (SL = 0.3). If under standard conditions, i.e. at 5 g starch/l and at an agitation rate of c. 100 strokes/min, the diffusion of starch into the floc, at least to the zones containing amylases, had been clearly limiting the overall reaction rate, variation of the above-mentioned factors would have affected the DA values observed. Even at 5–15 mg starch/l a clear limitation of DA by diffusion is unlikely to occur since the $K_{1/2}$ values of amylases in sludge and cell-free ultrasonicate were roughly the same (6.3).

3) Part of the amylolytic enzymes of activated sludge might be inaccessible to starch and to DE-9. This might be due to the particular location of part of these enzymes viz. in the slime layer surrounding the cell wall or inside the outer membrane of the cell in the case of Gram-negative bacteria. The former possibility is unlikely as non-slimy Zeist and Bennekom sludges showed also a pronounced increase of DA upon ultrasonic treatment, as contrasted to PA. The second possibility might include the location of the non-starch-accessible amylases in the cytoplasm or in the periplasmic space of the cell.

The cytoplasm of several bacteria that are actively secreting extracellular proteins shows either a total absence or only trace amounts of extracellular proteins (GLENN, 1976). Cytoplasmic enzymes are expected to have other properties than extracellular enzymes. In the case of degradation of polysaccharides of the starch-glycogen class inside the cell membrane, phosphorylases are generally involved rather than hydrolases. In section 3.2, Table 3.1, Fig. 3.3 and section 6.3 it has been demonstrated that the degradative capacities of amylolytic enzymes, DA/SA ratios, relative rates of starch, dextrin and maltotriose hydrolysis and $K_{1/2}$ values of the amylolytic enzymes were about the same in cell-free sludge ultrasonicates and whole sludge. From these data it may be concluded that cytoplasmic starch-degrading enzymes are not responsible for the increased amylolytic activity after ultrasonic treatment of sludge.

The cell wall of Gram-negative bacteria contains a variety of degradative enzymes, which are localized within the periplasmic space or at the cell surface (COSTERTON et al., 1974). At least one of the periplasmic enzymes, alkaline phosphatase, may occur also at the cell surface (COSTERTON et al., 1974). RAWLINGS and WOODS (1978) reported that ultrasonic treatment of activated sludge did not affect protease, urease and catalase activities, but that it markedly increased the alkaline phosphatase activity. Comparison of the results obtained in the present investigation with the literature data suggests a location of all amylolytic enzymes outside the cytoplasmic membrane of the Gram-negative bacteria found in activated sludge (Chapter 8). Part of these enzymes which is detectable in whole untreated sludge, is located on the cell surface (outer membrane or slime layer) and is freely accessible to macromolecular substrates like starch. The other part of these enzymes is probably located in the periplasmic space of the bacteria, i.e. inside the outer membrane which is functioning as a molecular sieve (COSTERTON et al., 1974). The latter enzymes are inaccessible to starch and likely to saccharides with a molecular weight of 900–1000 or more (NAKAE and NIKAIDO, 1975; section 6.5); they become accessible upon ultrasonic treatment. Both enzyme fractions show the same enzymic abilities, as stated above. From the results obtained (Figs. 7.1 and 7.2; other experiments) the periplasmic amylolytic activity may be estimated to be roughly equal to the activity of surface-bound amylolytic enzymes as estimated under standard conditions. The function of these periplasmic enzymes probably is to hydrolyse oligosaccharides with a $\overline{DP}$ of about 5 or less; complete hydrolysis of these oligosaccharides to glucose prior to permeation through the cytoplasmic membrane is unlikely to occur (6.4). The fact that upon ultrasonic treatment (Table 7.4) amylolytic enzymes of fraction (3) amounted to 70% (SA) and 79% (DA) of those of fraction (1), whereas the proteolytic enzymes of fraction (3) amounted to only 57% of those of fraction (1), may indicate that the periplasmic amylolytic enzymes are not or only loosely bound as compared to those bound to the cell-surface.

From the results with ultrasonicated sludge (Fig. 7.1) it can be concluded that the proteolytic enzymes of sludge bacteria are located on the cell surface of these organisms and not in the periplasmic space. This conclusion is in agreement with a number of literature recordings (PAYNE, 1976) stating that peptidases, in contrast to other degradative enzymes, are absent in the periplasm of *E. coli*.

Although about half of the amylolytic bacteria of moderate-loaded starch activated sludge were also proteolytic (5.2.2), all amylolytic enzymes occurring

TABLE 7.4. Proteolytic and amylolytic activities of different fractions of Zeist activated sludge (20/11/'75) submitted to ultrasonic disintegration for 20 min.

Ultrasonic treatment	Enzyme fraction	PA		DA		SA	
Before	Whole, untreated sludge[1]	100%		100%		100%	
	Sludge filtrate (0.45 μ)	0%		≤1%			
After	(1) Supernatant[2] of 60 min, 38,000 × g, 4°C	46%	100%	73%	160%	60%	143%
	(2) Pellet[2] of 60 min, 38,000 × g, 4°C	54%		87%		83%	
	(3) Supernatant[2] of 60 min, 129,000 × g, 9°C	26%		58%		42%	

[1] The PA, DA and SA values and the biomass concentration (g/l) of the original sludge were: 1.60, 4.01, 0.360 and 3.71, respectively; [2] fraction (3) is considered to contain soluble cell components only, fraction (1) in addition membrane residues and fraction (2) intact cells and cell debris.

TABLE 7.5. Distribution of amylolytic and proteolytic activities among dispersed and flocculated fractions of starch sludges I. Suspended sludge was obtained by filtration through Whatman N° 1 filter paper, sludge-free liquid by centrifugation for 30 min at 38,000 × g, at 4°C, sometimes followed by membrane filtration (0.45 μm). Suspension and cell-free liquid, if necessary, were concentrated by vacuum rotation evaporation at 30°C. Enzymic activities were measured in whole sludge (1), sludge suspension (2) and cell-free liquid (3). Activity of flocculated biomass: (1) minus (2); activity of suspended biomass: (2) minus (3). Biomass concentrations were estimated in (1) and (2).

SL	Days of loading	Biomass (g/l)		DA_{spec}			PA_{spec}		
		Suspended	Flocculated	Whole sludge	Suspended biomass	Flocculated biomass	Whole sludge	Suspended biomass	Flocculated biomass
0.075	48	0.0072	2.12	1.02	1.68[1]	1.02	0.37	0.21[1]	0.38
1.2	37	0.21	2.99	5.20	9.03[1]	4.93			
	41	0.40	2.81	3.03	5.40[1]	2.68	0.086	0.030[1]	0.093
	44	0.29	3.15				0.056	0.0081[1]	0.059
2.4	152	0.48	1.91	13.9	16.6	13.1	0.139	0.026	0.164
	164	0.071	2.33	12.1	4.70	12.1	0.204	0.096	0.203
	191	0.74	1.26	3.96	$\geq$1.91	$\leq$5.05	0.159	0.035	0.224
	200	0.17	2.71	3.04	2.57	3.07	0.332	0.056	0.337
	234	0.86	0.94	12.1	12.7	10.9	0.243	0.085	0.349
	263	0.43	1.58	8.35	10.9	7.65	0.244	0.058	0.270
	298	1.05	1.28	18.1	25.3	11.6	0.045	0.0025	0.077

[1] Determined in the presence of 1% toluene.

in the periplasmic space of these bacteria were protected against proteolytic attack. Only surface-bound amylases were degraded by proteolytic enzymes (5.5).

7.3. DISTRIBUTION OF AMYLOLYTIC AND PROTEOLYTIC ACTIVITIES AMONG DISPERSED AND FLOCCULATED SLUDGE BIOMASS

A minor part of the biomass of starch-grown sludge I consisted of suspended bacteria, as contrasted to starch-grown sludge of type II in which a major part of the bacteria was in suspension (4.6.1 and 5.2.3). To explain the differences observed between both systems, proteolytic and amylolytic activities of dispersed and flocculated sludge bacteria of the higher-loaded systems I were estimated (Table 7.5). Since the suspended bacteria are not retained by the spongy filter element applied in system I, their mean cell residence time (t_s) approaches the mean reactor hydraulic residence time ($t_h = D^{-1}$) i.e. 24 h (SL = 0.075–1.2) and 8 h (SL = 2.4). For a detailed description of the activated sludge, it is of interest to know to what extent suspended bacteria and flocculated biomass share in the amylolytic and proteolytic enzyme activities. As sludge flocs do not pass filter paper as suspended bacteria do, this way to separate the suspended fraction was used. The flocculated biomass was estimated indirectly (Table 7.5). If a more precise estimation of the suspended fraction of the total biomass is aimed at, centrifuging at a low gravity (e.g. about 150 × g) has to be preferred as after prolonged filtration part of the suspended bacteria can also be retained on the filter.

Generally the proportion of the dispersed bacteria of the total biomass increased with SL (when the t_h values reported above were applied). At SL = 2.4 the dispersed bacteria varied in the range from 3 to 50% of the total biomass (Table 7.5).

DA_{spec} of the suspended biomass on the average tended to be slightly higher than that of the sludge flocs. This suggests that on the average there was hardly any difference between the amylolytic activity of a viable suspended amylolytic bacterium and that of a viable amylolytic bacterium occurring in a floc, in spite of the different mean cell residence times of both types of bacteria. In this calculation it is assumed, that the biomass of both suspended and flocculated sludge consists for roughly equal % of living amylolytic bacteria. The conclusion that different cell residence times do not result in clearly different amylolytic activities per amylolytic bacterium is in agreement with the results plotted in Fig. 4.9. The apparently more favourable position of suspended amylolytic bacteria compared to those in flocs in their competition for starch utilization may therefore, amongst other things, be attributed to a higher substrate concentration in the liquid as compared to that in the floc rather than to a considerably higher amylolytic activity of the suspended cells.

In contrast to DA_{spec}, the average PA_{spec} of suspended biomass was significantly lower than that of flocculated biomass at all SL values studied, at

SL = 2.4 even 5–6 times. This suggests that proteolysis and extracellular turnover of biomass take place mainly in the flocs even in very high-loaded activated sludge. The higher PA_{spec} of flocculated biomass as compared to that of suspended biomass was apparently due to higher percentages of proteolytic bacteria rather than to a higher proteolytic activity per viable proteolytic bacterium in the former biomass. Although the about 3–6 times longer cell residence time of flocculated biomass as compared to that of suspended cells (at SL = 2.4) might suggest to cause a higher proteolytic activity per proteolytic cell, Fig. 5.4B shows that at least in sludge II even cell residence times differing with a factor 20 did not result in large differences in the proteolytic activity per (viable) proteolytic bacterium. The suspended biomass of sludge I after 234, 263 and especially 298 days of loading with SL = 2.4 (Table 7.5) consisted predominantly of branched *Nocardia*-like bacteria. This organism was previously shown to be non-proteolytic and to be unable to grow in flocs.

The observed high PA_{spec} of sludge I flocs in situ apparently results from relatively high rates of death and/or autolysis. It demonstrates that the growth conditions inside the floc are more unfavourable than those outside. Under the reactor conditions applied, the proteolytic activity is affected more seriously by the growth conditions (floc or suspension) than by the mean cell residence time. In section 5.2.3 it was concluded that retention of biomass (i.e. collection of flocs by physical methods) is an important condition to obtain a high PA_{spec} in continuously loaded systems (see e.g. Figs. 5.2B, 5.3B and 5.4B) which was correlated with the occurrence of flocculated mixed cultures. This conclusion is confirmed by the results of this section. Without retention of flocculated biomass, suspended bacteria as the above-mentioned non-proteolytic *Nocardia*-like bacterium win the competition. The suspended population tends to become a monoculture or a mixed culture dominated by a few types.

7.4. ADSORPTION OF ADDED EXTRACELLULAR ENZYMES BY ACTIVATED SLUDGE

'Biocatalysts', products of concentrated enzymes or dried bacteria, are sometimes added to sewage purification and sludge digestion systems in order to improve and accelerate the biological digestion of organic wastes. Most of the studies reported on this matter agree in their conclusions that commercial 'biocatalysts' have little if any effect (CHAMBERLIN, 1930; MCKINNEY and POLIAKOFF, 1954; VERSTRAETE et al., 1972). HEUKELEKIAN and BERGER (1953) observed a liquefying effect of purified enzymes (with e.g. amylolytic and proteolytic activity obtained from a strain of *Bac. subtilis* and an amylolytic enzyme from *Asp. oryzae*) on sterile wastes. Also reference was made of the successful use of 'Enzymatic' (a concentrated enzyme preparation) in the control of operational difficulties in sewage systems (User's report on enzymatic treatment of sewerage, 1954).

The fate of enzymes added to activated sludge has so far not been studied. Therefore an investigation was made of the fate of commercial amylase, proteinase and lysozyme added to activated sludge.

7.4.1. *α-Amylase*

A beneficial effect of the α-amylase of *Aspergillus oryzae* (Fungamyl 1600) on the respiration of starch by glucose activated sludge was observed in the short run experiments reported in 6.4.

Although Fungamyl 1600 (20 mg/l) was stable for several days (Fig. 7.3; 3.2.3), it was completely inactivated within 4 h when added to concentrated starch activated sludge (Fig. 7.3). The initial rate of inactivation of Fungamyl was more than 100 times greater than that of the amylolytic enzymes of starch sludge. When both inactivation rates are considered to be first order reactions, the (initial) specific amylase inactivation rate of Fungamyl would roughly amount to 32 day^{-1} and that of starch sludge to 1.93 day^{-1}; cf. data of Fig. 5.14. The high inactivation rate of Fungamyl cannot be attributed to a ready degradation of free amylases by the sludge-bound proteolytic enzymes since the fungal α-amylase was quite stable in the presence of cell-free ultrasonicate of starch sludge which showed considerable proteolytic activity (Fig. 7.4). This experiment shows that the α-amylase of *Aspergillus oryzae*, in contrast to the sludge amylases, is resistant to sludge proteolytic enzymes, like it is to trypsin and chymotrypsin (5.1.4).

From Figs. 7.3 and 7.4 it is concluded that adsorption of Fungamyl to sludge was probably involved in the inactivation of added α-amylase. Fig. 7.5 shows

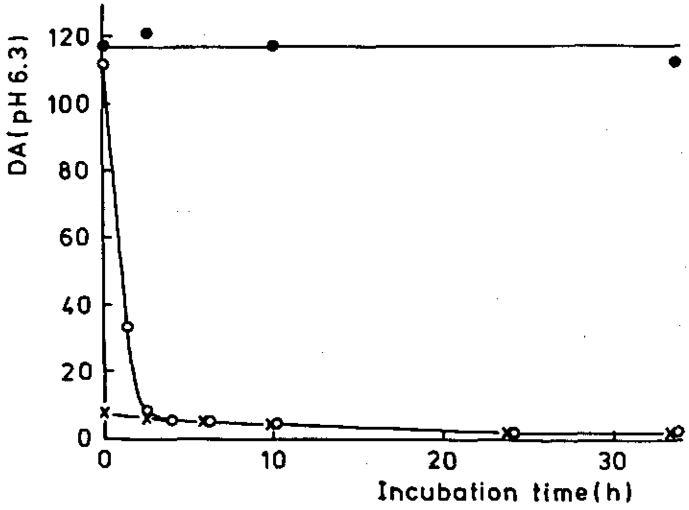

FIG. 7.3. Inactivation of sludge and Fungamyl 1600 amylases in starch activated sludge at 30°C. The sludge (DA_{spec} = 1.95 and PA_{spec} = about 0.87) had been loaded during 325 days at SL = 0.3. Deviations from standard procedure (2.6.13): after centrifugation the sludge was washed once with demineralized water; the inactivation mixtures contained 5 mM potassium phosphate and 1 mM $CaCl_2.2H_2O$; the complete mixtures were incubated for 15–20 min before the measurements of enzyme activities were started (t = 0 h); DA was determined at pH 6.3. x, Sludge (3.87 g/l); ●, Fungamyl 1600 (20 mg/l) without sludge (the DA of 1 mg Fungamyl/l at 30°C and pH 6.3 was about 6, that at pH 7.0 about 3); ○, Fungamyl 1600 (20 mg/l) with sludge (total activity).

that Fungamyl inactivation coincided exactly with the adsorption to Bennekom sludge. This adsorption process proceeded relatively slowly. Adsorption of Fungamyl resulting in inactivation was shown to occur in two further sludges tested (glucose and starch activated sludges). The biomass concentrations of both sludges shown in Fig. 7.5 were lower than that of the experiment of Fig. 7.3 and resulted in an incomplete α-amylase inactivation. The adsorption rate in Bennekom sludge was clearly lower than that in starch sludge. The residual α-amylase kept its activity during prolonged incubation. From the residual activities in these and other experiments it was calculated that both sludges had adsorbed about 6–8 mg Fungamyl per g of biomass if 20 mg of the amylase preparation had been added per litre under the conditions reported. Fungamyl contained 30% protein of which less than 1/10 was active α-amylase (4.6.3). However, the amount of Fungamyl adsorbed increased clearly with the amount added, at least within the range 10–40 mg added per litre (data not shown).

The amount of Fungamyl finally inactivated by activated sludge as well as the rate of inactivation of this amylase preparation were affected by the amount of potassium phosphate (pH 7.0) in the mixture (Fig. 7.6). The supply of 0–5 mM phosphate buffer prior to the addition of Fungamyl had no clear effect; 20 mM buffer inhibited α-amylase inactivation partly and 100 mM completely. At the latter buffer concentration the original Fungamyl activity was entirely present in

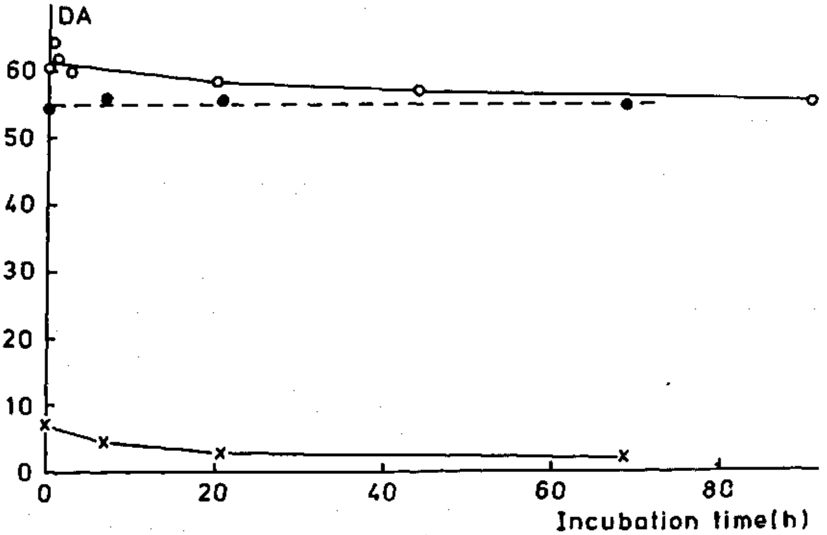

FIG. 7.4. Inactivation of sludge-derived and Fungamyl 1600 amylases in cell-free ultrasonicate (sonication time: 10 min) of starch activated sludge at 30°C. The sludge had been loaded during 393 days at SL = 0.3. Deviations from standard procedure (2.6.13): after centrifugation the sludge was washed once with demineralized water; stability assay mixtures contained 50 mM potassium phosphate. x, Cell-free ultrasonicate; ○, Fungamyl 1600 (20 mg/l) in cell-free ultrasonicate (total activity); ●, Fungamyl 1600 (20 mg/l), calculated by subtracting ultrasonicate activity from total activity.

the cell-free liquids of both Bennekom and Zeist sludge. The protecting effect of potassium phosphate, likely due to electrostatic interaction, is additional evidence that fungal α-amylase inactivation depends on adsorption to the sludges. If, however, Bennekom sludge that had adsorbed Fungamyl was extracted with 100 mM potassium phosphate, pH 7.0, no α-amylase activity was released from the sludge (see e.g. Fig. 7.5). This buffer, at a concentration of 100 mM, was previously found to extract sludge amylases from several sludges (Table 7.2). Apparently, the adsorption of Fungamyl is either completely irreversible with loss of activity or reversible with the released enzyme having lost its activity (denaturation). The latter possibility is unlikely to occur, since the residual α-amylase activity in the sludge Fungamyl mixture was quite stable during prolonged incubation (Fig. 7.5). Adsorption of macromolecules is usually an irreversible process since statistically it is highly improbable that all the attached segments of one molecule would desorb simultaneously (NORDE, 1976). Binding

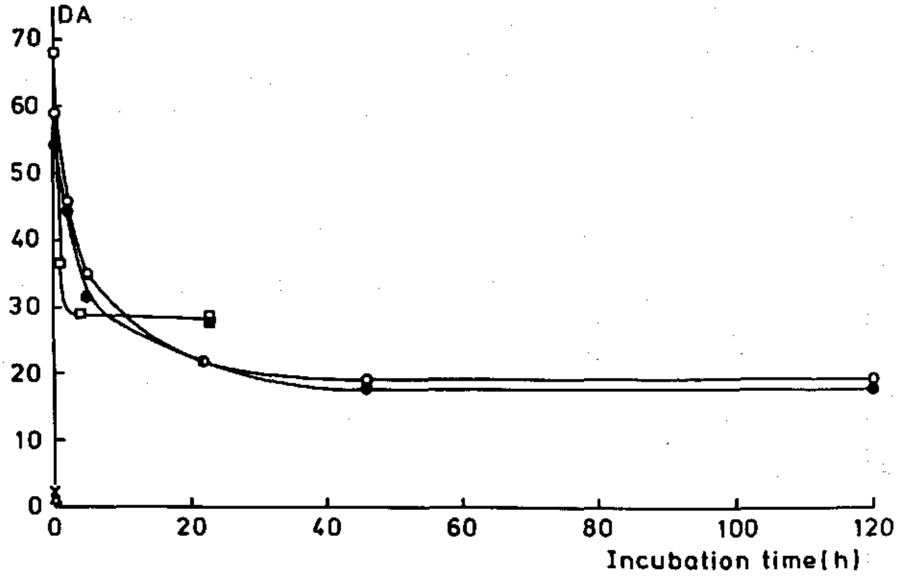

FIG. 7.5. Inactivation of Fungamyl 1600 in starch activated sludge and in Bennekom activated sludge at 30°C. The starch sludge ($\mathrm{DA}_{\mathrm{spec}}$ = 1.48) had been loaded during 171 days at SL = 2.4; the Bennekom sludge ($\mathrm{DA}_{\mathrm{spec}}$ = 0.43) dated form 14/7/1976. Deviations from standard procedure (2.6.13): the starch sludge and the Bennekom sludge were incubated at 66 and 40 %, respectively, of their original concentrations; the inactivation mixtures contained 5 mM potassium phosphate. The enzyme activities were computed at the original Fungamyl concentration (20 mg/l). □, Fungamyl 1600 (20 mg/l) with starch sludge (1.47 g/l), total activity; ■, supernatant of the centrifuged sludge-Fungamyl mixture; ○, Fungamyl 1600 (20 mg/l) with Bennekom sludge (1.71 g/l), total activity; ●, supernatant of the centrifuged sludge – Fungamyl mixture; x, starch sludge; △, Bennekom sludge. After 46 h of incubation of Fungamyl with Bennekom sludge in 5 mM buffer a sample was withdrawn and incubated for an additional period of 3 days in 100 mM buffer. After this period the DA of the whole mixture and of the supernatant were both 18.35 which is equal to the DA of the sample incubated during 46 h in 5 mM buffer.

of a protein molecule in its native conformation at an interface may render the active site(s) less accessible (NORDE, 1976). Therefore, the loss of amylolytic activity is not necessarily the result of conformational changes.

7.4.2. *Lysozyme*

Commercial egg white lysozyme lost its activity almost immediately and completely after addition to starch sludge (Table 7.6). This was deduced from the absence of lytic activity on *Sarcina lutea* (2.6.14) in resuspended centrifuged sludge (tested after the experiment) and its cell-free supernatant. The same result was obtained with boiled sludge (Table 7.6). These results strongly suggest that adsorption rather than proteolysis was the cause of lysozyme inactivation. Lysozyme is adsorbed to sludge at a rate much faster than that of Fungamyl adsorption. According to W. NORDE (personal communication) lysozyme is adsorbed to polystyrene latices also relatively quickly as compared with other proteins.

When a suspension of *Sarcina lutea* was added after 1 min of mixing lysozyme (20 mg/l) with starch or glucose sludge and buffer (3 mM), only after prolonged incubation a slight lysis was observed which was attributed to a slight residual lytic activity in the liquid (cf. Table 7.6). This shows that lysozyme is immediately inactivated by adsorption to sludge. However, when lysozyme was

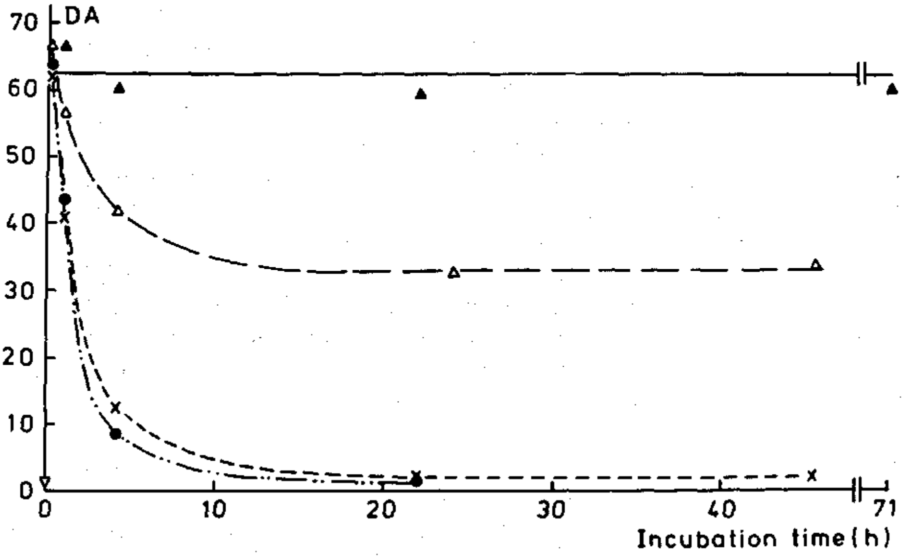

FIG. 7.6. Effect of potassium phosphate concentration on the adsorption of fungal amylase by Bennekom activated sludge (at 30°C). The DA_{spec} of the sludge (28/6/1976) was 0.45 and the pH 7.0. Deviations from the standard procedure (2.6.13): the sludge was incubated at 66% of its original concentration. The estimated enzyme activities were the total activities of 20 mg Fungamyl/l and diluted sludge. ▲ (———), 100 mM phosphate buffer in inactivation mixture; △ (– – –), 20 mM buffer; x (- - - - -), 2.5 mM buffer; ● (—.—..), 0 mM buffer; ∇ Bennekom sludge (of 3.23 g/l) without Fungamyl 1600. In the case of 1 and 5 mM phosphate buffer the rate and degree of inactivation of Fungamyl closely resembled those observed in 0 and 2.5 mM buffer.

TABLE 7.6. Inactivation of lysozyme in starch activated sludge. The starch sludge had been loaded during 310 days at SL = 0.3 and contained about 4.5 g dry wt/l. One sample was washed once, a second was boiled for ½ h and washed 3 times. Both samples were incubated at half of the original sludge concentration in a water bath (30°C, 3 mM potassium phosphate, pH 7.0, rate of shaking: c. 100 strokes/min) in the presence of 40 mg lysozyme/l. Samples withdrawn from the inactivation mixtures were immediately ice-cooled and centrifuged at 38,000 × g. The resulting supernatants were tested for the presence of lysozyme with a suspension of *Sarcina lutea* as substrate (2.6.14).

Time of incubation (min)	Decrease of nephelometric units per min in supernatant		
	Control without sludge	Boiled sludge	Untreated sludge
0.5[1]	55[2]	1.8	3.0
30		0.4	0.6
60	60[2]	0.1	0.1

[1] After one h the resuspended precipitates of both sludges were tested for lysozyme activity by adding buffer and a suspension of *Sarcina lutea*. Even after 17 h no lysis of *S. lutea* was observed neither microscopically nor turbidimetrically; [2] 20 mg lysozyme/l.

added to a mixture of *Sarcina* cells and sludge, it partially escaped inactivation, apparently due to binding to *Sarcina* cells which lysed quickly.

7.4.3. *Pronase*

Experiments with Pronase E, a mixture of proteolytic enzymes produced by *Streptomyces griseus*, were less easily to interpret due to the relatively high proteolytic activity of the sludge itself and to the instability of the enzymes. At least part of the proteolytic enzymes was labile in 100 mM and in 5 mM potassium phosphate pH 7.0, also after addition of 2.5 mM $CaCl_2$ (conditions of 2.6.13). Nevertheless, Pronase E (20 mg/l) inactivation was shown to be promoted by the presence of starch activated sludge (SL = 0.3). Although this inactivation was retarded by the presence of potassium phosphate pH 7.0 in the range of 0–100 mM, the latter concentration likely did not prevent ultimate inactivation of Pronase. The results suggest that Pronase was inactivated by sludge, probably due to adsorption, at a rate comparable with that of Fungamyl rather than with that of lysozyme (results not shown).

7.4.4. *Fate of added and sludge-produced extracellular enzymes*

Extracellular enzymes added to activated sludge appeared to loose their activity completely, unless an excessive amount of enzyme was added, and relatively readily (cf. 5.5). This loss was probably due to adsorption to the sludge flocs. Different enzymes responded differently to added potassium phosphate and were inactivated at different rates.

Although the adsorption capacity of activated sludge for proteins was shown to approximate only some mg per g sludge biomass, both for Fungamyl and likely for casein (6.6), the adsorption of proteins to sludge interferes with the beneficial action of enzymes added to activated sludge. Any advantageous effect of added enzymes is to be expected only if the enzymes are administered in excess of the sludge's adsorption capacity. However, in the case of Fungamyl this capacity was shown to increase clearly with the amount of amylase added. Moreover, such an excessive amount of added enzyme will soon wash out due to the high hydraulic space loading usually applied in the activated-sludge process.

The fate of enzymes, possibly set free by sludge bacteria to the cell-free sludge fluid, is expected to be the same as that of the added enzymes. Hence, the sludge-bound condition of the bacterial amylases in activated sludge as was observed in the present study is probably not the result of re-adsorption of released enzymes, but depends on the maintenance of a close contact of the amylases with the bacterial cells producing these extracellular enzymes. Bacteria isolated from starch activated sludge (SL = 0.3) excreted at most small amounts of amylases into the medium when grown in batch culture (Chapter 8).

7.5. SUMMARY

1. Amylolytic and proteolytic activities of starch-grown sludges I (SL = 0.075 and 0.3) and II (SL = c.0.12) and of activated sludges from outdoor plants were shown to be completely bound to the solid fraction. In starch-grown sludges I with SL = 1.2 and 2.4 usually less than 3% of the total enzyme activities was present in the cell-free liquids. Treatment of activated sludge with various salt solutions released only rather low percentages of amylolytic and proteolytic enzyme activities from the sludges. The enzyme-sludge binding prevents the continuous wash-out of these enzymes from the sludge tanks.
2. Ultrasonic treatment of sludge I released a considerable enzyme activity into the sludge liquid; proteolytic and amylolytic activities responded differently to this treatment.
3. The proteolytic enzymes of sludge I appeared to be rather stable during 30–60 min of ultrasonic treatment; they were gradually released until 50 to 60% of their activity was set free; in the case of very high-loaded starch activated sludge this percentage amounted even to 80–90. It was concluded that the sludge proteolytic activity is located on the cell surface where it is directly accessible to protein substrates.
4. In contrast to proteolytic activity, standard amylolytic activity sharply increased during ultrasonic treatment to at most twice the original activity. Especially in starch activated sludge (SL = 2.4) the enzymes were labile during this treatment.
5. An increase of amylolytic activity, but not of proteolytic activity was frequently also seen under other conditions, viz. toluene treatment (3.3.6 and 3.4.5), freezing and thawing (3.3.5 and 3.4.4) and addition of casein (5.5).
6. Mass transfer resistance of polymers to extracellular amylolytic and proteolytic enzymes in sludge flocs and steric hindrance due to binding of the enzymes to slimy compounds or cell wall components were concluded not to

be clearly limiting standard amylolytic and proteolytic reaction rates in whole activated sludge.

7. It is concluded that part of the amylolytic enzymes in whole activated sludge is inaccessible to starch and to dextrin DE-9 due to its presumed location in the periplasmic space. The inaccessible fraction of the amylolytic enzymes is made accessible and largely released upon ultrasonic treatment and was shown to have about the same enzymic abilities (degradative capacities, $K_{1/2}$ values, DA/SA ratios, relative reaction rates with starch, dextrins and maltotriose) as the directly accessible fraction (see also experiments reported in 3). The function of the periplasmic fraction probably is the (incomplete) breakdown of dextrins with a $\overline{DP}$ of about 5, prior to their permeation through the cytoplasmic cell membrane. Due to its location this fraction of the amylolytic enzymes is protected against proteolytic attack.
8. The average DA_{spec} of the suspended and the flocculated biomass fractions of the higher-loaded starch sludges I was about the same, but PA_{spec} of the flocculated fraction was severalfold higher than that of the suspended fraction. Thus, extracellular proteolysis and turnover of biomass take place mainly in the flocs, even in very high-loaded activated sludge. Although flocs are distinguished from suspended bacteria by a longer mean cell residence time, their high PA_{spec} apparently results from relatively high rates of death and/or autolysis in flocs due to relatively unfavourable growth conditions.
9. Enzymes added to whole activated sludge were readily (cf. 5.5) adsorbed and concomitantly inactivated. The adsorption of Fungamyl 1600 was completely inhibited by 100 mM potassium phosphate, pH 7.0. Fungamyl activity that was lost by adsorption to sludge was not restored by adding this phosphate buffer. Egg white lysozyme was inactivated almost immediately by sludge (boiled or unboiled) whereas cell-free ultrasonicates of starch sludge did not inactivate fungal α-amylase (Fungamyl 1600). Therefore proteolysis was concluded not to play a role in this type of enzyme inactivation.

8. AMYLOLYTIC BACTERIA ISOLATED FROM ACTIVATED SLUDGE LOADED WITH MALTOSE OR STARCH

8.1. INTRODUCTION AND PRELIMINARY EXPERIMENTS

The bacterial population of sludge I was found to consist of many species, in contrast to that of sludge II. Competitive selection of species out of the immense pool of metabolic types of micro-organisms is likely to be the most important mechanism of acclimatization of entire microbial associations in nature (WUHRMANN, 1964). In section 4.4 it has been demonstrated that the acclimatization of maltose- or glucose-grown activated sludge to starch loading is due to an increase of the number of amylolytic bacteria per unit of biomass. Furthermore, it was shown that the amylolytic activity per amylolytic cell in glucose and maltose activated sludge was about equal to that in starch sludge (4.6.2). These observations raised several questions. Which amylolytic bacterial species are predominant in activated sludge during acclimatization to starch loading and which under presumed steady-state conditions? Are true steady-state conditions attained after prolonged loading with starch? In which way is amylase synthesis controlled in pure cultures of predominant amylolytic species? Do the conditions in continuously-fed starch activated sludge favour the selection of species or mutant strains showing a particular regulation mechanism, e.g. constitutive amylase synthesis? Continuous cultivation of a micro-organism in a chemostat with the carbon source as the limiting nutrient is throught to favour the selection of mutants showing constitutive synthesis of enzymes involved in the degradation of this carbon substrate.

Constitutive strains, in contrast with inducible strains, do not require the presence of inducing substances in the growth medium for synthesis of an enzyme. Both constitutive and inducible enzyme synthesis may be subject to catabolite repression. By this regulatory mechanism, enzyme synthesis is efficiently prevented by the presence of a readily assimilable carbon and energy source, e.g. glucose or, in the case of enzymes degrading organic N compounds, ammonia. Due to catabolite repression, the organism is able to consume the more readily assimilable substrate first and to delay consumption of the less favourable carbon source. During growth on the former substrate, enzymes degrading the latter are superfluous. Strains that are able to repress synthesis of the degrading enzymes in the presence of the repressing substrate have a selective advantage over strains that are lacking this ability.

A detailed study of the mechanism of amylase synthesis in all of the isolated pure cultures was not carried out by lack of time; furthermore in many cases it was hampered by difficulties regarding cultivation of the organism. In order to obtain an indication of the control mechanisms in starch degradation, DA_{spec} values, preferably estimated in young and in stationary growth phase cultures, in glucose yeast extract medium were compared with those in starch yeast extract medium. Constitutive amylase synthesis insensitive to catabolite repression was assumed if DA_{spec} of cultures grown on glucose was higher than about 70% of that of cultures grown on starch. If DA_{spec} in glucose cultures was lower than about 10% of that in starch cultures, amylase synthesis was assumed to require an inducer, probably some dextrin-like substance (or to be constitutive but highly sensitive to catabolite repression by glucose or to be both inducible and subject to catabolite repression). When DA_{spec} was low in young glucose-grown cultures but high in the stationary growth phase, amylase synthesis in such strains was considered to be constitutive and subject to repression by glucose, the latter repression being relieved when the glucose was exhausted. Strains showing a DA_{spec} in glucose cultures of about 10–70% of that in starch cultures were tentatively designated as partially constitutive. Amylase synthesis in these strains may be controlled by induction, by catabolite repression or by both, but not very strictly.

Preliminary experiments on bacteria isolated from the highest dilution tubes showing growth after inoculation with serial dilutions of sludges I from laboratory or outdoor plants learnt that many of these organisms grew poorly with prolonged lag phases whilst inoculations often failed to develop. Different sludge bacteria were found to have different substrate requirements. For that reason yeast extract was incorporated in all growth media used for the isolation and further cultivation of sludge bacteria although it might affect enzyme regulation.

In the preliminary experiments about 50 pure cultures of bacteria were isolated from different sludges. Ten of these isolates growing rather well in liquid media on starch in the presence of yeast extract, were used for regulation experiments. Two of the bacteria originated from Zeist sludge, 4 from maltose sludge and 4 from starch sludge; they also grew on glucose. This suggests that the amylolytic bacteria in activated sludge can also take up glucose, although in situ starch is taken up probably as dextrins (6.4). The synthesis of amylase appeared to be almost completely inducible in 2–3 of these strains, partly constitutive in 6–7 strains and completely constitutive and insensitive to catabolic repression in 1 strain. The distribution of these strains over the 3 sludges did not suggest that a distinct sludge is characterized by the occurrence of a prevailing regulation mechanism.

8.2. ISOLATION OF AMYLOLYTIC BACTERIA

The experiments on loading of laboratory-grown sludges with maltose or starch (Chapter 4) included isolation of amylolytic bacteria from these sludges by spreading serial dilutions of homogenized sludge on starch agar plates. The plates were incubated at 25°C for at least 12 days (see 2.5.2). All apparently different colony types developing on plates with 45–220 colonies, mostly representing amylolytic bacteria, were counted and obtained in pure culture by streaking on the same low-concentrated starch agar media (starch yeast extract medium A_2 or A_4, with no more than 10 g of agar per litre). Pin-point colonies which developed only after a prolonged incubation time were included in these enumerations. PRAKASAM and DONDERO (1967a) found that low levels of nutrients enabling growth of very small colonies resulted in higher counts of activated-sludge bacteria. FOOT and TAYLOR (1949) also found that counts of freshwater bacteria were higher when obtained on a dilute than on a concentrated peptone medium. The appearance of pin-point colonies after an incubation period of 10 days was a particular feature of the relatively low-concentrated standard medium used by those authors (peptone 0.5 g; soluble casein 0.5 g; soluble starch 0.5 g; glycerol 1.0 ml and salts). Even on plain agar supplied with salts 80% of the colonies found on the standard medium was able to grow.

In the present study it was difficult to keep the isolated bacteria on agar media; the growth of many strains was poor already during isolation. Maintaining these strains seemed to be more difficult than is already the general experience with water bacteria. After a few transfers several strains failed to grow. This seemed to be especially the case with the predominant bacteria in activated sludge (e.g. strains N° 3-2, 3-3, 3-5, 3-9, 3-1). The use of well-balanced, complex, low-concentrated media (e.g. A_4 and A_2) or higher-concentrated media (e.g. B_3, C and E) in agar slants supplied with water did not prevent death of the strains. Also for this reason, direct counting of specific bacteria in serial sludge dilutions incubated with different substrates as glucose, starch, casein or yeast extract (4.2), was preferred to isolation of representative pure cultures and subsequent testing of their properties.

Sometimes the highest dilution tubes showing growth are used to obtain pure cultures from a mixed suspension. This was also done in the very beginning of our research (2.5.2, 8.1). This method was rejected for isolation of bacteria from the mixed sludge population since representative isolation was aimed at.

8.3. POPULATION SHIFT OBSERVED UPON LOADING OF MALTOSE ACTIVATED SLUDGE WITH STARCH

The principle mechanism operating in sludge acclimatization to starch was shown to be a population shift rather than enzyme induction in bacteria already present before the starch loading. The increase of DA observed during the acclimatization of maltose- or glucose-grown activated sludge to starch feeding (planned SL = 0.3) was associated with an increase of the number of amylolytic bacteria (Figs. 4.3B and E; 4.4 and 4.5B). The maltose sludge of the experiment shown in Figs. 4.3B and 4.6A had a relatively high DA enabling an immediate and complete utilization of added starch. Nevertheless, numbers of amylolytic bacteria were shown to increase considerably within a few days (Fig. 4.4). The shift was analysed by direct spreading of 0.1 ml (in duplicate) from the sludge dilutions 10^{-5} and 10^{-6} on starch yeast extract medium A_2 (also used for the

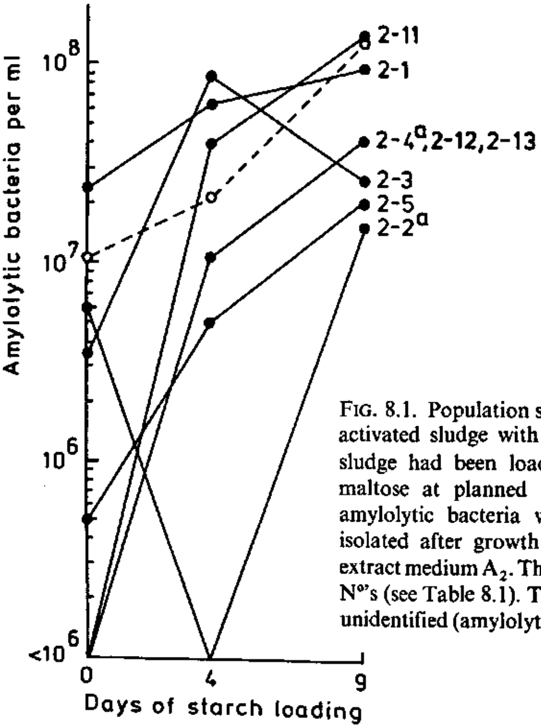

FIG. 8.1. Population shift observed upon loading of maltose activated sludge with starch (cf. Figs. 4.3B and 4.4). The sludge had been loaded previously during 84 days with maltose at planned SL = 0.3. The most predominant amylolytic bacteria were enumerated (in duplicate) and isolated after growth of sludge dilutions on starch yeast extract medium A_2. The figures in the graph denote the isolate N°'s (see Table 8.1). The broken line represents a mixture of unidentified (amylolytic?) strains (cocci and rods).

countings plotted in Fig. 4.4) and enumerating and isolating the different types of (mostly) amylolytic bacteria (2.5.2 and 8.2). These bacteria were identified according to generally accepted taxonomic criteria (BERGEY'S Manual, 1957 and 1974; SKERMAN, 1959). Some isolates were studied with respect to the regulation of amylase synthesis. Isolations were made in the beginning of the experiment and after 4 days of loading with starch (SL = 0.3) (Fig. 8.1 and Tables 8.1, 8.2 and 8.3).

Fig. 8.1 shows that the amylolytic microflora of sludge I consisted of several bacterial species. Some properties of representative isolates are listed in Table 8.1. In maltose sludge the amylolytic bacteria amounted to 4–6 % of the total bacteria (Fig. 4.4). Some types, tentatively designated as a *Flavobacterium* sp. (N° 2-1), a rod embedded in slime (N° $2\text{-}2^a$) and *Microcyclus major* (N° 2-3) made up about 53, 14 and 8 % of the amylolytic species in maltose sludge. After 9 days of starch loading the numbers of these bacteria had increased about 2.5- to 7-fold; after 4 days *Microcyclus major* even 25-fold. Other types, e.g. coryneforms (N° $2\text{-}4^a$, 2-12, 2-13, all showing turbid growth in media B_2–B_3), a filamentous *Flavobacterium* sp. (N° 2-5) and a different non-filamentous *Flavobacterium* sp. (N° 2-11, growing in flocs in medium B_3) occurred in maltose sludge in numbers of about 1 % of the amylolytic cell count. The numbers of these bacteria had increased 40- to more than 200-fold after 9 days of starch

TABLE 8.1. Different types of amylolytic bacteria, counted on starch yeast extract medium A_2, as % of total number of starch-degrading bacteria[1] (on this medium) of maltose activated sludge at 0, 4 and 9 days of starch loading.

Isolate[2] N°	(Tentative) name of bacterial type	As % of amylolytic bacteria at day			Morphology; colony appearance on A_2	Motility	Pigmentation[3]	Gelatine	Casein	Acid from			
		0	4	9						glucose	lactose	sucrose	maltose
2-1	*Flavobacterium* sp.	53	28	20	chains of 2-12 short rods, occasionally filaments of cells without visible septa	–	yo	–	–	+	+	+	+
2-2[a]	?	14	≤1	3	rods embedded in slime		yg						
2-3	*Microcyclus major*[4]	8	38	5	curved and horse-shoe rods, sometimes filamentous rods; large, smooth colonies		pp						
2-4[a]	Coryneform sp.	≤c. 1[6]	5[6]	9[6]	branched, quickly growing cells; large colonies		yw						
2-4[b]	Coryneform sp.	≤c. 1			rods and cocci; very small colonies		–						
2-5	Filamentous *Flavobacterium* sp.[5]	1	2	4	large, straight rods in rather short chains; small colonies		p		+[7]				
2-11	*Flavobacterium* sp.	≤c. 1	17	28	rather long, slender, readily growing rods	–	yo	+	+	+	–	+	+
2-12	Coryneform sp.	cf. foot-note 6			branched cells; good growth; large colonies	–	–	–	–	+	±	+	+
2-13	Coryneform sp.	cf. foot-note 6			branches cells; good growth; large colonies		yw						

The following properties were additionally determined: strains 2-1 and 2-11 Gram-negative, strain 2-12 Gram-positive; 2-1 and 2-12 no growth, 2-11 uncertain growth at 37°C; 2-1 and 2-11 catalase-positive, not halotolerant (1.5% NaCl) and no utilization of citrate; 2-11 did, 2-1 did not reduce NO_3^- to NO_2^-; 2-12 no anaerobic growth. [1] Cf. Fig. 4.4; [2] strains 2-1 up to 2-5 inclusive were isolated at 0 days, the other strains after 4 days of loading; [3] yo = yellow-orange, yg = yellow-green, yw = yellow-white and (p)p = (pale)-pink; [4] the species '*major*' is the only amylolytic and pale-pink pigmented *Microcyclus* mentioned by RAJ (1977), and in BERGEY's Manual 8th ed., 1974; [5] cf. VAN VEEN (1973) and EIKELBOOM (1975); [6] inclusive the numbers of strains 2-12 and 2-13; [7] filamentous flavobacteria were shown to be caseinolytic in liquid cultures of medium A_3.

loading. At that time the population was dominated by two distinct types of *Flavobacterium* spp. (N° 2-11, 28% and N° 2-1, 20%) and to a less extent consisted of coryneform bacteria (N° 2-4^{a}, 2-12, 2-13, 9%), *Microcyclus major* (N° 2-3, 5%) and a filamentous *Flavobacterium* (N° 2-5, 4%). Part of the strains (10–27%) isolated after 0,4 and 9 days of starch loading was not identified. During prolonged loading with starch the population composition continued to be variable. After 20 days *M. major* (N° 2-3) had disappeared whereas *Flavobacterium* type 2-11 predominated completely. Also the filamentous *Flavobacterium* sp. (N° 2-5), coryneforms and rods were present as appeared after microscopic observation of tubes used for counting. After 78 days *Flavobacterium* type 2-11 and *M. major* were absent. The microscopic picture revealed many filamentous bacteria, rods embedded in slime and rosette-clustered rods. Bacteria provisionally named *Nostocoida* (VAN VEEN, 1973) and rotifers were minor components of the total biomass, as usually (Plates 4.2, 4.6 and 4.5). After 281 days of starch loading the bacterial population was studied in detail (8.4). The population was dominated by two morphologically distinct flavobacteria (rods) and, to a less extent, by two morphologically distinct filamentous flavobacteria.

The regulation of amylase synthesis was studied by comparison of the enzyme activities of glucose- and starch-grown cultures (Tables 8.2 and 8.3). In Table 8.2 the results obtained with 5 bacteria isolated from maltose-grown sludge prior to loading with starch are shown. The predominant amylolytic types 2-1

TABLE 8.2. DA_{spec} values of amylolytic bacteria isolated from maltose activated sludge. The organisms were grown in batch cultures containing glucose or starch yeast extract (GY or SY, respectively) media B_2 (100 mM potassium phosphate, pH 7.0). The sludge had been loaded with maltose (SL = 0.3) for 84 days. Inoculum: liquid (or slant) cultures pregrown on starch.

Isolate N°	Medium	Growth period (h)			I		II		III	
		I	II	III	Dry wt (mg/ml)	DA_{spec}	Dry wt (mg/ml)	DA_{spec}	Dry wt (mg/ml)	DA_{spec}
2-1	GY		60				1.05	≤ 0.066		
	SY	35	60		1.07	0.34	1.05	0.30		
2-2^{a}	GY	60	132.5		1.32	≤0.049	2.05	0.06		
	SY	60	132.5		1.39	0.29	2.16	0.75		
2-3	GY[1]	60	132.5		0.57	1.65	0.35	1.97		
	GY		132.5				0.70	1.42		
	SY		132.5				0.75	2.08		
2-4^{a}	GY[1]	35	60	132.5	1.88	1.05	2.93	1.25	2.89	1.28
	GY	35	60	132.5	1.56	1.35	2.80	1.18	2.60	0.96
	SY	35	60	132.5	2.10	0.95	2.86	2.36	2.81	2.35
2-4^{b}	GY		185				0.51	c. 0		
	SY	132.5	185		2.27	0.58	2.20	0.59		

[1] 50 mM potassium phosphate (pH 7.0) in growth medium, instead of 100 mM.

(*Flavobacterium* sp.) and 2-2^a produced DA in starch medium but hardly in glucose medium. Amylase synthesis in these strains likely was inducible or subject to catabolite repression or both. DA values of starch-grown cultures of both types were low. Strain 2-1 only partly degraded the starch present in the culture medium. Two of the three other types studied (2-3, *Microcyclus major* and 2-4^a, a coryneform strain) occurring less frequently than the former two, likely showed a constitutive amylase synthesis. Stationary phase cultures of coryneform 2-4^a in starch medium showed a DA increase which was not found in glucose-grown cultures. Type 2-5, also isolated from maltose sludge, is not inserted in Table 8.2 because this organism did not grow with 50–100 mM potassium phosphate. A similar strain (3-5), tested in the presence of 5 mM phosphate, was found to be constitutive as to amylase synthesis (Table 8.5).

The low DA_{spec} of the predominant amylolytic bacterial strains isolated from maltose sludge (Table 8.2) is neither in agreement with the considerable DA_{spec} (0.52) measured in this sludge (Fig. 4.3B) nor with the observation that the amylolytic activity per viable amylolytic cell in this maltose sludge was about the same as that in starch sludge (Tables 4.6 and 4.5).

Amylolytic bacteria isolated from maltose sludge that had been loaded with starch for 4 days, showed somewhat higher DA_{spec} values (Table 8.3) than bacteria isolated from maltose sludge (Table 8.2). However, activity units and growth media of both series of estimations were different (DA values of Table 8.2 were computed at dry matter and those of Table 8.3 at protein; growth medium of the former set of strains contained 100 mM phosphate buffer, that of the latter 5 mM). Amylase synthesis in *Flavobacterium* type 2-11 markedly

TABLE 8.3. DA_{spec}[1] values of amylolytic bacteria isolated from maltose activated sludge loaded with starch (SL = 0.3) during 4 days. The organisms were grown in batch cultures containing glucose or starch yeast extract (GY or SY, respectively) media B_3 (20 mM potassium phosphate, pH 7.0, 0.5 g $CaCO_3$/l). Inoculum: liquid (or slant) cultures pregrown on starch. Figures in brackets are the 'free' DA values of the supernatants of centrifuged cultures, as percentages of the entire culture DA values.

Isolate N°	Medium	Growth period (h)		I		II	
		I	II	Protein (mg/ml)	DA_{spec}[1]	Protein (mg/ml)	DA_{spec}[1]
2-11[2]	GY	20.5	45.5	0.90	0.59	0.95	5.17
	SY	20.5	45.5	1.00	6.94	0.93	7.13 (c. 25%)
2-11[3]	GY	22	49	0.78	1.03	0.96	2.57
	SY	22	49	0.62	9.64 (31%)	0.99	6.63
2-12	GY	45.5	69.5	0.40	2.50	0.46	2.37
	SY	45.5	69.5	0.40	6.60 (26%)	0.40	7.68 (37%)
2-13	GY	93.5	123.5	0.36	2.89	0.44	1.77
	SY	93.5	123.5	0.09	8.1	0.39	4.56

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[1] DA_{spec} expressed as mg starch (equivalent)/mg protein.h. The protein in the cell material was determined by the biuret method (2.6.10); [2] 5 mM potassium phosphate in growth medium; [3] 20 mM potassium phosphate in growth medium; inoculum: batch culture pregrown on glucose (GY medium B_3).

responded to growth conditions. In young glucose-grown cultures relatively little DA was produced, but in old glucose-grown cultures DA was derepressed. Since this derepression was most pronounced in the weakly buffered culture, it was apparently favoured by a low pH. From the results obtained with 20 mM phosphate buffer it might be concluded that the amylase synthesis in this strain is (partly) constitutive and subject to catabolite repression by glucose. Amylase synthesis by the coryneform types 2-12 and 2-13 was hardly affected by the age of the culture. DA values in starch-grown cultures were about 3 times as high as those in glucose-grown cultures. It is not known whether this difference in amylase synthesis was due to a weak repression of the synthesis of (partially) constitutive amylases by glucose or to an inducing effect of starch metabolites.

In contrast to whole activated sludge (7.2), some pure cultures released a DA fraction of about 30% to the medium (Table 8.3). However, comparison with Table 8.5 and Plate 8.1 learns that the predominant amylolytic bacteria usually do not excrete amylolytic enzymes.

The amylolytic bacteria frequently showed prolonged lag phases which necessitated repeated inoculation, and their growth rates usually were relatively low (Tables 8.2 and 8.3; strain 2-11 is an exception). Starting with an inoculum of about 1% (v/v), 6 to 7 generation times are required to attain the stationary growth phase. Cell yields generally were low, i.e. 0.7–2.9 mg dry wt/ml, in

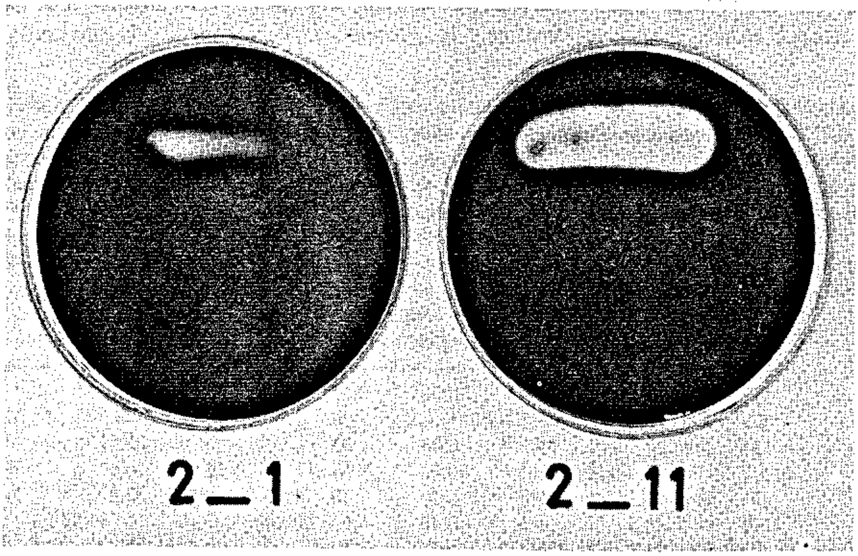


PLATE 8.1. Starch hydrolysis by two distinct types of *Flavobacterium* (2-1 and 2-11) on agar medium C. After several days of growth the grown bacteria of type 2-1 were removed, those of type 2-11 were left on the agar; both plates were flooded with Lugol's iodine solution (4.2). Type 2-1 showed a decolourized zone only under the streak, type 2-11 also around the streak. The latter indicates digestion of starch by excreted amylases which diffused (rapidly) from the bacteria into the surrounding medium (cf. Table 8.3).

growth medium containing 5 mg carbohydrate COD and 5 mg yeast extract per ml (Table 8.2) and 0.4–1.0 mg cell protein/ml in growth medium containing 5 mg carbohydrate COD and 2.5 mg yeast extract per ml (Table 8.3). Representatives of well-known amylolytic bacteria, e.g. bacilli or pseudomonads, were not found among the predominant amylolytic strains isolated from activated sludge.

8.4. THE BACTERIAL POPULATION OF STARCH SLUDGES I AND II

In section 8.3 it has been reported that maltose activated sludge after loading with starch (SL = 0.24) during 78 days did not reach a steady state with respect to its bacterial population. After 281 days of loading the bacterial microflora was analysed again. However, steady-state conditions were not expected to have been attained since alterations of sludge characteristics like colour and floc structure were observed at irregular intervals during starch loading. During the experiment SL varied with the factor two and was about 0.15–0.20 during the period of the isolation. The sludge was slimy and did not show any settling, as was always observed with starch sludge at low loadings. DA_{spec} was about 1.5 and fluctuated moderately (Fig. 4.6A).

Serial dilutions of the sludge (viz. 0.1 ml of the dilutions 10^{-5}, 10^{-6} and 10^{-7}) were spread in triplicate on starch yeast extract trypticase soy broth agar A_4 plates. The sludge contained 8.1×10^8 colony-forming units (viable bacteria) per ml, i.e. 1.8×10^8 per mg dry wt (counted after 25 days of incubation). Representatives of all of the different types of bacteria found among the 81 colonies that had developed after more than 12 days of incubation on one plate, inoculated with 0.1 ml of $10^6 \times$ diluted sludge, were examined and classified (2.5.2, 8.2). The well-identifiable bacterial types 3-1, 3-4, 3-5, 3-6, 3-8 and 3-9 were counted in triplicate, the other strains only once. Some bacteria with deviating colony types which developed only on the plates inoculated with 0.1 ml of $10^5 \times$ diluted sludge were included in the study in spite of their relatively small numbers.

Amylolysis by pure cultures was deduced from the decrease of iodine-stainable compounds in liquid starch media B_2 or B_3. A different criterion, viz. an improved growth of an amylolytic bacterium on agar medium A_4 containing starch compared to the growth on this medium without starch, was not satisfactory. Since several isolates died after a few transfers on agar media before they could be identified, the study of the regulation of amylase synthesis was started as soon as possible.

From the results obtained (Table 8.4; Plate 8.2) it can be seen that amylolytic bacteria accounted for at least 93% of the total population growing on starch yeast extract trypticase soy broth-containing plates; this is in agreement with data reported in Table 4.5. The predominant non-amylolytic type (3-7; turbid growth in medium B_3), probably not growing on glucose either, resembled *Xanthomonas*. The (non-amylolytic) type 3-16 could not be isolated possibly

Table 8.4. Bacterial composition of activated sludge (loaded during 281 days with starch; $DA_{spec} = 1.55$, dry wt = 4.61 g/l) as % of total number of colony-forming units on starch-containing medium A_4.

Isolate N°	(Tentative) name of bacterial type	Amylolytic	As % of counted bacteria[1]	Morphology; colony appearance on A_4	Motility	Pigmentation[2]	Gelatine	Casein	Acid from			
									glucose	lactose	sucrose	maltose
3-1	*Acinetobacter* sp.	+	1	short rods, embedded in slime; ready formation of large colonies	−	−	+	+	+	−	+	−
3-2	*Flavobacterium* sp.	+	36	slender rods, sometimes in rosettes; retarded formation of very small colonies	−	yo						
3-3	*Flavobacterium* sp.	+	33	long, slender rods; retarded formation of small colonies	−?	yo	−		−	−	+	−
3-4	*Acinetobacter* sp.	+	1	slightly curved rods, often in rosettes; good growth on all media tested	−	−	−	−				
3-5	Filamentous *Flavobacterium* sp.[3]	+	6	large straight rods, often in filaments with clearly visible septa, small colonies with curled margin		p		+[7]				
3-6	*Flavobacterium* sp.	−	0	highly motile rods; good growth	+[5]	o		−	+	−	+	−
3-7	*Xanthomonas* sp.	−	c. 5	highly motile rods; good growth	+[6]	y	−	−	−	−	−	±
3-8	*Acinetobacter* sp.	+	6	rods, often in pairs, in liquid media embedded in slime; rather large, rough colonies	−	−	−	−				
3-9	Filamentous *Flavobacterium* sp.[3]	+	2.5	large rods, mostly in long, coiled filaments; small colonies		pp		+[7]				
3-10	*Flavobacterium* sp.	−	0	small slender rods; good growth	−	yo	−	−	−	−	−	+
3-11	Coryneform sp.	−	0	moderately pleomorphic (rods and cocci); good growth	−	y	−	−				
3-13	?	+	0	rather large rods; colonies attached to agar; good growth								
3-14	*Flavobacterium* sp.	−	0	very motile slender rods	+[5]	y	+	+	+	+	−	+
3-15	?	+	c. 7	rather large rods		y						
3-16	?	−[4]	c. 2.5	long, very slender, difficultly visible rods; very small colonies								
3-17	?	?	0	fusiform cells, sometimes filamentous, with many bright inclusions								
3-18	?	?	0	heavy, fungus-like filaments								
Total		92.5%	100%									

Almost all isolates occasionally showed filamentous growth on plates or in liquid cultures. All bacteria tested (3-1, 3-2, 3-3, 3-4, 3-6, 3-7, 3-8, 3-10, 3-11 and 3-14) were shown to be Gram-negative with the exception of strain 3-11, which was Gram-positive. They did not grow at 37°C (growth of strain 3-4 was uncertain) and were catalase-positive (strain 3-7 negative, 3-2 and 3-11 not tested). Strain 3-7 was halotolerant (1.5% NaCl), 3-3, 3-6, 3-10 and 3-14 were not. Strains 3-1, 3-4, 3-8 and 3-11 did not grow anaerobically, other strains not tested. Strains 3-3, 3-6, 3-7, 3-10 and 3-14 did not reduce NO_3^- to NO_2^-, strains 3-6, 3-7, 3-10 and 3-14 did not utilize citrate, others strains not tested. [1] 0: no growth on A_4 plates inoculated with 0.1 ml of 10^6 × diluted sludge; [2] y = yellow, yo = yellow-orange, o = orange, (p)p = (pale)pink; [3] cf. VAN VEEN (1973) and EIKELBOOM (1975); [4] absence of growth difference on agar A_4 plates with and without starch; [5] degenerately peritrichous and [6] polair flagellar arrangement; [7] filamentous flavobacteria were shown to be caseinolytic in liquid cultures of medium A_3.

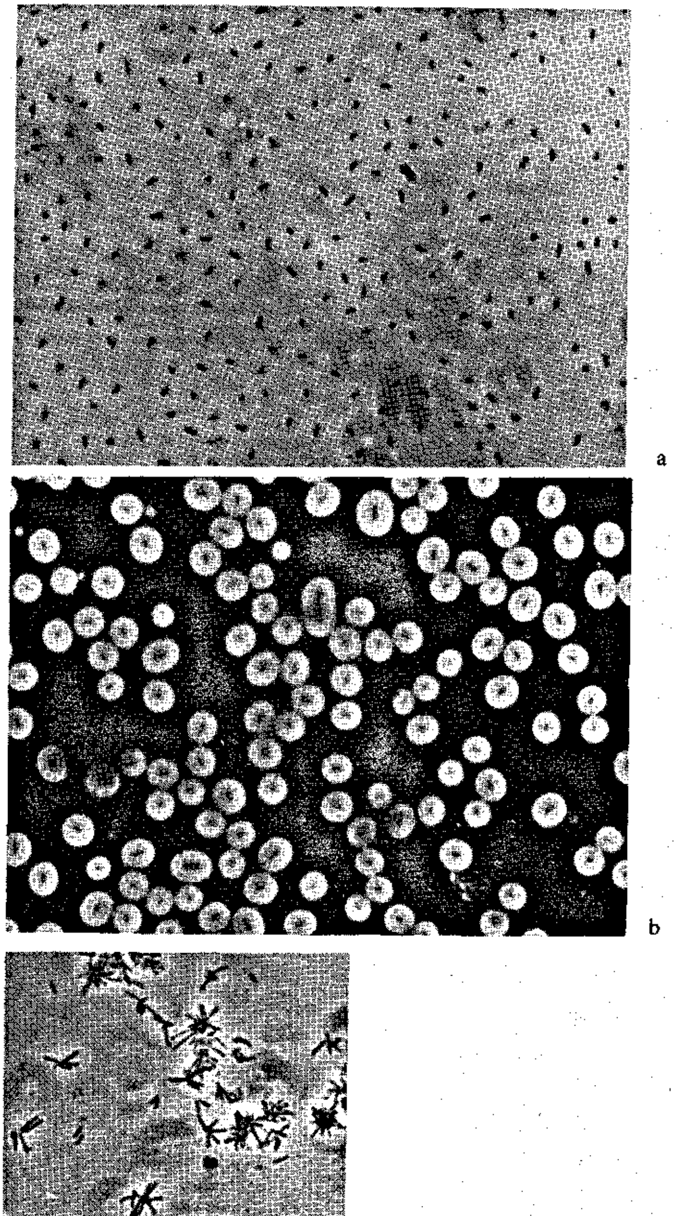
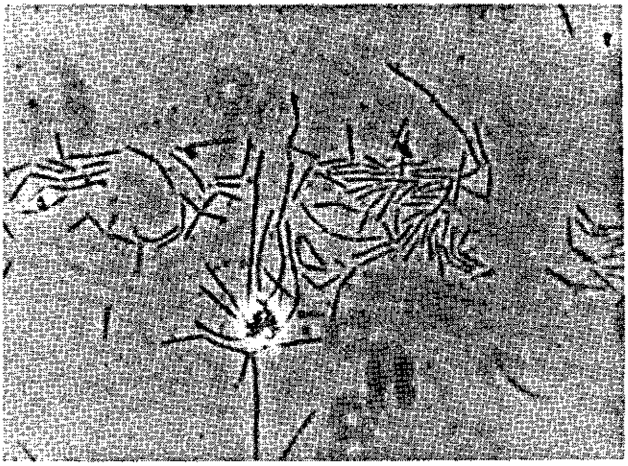


PLATE 8.2, a–f. Phase-contrast photomicrographs, 1250×, of amylolytic bacterial types isolated from activated sludge after 281 days of starch loading (Table 8.4), and grown in batch culture, in starch medium B_3. (a) Isolate N° 3-1; (b) isolate N° 3-1, dispersed in Indian ink; (c) isolate N° 3-2; (d) isolate N° 3-3; (e) isolate N° 3-5; (f) isolate N° 3-9.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e


f

TABLE 8.5. DA_{spec}[1] values of amylolytic bacteria isolated from activated sludge loaded with starch (SL = 0.3) during 281 days. The organisms were grown in batch cultures containing glucose or starch yeast extract (GY and SY, respectively) media B_3 (5 mM potassium phosphate, pH 7.0, 0.5 g $CaCO_3$/l). Inoculum: liquid (or slant) cultures pregrown on starch during 3–8 days. Figures in brackets are the 'free' DA values of the supernatants of centrifuged cultures, as percentages of the entire culture DA values.

Isolate N°	Medium	Growth period (h)			I		II		III	
		I	II	III	Protein (mg/ml)	DA_{spec}[1]	Protein (mg/ml)	DA_{spec}[1]	Protein (mg/ml)	DA_{spec}[1]
3-1	GY	22.5	45		0.17	0.53	0.29	0.55		
	SY	22.5	45		0.29	4.72	0.48	5.48		
3-1[2,3]	GY	91	115		0.12	1.42	0.50	0.52		
	SY	91	115		0.23	6.09 (0%)	0.59	3.61		
3-2	GY	144	169		0.37	0.68	1.09	0.33		
	SY	144	169		1.13	2.04	1.30	1.92 (≤2%)		
3-3	GY	45	76		0.22	0.64	0.79	0.41		
	SY	45	76		0.96	1.95	1.09	1.70 (≤0.8%)		
3-4	GY	22.5	45		1.17	0	1.12	0		
	SY	22.5	45	185	0.83	1.57	0.80	1.40	0.81	2.08 (≤4%)
3-5	GY	45	76		0.57	c. 0.10	1.09	0.25		
	SY	45	76		0.66	0.27	0.79	0.25 (0%)		
3-8	GY	22.5	45		0.15	1.80	0.37	1.84		
	SY	22.5	45		0.21	4.67	0.62	3.06		
3-8[3,4]	GY	91	115		0.20	0.65	0.38	0.66		
	SY	91	115		c. 0.02	c. 8.3 (c. 0%)	0.14	8.64 (≤2.6%)		
3-9	GY	76	91		0.32	0.69	0.72	0.46		
	SY	76	91		0.50	1.30	0.74	0.57 (0%)		
3-13	GY	20.5	45.5		0.43	1.14	0.48	1.54		
	SY	20.5	45.5		0.61	5.92	0.92	≥6.21 (c. 19%)		
3-13[3,5]	GY	24	54		0.11	3.55	0.28	2.50		
	SY	24	54		0.36	5.92	1.02	7.46		
3-15[6]	GY	20.5	45.5		0.22	4.00	0.69	3.46		
	SY	20.5	45.5		0.28	9.68	0.69	5.07 (18%)		

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[1] DA_{spec} expressed as mg starch (equivalents)/mg protein.h; [2] different inoculum (from plate). Liquid cultures of strain 3-1 were hardly washed due to the extreme viscosity of the cultures (not precipitable) before protein and dry wt were determined. Dry wt was determined on membranes after adding 10 ml 0.01 N HCl to the 'pellet' of centrifuged culture samples of 10 ml to dissolve $CaCO_3$. DA_{spec} (based on dry wt) after 91 h was 0.36 (GY) and 1.28 (SY); after 115 h it was 0.066 (GY); [3] 20 mM potassium phosphate in growth medium; [4] different inoculum (from plate). DA_{spec} (based on dry wt) after 115 h was 0.13 (GY) and 1.98 (SY); [5] inoculum: batch culture pregrown on glucose (GY medium B_3); [6] DA_{spec} (based on dry wt) after 45.5 h was 1.94 (GY) and 2.08 (SY).

because it grew only in the presence of other bacteria. The population was dominated by two morphologically distinct yellow flavobacteria (3-2 and 3-3), accounting for 70 % of the total (viable) number. Strain 3-2 grew in medium B_3 as very small flocs, the culture of strain 3-3 was turbid and slightly slimy. Two distinct filamentous pink flavobacteria (3-5 and 3-9) accounted for 9 % of the total number of colonies. The latter count may have be underrated due to doubtful separation of the cells in sample dilutions. Liquid cultures (medium B_3) of type 3-5 were turbid, those of type 3-9 contained large filamentous masses of slime. Three distinct colourless *Acinetobacter* species made up about 8 % of the viable cells (3-1, 3-4 and 3-8). Two of these (3-1 and 3-8) produced great masses of cell-bound slime (Plate 8.2), resulting in low protein contents of 13–26 % (Table 8.5) when grown in medium B_3. Strain 3-8 formed large, slimy flocs; liquid cultures of strain 3-1 were so viscous that no precipitation was observed at 38,000 × g. The culture of strain 3-4 was turbid (medium B_3). Another yellow amylolytic strain (3-15) gave slightly slimy and turbid appearance in medium B_3. The other isolates were quantitatively of less importance ($\leqslant 1$ %). Among these strains were 3 non-amylolytic flavobacteria, of which 2 were yellow (3-10 and 3-14) and one was orange (3-6; turbid growth in medium B_3). All of these three flavobacteria likely grew on glucose. The last property was also shown by a non-amylolytic coryneform bacterium (3-11), which was the only organism in this population shown to be Gram-positive. The non-amylolytic, glucose-utilizing bacteria of the sludge were expected to utilize degradation products of starch in situ. Their almost negligible numbers confirm the results of section 4.6.2.

The regulation of amylase synthesis (Table 8.5) was studied as reported in 8.1 and 8.3. Only one amylolytic strain (3-4), accounting for about 1 % of the population, synthesized no amylase in glucose medium B_3. The starch in medium B_3, even after 8 days, was not completely degraded. All of the other strains showed a considerable amylolytic activity in glucose-grown cultures; as to strain 3-1 (1 % of the population) this activity was amounted to 10–25 % of the activity in starch-grown cultures. In the other isolates, including the predominant species 3-2 and 3-3, DA values in glucose-grown cultures were 20–100 % of those in starch-grown cultures, indicating that amylase synthesis in these strains was not strictly controlled. Amylase synthesis appeared to be constitutive (without pronounced catabolite repression) in the proteolytic filamentous flavobacteria 3-5 and 3-9, which belonged to the only types that almost always occurred in starch activated sludge (SL = 0.3) (cf. HOUTMEYERS, 1978). Their low amylolytic activity, especially that of strain 3-5, was responsible for the slow decrease of iodine-stainable compounds in medium B_3. In the predominant flavobacteria 3-2 and 3-3 which constituted 70 % of the population, DA in the glucose medium was 20–30 % of that in the starch medium. This suggests that in these strains amylase synthesis is partially constitutive. These strains had relatively low amylolytic enzyme activities (1.7–2.0 mg SE per h per mg cell protein) which were bound to the cells. Binding of the amylases to the biomass was found to occur in all amylolytic strains tested except in isolates 3-13 and 3-15. In these two bacteria 20 % of the amylases was set free into the medium. The absence of excretion of amylases by almost all of the pure cultures is in agreement with the complete binding of amylases to activated sludge.

Most amylolytic strains isolated on starch medium from starch-grown activated sludge, including the predominant types 3-2 and 3-3, showed a (partially) constitutive amylase synthesis (Tables 8.3 and 8.5). The question arose whether this constitutive amylase synthesis was a property of the bacterial type or of a mutant strain selected in the sludge population during (continuous) starch loading. In the latter case, back mutants may be expected. Cultivation on glucose agar for several generations may favour the selection of back mutants showing a more pronounced regulation of amylase synthesis. In order to gain more information concerning this phenomenon, some strains showing partially constitutive amylase synthesis, were transferred to glucose yeast extract slants (medium B_3); after growth had occurred this transfer was repeated 4 times. A similar serial transfer on starch medium, presumably not favouring the selection of back mutants, served as the control. Unfortunately, only a limited number of strains, being not completely constitutive, had been kept alive when these serial transfers started, viz. 2-12, 3-1 and 3-8. A strain of *Microcyclus major* isolated by A. D. ADAMSE from activated sludge and maintained on glucose-containing slants for years, was additionally tested (cf. our strain, 2-3, of this species).

The regulation of amylase synthesis in these strains was not affected by the serial transfer (results not shown). DA_{spec} values of cultures grown in glucose medium, calculated as % of those of starch-grown cultures, were about equal to the relative values of freshly isolated strains (Tables 8.3 and 8.5). ADAMSE's strain of *M. major* showed to be highly consitutive as to amylase synthesis. These results suggest that the (partially) constitutive amylase synthesis should be considered to be a property of the bacterial type. The hypothesis that the freshly isolated strains are regulatory mutants producing DA either (partially) constitutively (or in larger amounts than the parent strains), should be rejected. If this hypothesis were valid, back mutants would have been selected during the serial transfers on glucose agar medium.

That inducibility of amylase synthesis is also found as a property of distinct starch-degrading bacteria is indicated by the occurrence of equal regulation mechanisms in two isolates of Flavobacterium type 2-1. One isolate originated from Zeist sludge loaded during 12 days with maltose (SL = 0.3), another from laboratory-grown sludge loaded during 41 days with starch and subsequently during 84 days with maltose (SL = 0.3; Table 8.2).

From the results obtained it is concluded that selection of mutants showing constitutive amylase synthesis is not a mechanism explaining sludge acclimatization to starch loading. In fact, a constitutive amylase synthesis was shown to occur in at least 2 bacteria (2-3 and 2-4[a]) isolated from maltose sludge before starch loading was applied.

Sludge II fed with starch (D = 0.1 or 0.05 day^{-1}) tended to develop an amylolytic monoculture, usually growing in suspension outside the few flocs present. Examples of such bacteria were sarcina's (during a limited period) and actinomycete-like bacteria. A branched, non-proteolytic *Nocardia*-like bac-

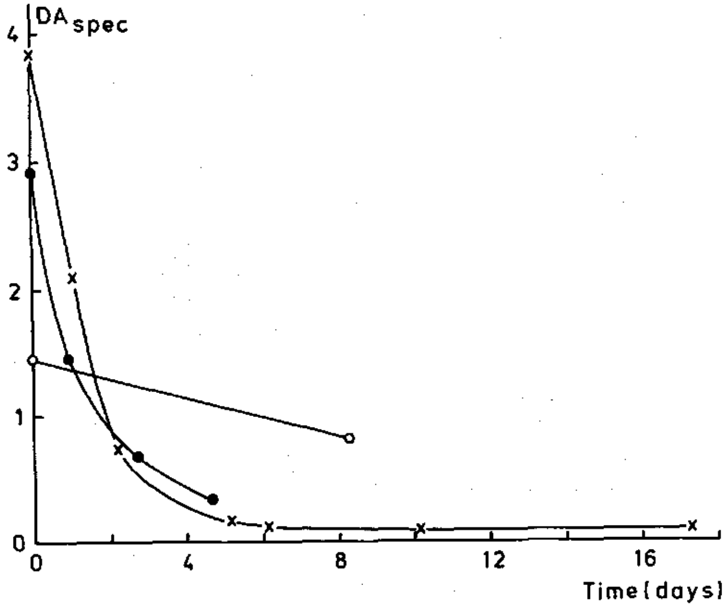
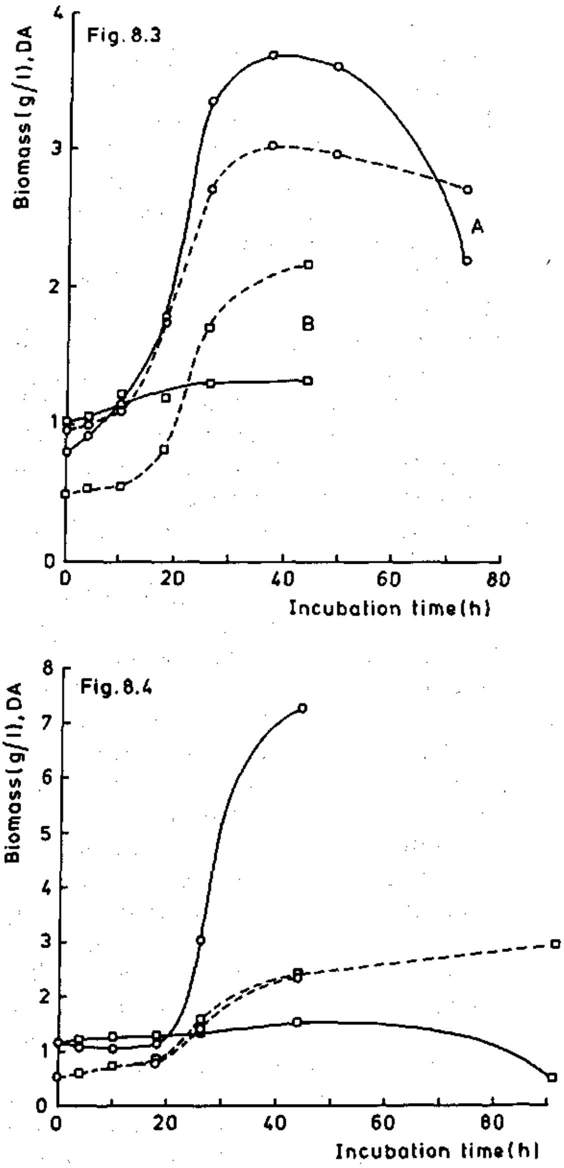


FIG. 8.2. Amylolytic activity of 3 different starch activated sludges (originating from the same Zeist sludge), contained in the reactor (20°C), upon omission of substrate supply (SL = 0, D = 0 day^{-1}) or substitution of maltose for starch (SL = 0.3, D = 1 day^{-1}) at t = 0. ●, Starvation (control); history: Zeist sludge, loaded with maltose during 55 days and finally with starch during 17 days; x, maltose loading; history: Zeist sludge, loaded with starch during 55 days; ○, maltose loading; history: Zeist sludge, loaded with starch during 55 days, with maltose 17, without substrate 5 and finally with starch during 5 days.

terium (4.6.1) even predominated during 500 days. *Nostocoida*-like bacteria were observed very frequently in rather low numbers. Maximum DA$_{spec}$ values of 7, 17 and 69 were found for sarcina, an actinomycete and the *Nocardia* sp., respectively.

8.5. REGULATION OF AMYLASE SYNTHESIS IN WHOLE STARCH ACTIVATED SLUDGE

Since experiments with pure cultures isolated from starch activated sludge did not allow to draw definite conclusions on the predominant regulatory mechanism of amylase synthesis in whole sludge after different periods of loading, some experiments were carried out with sludge in reactor or in batch cultures. In the reactor experiments (20°C) shown in Fig. 8.2 the substrate supply was either ceased (SL = 0, D = 0 day^{-1}) or starch substrate solution was replaced by a maltose solution of similar concentration (SL = 0.3, D = 1 day^{-1}). Like in the experiments shown in 5.5, starvation (●) of starch activated sludge (SL = 0.3) resulted in a rapid decrease of DA$_{spec}$. Maltose loading of sludge (x) which previously had been loaded with starch during 55 days resulted in a comparable



FIGS. 8.3 and 8.4. Synthesis of amylolytic enzymes and biomass by starch activated sludge (SL = 0.3) incubated as batch culture at 25°C. The sludge was centrifuged at 2500 × g, washed once with 20 mM potassium phosphate, pH 7.0, resuspended in this buffer solution and incubated at 1/5 of its original concentration in unsterilized medium B_1 (100 mM potassium phosphate, pH 7.0). A and B denote the same sludge loaded during 125 and 141 days, respectively, with starch prior to starting the batch cultures. Closed and broken lines denote DA and dry wt of biomass, respectively. Fig. 8.3: sludge A and B in glucose medium B_1 (sludge A additionally supplied with 1 g tryptone/l). Fig. 8.4: sludge B in starch medium B_1 with (square symbols) and without (spheric symbols) additional supply of 20 g glucose/l.

rate of decrease of DA_{spec}. Such a rapid drop of amylolytic activity after substitution of maltose for starch was observed only once. The decrease of DA_{spec} was much slower in some other experiments, e.g. in the sludge which had been loaded during 5 days with starch prior to initiating maltose loading (o). In the latter experiment the same sludge was used as in the preceding one with the exception of a different pretreatment. Apparently, overall amylase synthesis in sludge (i.e. in the predominant microflora) grown during 55 days on starch required an inducer, probably a dextrin compound, whereas the amylolytic microflora of sludge grown for 5 days on starch was (at least) partly constitutive.

In the experiments shown in Figs. 8.3 and 8.4, the same sludge loaded with starch (SL = 0.3) during 125 (A) and 141 days (B) was harvested and grown as batch cultures in medium B_1 for periods up to 4 days. In glucose (tryptone) media, DA_{spec} (i.e. DA/biomass concentration) of sludge A increased from 0.83 (at inoculation) to 1.22 (at the onset of the stationary phase), whereas that of sludge B decreased from 2.08 to 0.61 (Fig. 8.3). Sludge B produced amylolytic enzymes to a significant extent only in starch medium, but not in starch medium supplied with an excess of glucose (Fig. 8.4).

The large amounts of amylolytic enzymes synthesized concomitantly with growth of sludge A (Fig. 8.3) in glucose-tryptone medium suggest a (partly) constitutive overall DA synthesis insensitive to catabolite repression. Although sludge B had been loaded with starch during an only 16 days longer period, it behaved differently from sludge A, amylase synthesis being controlled. In glucose medium only small amounts of amylases were produced in the presence of starch (Fig. 8.4) as well as in its absence (Fig. 8.3). This suggests a catabolite repression exerted by glucose or its metabolites. In starch medium without glucose a considerable amylase synthesis was observed (Fig. 8.4). This enzyme synthesis possibly required the presence of inducing dextrins.

The different behaviour of sludges A and B as to the development of amylolytic activity in the batch cultures (Figs. 8.3 and 8.4) is attributed to a difference in the predominant amylolytic microflora in the original sludges. It is improbable that large population shifts have occurred during the batch culture experiments. The biomass increased only 3–4½-fold during the experiments, the percentage of amylolytic bacteria in starch sludge (SL = 0.3) was about 90%, the percentage of non-amylolytic glucose-utilizing bacteria was very small (4.6.2 and 8.4) and the amylolytic bacteria tested could also grow on glucose (8.1). Hence, only a limited population shift was expected in the experiments shown in Figs. 8.3 and 8.4.

The experiments on amylase synthesis in starch activated sludges suggest that no steady-state conditions as to a predominant regulatory mechanism were attained. In sludge A overall amylase synthesis was probably (partly) constitutive and insensitive to catabolite repression, in sludge B susceptibility to repression by glucose and, possibly, the requirement for inducing compounds were characteristic features. From the results of this section no evidence was obtained of the selection of a starch sludge population showing completely constitutive amylase synthesis insensitive to catabolite repression. Apparently, regulation mechanisms do not have a predominant effect on the competition between amylolytic bacteria in activated sludge.

8.6. SUMMARY

1. Enumerations and isolations were made of all predominant and apparently different bacterial types grown on agar media with low concentrations of starch (A_2 or A_4) after spreading high dilutions of activated sludge. Identification and characterization of the isolates were hampered due to poor growth. In spite of the precautions taken, most strains of the predominant types died after a few transfers.
2. Alterations of several activated-sludge characteristics observed at irregular intervals during more or less continuous starch loading, particularly at high loadings, indicated that a steady state with respect to the bacterial population was never attained. A population analysis of sludge I (SL = 0.3) initially loaded with maltose and subsequently with starch confirmed this observation. Several morphologically distinct flavobacteria usually dominated the amylolytic flora of this sludge. After 281 days of starch loading, amylolytic bacteria accounted for at least 93% of the colony-forming units, which confirms the results of Chapter 4; 70% of the total count was made up by 2 distinct non-filamentous yellow flavobacteria, at least 9% by 2 distinct filamentous pink flavobacteria and 8% by 3 distinct colourless *Acinetobacter* species. Three other distinct flavobacteria (2 yellow, 1 orange) and 1 yellow coryneform organism were non-amylolytic, but likely grew on glucose. Their negligible numbers confirm that spill over of starch degradation products by amylolytic bacteria does hardly occur in continuously loaded starch activated sludge (4.6.2).
3. The microflora of maltose sludge consisted for about 5% of amylolytic bacteria. Two predominant bacteria isolated from this sludge, and making up about $\frac{2}{3}$ of the amylolytic flora, produced amylases in starch medium but hardly in glucose medium. Two other isolates accounting for about 10% of the amylolytic flora, likely showed constitutive amylase synthesis. Bacteria isolated after 281 days of starch loading produced different amounts of amylases in glucose medium (usually 20–100% of those in starch medium). This suggests that the amylase synthesis in the majority of the bacteria of starch activated sludge in partly constitutive (i.e. not strictly controlled).
4. Experiments on the regulation of amylase synthesis in whole starch-grown sludge indicated that this sludge did not attain a steady state with respect to the predominant regulatory mechanism. Apparently, regulation mechanisms have no predominant effect on the competition between amylolytic bacteria in activated sludge.
5. Partial constitutive amylase synthesis is considered to be a property characteristic of the isolated predominant amylolytic bacterial types and not to be the result of mutation. This conclusion was derived from the observation that prolonged cultivation (on glucose agar) did not affect regulation of amylase synthesis, i.e. did not result in the selection of back mutants.
6. The rather low amylase activity of the predominant amylolytic bacteria

isolated after 281 days of starch loading (I, SL = 0.3) was bound to the cells. In some strains isolated after 4 days of starch loading it was partly (about 30%) set free to the medium.

7. Low-loaded starch sludge II tended to develop an amylolytic monoculture occurring in suspended form. A non-proteolytic *Nocardia*-like bacterium even predominated during 500 days of loading.

GENERAL SUMMARY

An investigation has been made of the enzymic degradation of biopolymers by activated sludge. Starch was chosen as the model substrate; it was administered continuously at different sludge loading values which covered the entire range of loadings applied in sewage purification plants. The acclimatization of activated sludge to loading with starch as the sole source of carbon and energy was followed with regard to substrate removal, growth, amylase activity and numbers and types of amylolytic bacteria. Attention was paid to the nature and the location of the amylolytic enzymes and to the mechanism and kinetics of the amylolysis. The production and degradation of biomass and of amylases, the latter including their kinetics, were studied in relation to the sludge loading with starch and the proteolytic activity of the sludge. The influence of biomass retention on these phenomena was studied by comparing laboratory-grown activated sludge (i.e. sludge I, with retention of flocculated biomass) with sludge II (without retention of biomass).

Sludges I and II, acclimatized to starch, were able to degrade this compound almost completely when it was administered as the sole carbon nutrient over the sludge loading range 0.075 to 2.4 g COD/g sludge.day (Table 4.3). Immediate and nearly complete removal of starch and other carbohydrates by sludges I grown on glucose or maltose was observed when the initial, preformed, amylolytic activity of the sludge was higher than a distinct critical value; when it was lower, a lag phase was observed in the increase of biomass and of amylolytic activity (Fig. 4.3). Under such conditions incomplete starch removal was observed to occur during a period of maximally one week (Fig. 4.3E).

The concerted action of α-amylases and debranching enzymes was probably the most important mechanism of extracellular starch degradation by sludge bacteria. The starch hydrolases catalyse also the breakdown of dextrins. Enzymes specifically hydrolysing lower oligosaccharides such as maltotriose were not clearly detected in whole starch sludge or in its ultrasonicate (3.2).

The average amylolytic activity calculated per (viable) amylolytic bacterial cell present in starch sludge I, cultivated at pH 7.0, did not vary clearly over the SL range 0.3–2.4 and was about equal to the amylolytic activity of amylolytic bacteria in maltose and glucose sludge I (SL = 0.3). Only at SL = 0.075 with starch it was higher, probably due to active amylases associated with dead cells and cell debris which make up the bulk of the biomass of low-loaded sludge (Tables 4.5 and 4.6; Fig. 4.9).

Enzymes hydrolysing macromolecular substrates like protein and starch were completely associated with the sludge biomass. Only in high-loaded laboratory sludges 0,5–3% of the activity was detected in the sludge-free liquid (Table 7.1). This result is in agreement with the observation that the predominant amylolytic bacteria isolated from continuously loaded starch sludge I (SL = 0.3) did not excrete amylases into the media of batch cultures (Table 8.5). The enzyme-sludge association prevents the continuous wash-out of these enzymes from the sludge tanks. The proteinases are located at the outside of the cell wall of the sludge bacteria and are freely accessible to macromolecular substrates, as it is true of about 50 % of the amylases. Mass transfer resistance of polymers to cell surface-bound amylases and proteinases in sludge flocs, and steric hindrance due to binding of these enzymes to the bacteria were not to a large extent limiting the standard reaction rates with whole activated sludge (7.2).

The remaining 50 % of the amylases is probably located within the periplasmic space i.e. outside the cytoplasmic membrane but inside the molecular sieve constituted by the outer membrane of the cell wall of the Gram-negative bacteria occurring in activated sludge (Chapter 8). The periplasmic amylases are accessible to oligosaccharides which are able to pass through the outer membrane and are protected against proteolytic attack (7.2).

The relationship between dextrinogenic reaction rate of amylases from activated sludge and starch concentration strictly obeyed Michaelis-Menten kinetics only seldom (cf. the experiment of Fig. 6.4). In almost all of the other experiments (SL = 0.075–2.4) a curvilinear relationship was observed in the Lineweaver-Burk plot (Figs. 6.5 and 6.6), indicating rather large differences between the half-saturating substrate concentrations of the amylases present (6.1). Half-maximum reaction rate constants of the amylases with the greatest substrate affinity were estimated to approximate unrivalled low values, viz. 15 to 20 mg of starch/l (cf. data of Table 6.1 and Fig. 6.7). The $K_{1/2}$ values were not appreciably affected by SL (0.075–2.4), by the period of starch loading, and by the association of the amylases with the sludge (6.2, 6.3). Starch adsorption by starch sludge was observed to occur (Figs. 6.1 and 6.2) but did not initiate amylolysis at the surface of bacterial cell walls; it is unlikely to play a significant role in continuously operated starch activated sludge (6.2, 6.3).

A comparison of the respiration rates of starch-grown sludge supplied with glucose or starch revealed that dextrins rather than glucose are the end products of extracellular starch hydrolysis (Fig. 6.10). The same conclusion was drawn on the basis of the kinetics of starch hydrolysis in sludge, viz. of the ratio of the critical (potential) to the actual amylase activity being only 1.2 (Fig. 4.3E; 6.5).

Assuming that dextrins passing through the outer membrane of Gram-negative (amylolytic) bacteria consisted on the average of 5 glucose residues, that the kinetic parameters of sludge amylases were the same with starch and with dextrins with a DP of 6 or higher as the substrate, and that $K_{1/2}$ approached K_{diss}, the following statements relating to continuous starch loading of sludge I could be made (Table 6.4, section 6.5). a) Inadequate starch degradation occurred only if more than about $\frac{1}{6}$ of the amylases was occupied with substrate. All amylolytic activities observed at SL = 0.075 and 0.24 far exceeded the critical values, but those at SL = 1.2 and 2.4 incidentally were lower than the critical activities (Figs. 4.6A and 4.7). b) On the average about 2 % (SL = 0.075) to 5.5 % (SL = 2.4) of the surface-bound amylases was occupied with substrate. c) Average concentrations of higher dextrins (with a DP of about 6 or higher) ranged from only 0.4–0.5 (SL = 0.075) to 1.2–1.5 mg (SL = 2.4) glucose equivalents/litre of sludge-free liquid (cf. Table 4.3). d) The average residence times of these dextrins ranged from about 4 min (SL = 0.075) to 0.4 min (SL = 2.4). e) Average concentrations of total residual substrate (lower and higher dextrins) likely amounted to 5–10% or less of the COD present in the sludge-free liquid, over the whole range of loadings applied; the remainder, corresponding with several per cents of the COD of the influent, probably consisted of refractory bacterial products which limited the process of purification (cf. Table 4.3).

The acclimatization of glucose- or maltose-grown sludge I to starch loading was achieved by a population shift rather than by induction of enzyme synthesis of an existing population. When the activity of the preformed amylases was high enough to ensure immediate and complete starch removal, i.e. was higher than the critical value, the amylolytic bacteria increased from about 5% of the total (viable) cell count to almost 100% within 4 days (Figs. 4.3B and 4.4). DA_{spec} of this sludge attained the value of starch-grown sludge after 3–5 days (Figs. 4.3 and 4.6A). When the preformed amylase activity was lower than the critical value, the increase of the amylolytic bacteria to almost 100% was attained after 7–10 days. This retardation was probably due to concomitant growth of non-amylolytic bacteria at the expense of accumulated dextrins (Figs. 4.3E and 4.5). At continuous starch loading (SL = 0.3–2.4) the proportion of the amylolytic bacteria remained at 80–90% of the total (viable) bacteria. At SL = 0.075 only about 60% was amylolytic and about $\frac{1}{3}$ of the viable bacteria apparently utilized only substrates derived from decaying cell material (entirely cryptic growth). Spill over of starch degradation products by amylolytic bacteria was concluded hardly to occur under conditions of complete starch removal (Tables 4.5 and 8.4).

Starch sludge I did not attain a steady state with respect to its population composition (8.3 and 8.4), predominant regulation mechanism of amylase synthesis (8.5) and, dependent on SL, amylolytic enzyme activity (Fig. 4.6A). Several morphologically distinct flavobacteria usually succeeded each other as the predominant bacteria (8.4). The control of amylase synthesis in pure cultures of these bacteria was not very strict (8.4). Apparently, the amylase synthesis in the majority of the bacteria of starch sludge I was partly constitutive. However, experiments with whole starch sludge I indicated that the predominant regulatory mechanism of amylase synthesis can vary during the time of starch loading (8.5). Hence it was concluded that the regulation mechanisms of amylase synthesis had no predominant effect on the competition between amylolytic bacteria in activated sludge.

Sludge I, deprived of added protein for a long period and supplied with starch and ammonium sulphate as only carbon and nitrogen nutrients (C/N = 5), displayed a high proteolytic activity which on the average decreased by 50% with increasing SL with starch (Fig. 5.2), whereas the average amylolytic activity increased more than tenfold (Table 5.2). Therefore PA_{spec}, at least in non-protein-fed activated-sludge systems, and DA_{spec} can be regarded as intrinsic and extrinsic parameters, respectively (5.2.1). Proteolytic bacteria on the average amounted to about 50% of the total (viable) count at SL = 0.075 and 0.3, and to about 20% at SL = 2.4 (Table 5.5A; Fig. 5.3). At SL = 0.3, about half of the amylolytic bacteria was also proteolytic and the majority of the latter was also amylolytic (partly cryptic growth). The average proteolytic activity, calculated per (viable) proteolytic cell, sharply increased with decreasing SL (Fig. 5.4), probably due to the great stability of proteinases (as compared to that of amylases) in combination with large numbers of dead cells at low loadings. It is concluded that degradation of cells and reutilization of released cell material (turnover) play an important role in the metabolism of sludge I. PA_{spec} can be regarded as a measure of the intensity of extracellular turnover of cell protein. An important part of the proteolytic enzymes in activated sludge of outdoor plants apparently served also turnover as was concluded from the fact that low-loaded Bennekom sludge showed the same PA_{spec} as high-loaded Zeist sludge, whereas DA_{spec} of the former was half of that of the latter (Table 5.3). The adverse effect of winter conditions on PA_{spec} of these sludges did not or only to some extent result in the production of greater amounts of proteolytic enzymes, as contrasted to the compensation for the low-temperature effect on the amylolytic activity by the production of greater amounts of amylases (Fig. 4.12).

Starch sludge II, consisting mainly of bacteria growing in suspension, showed a low PA_{spec} and contained about 15% proteolytic bacteria. Both parameters were hardly affected by SL with starch and mean cell residence time (Figs. 5.2 and 5.3; Table 5.5B). This indicates that PA_{spec} cannot be increased by rising sludge age only, but that retention of flocculated biomass, as it occurs in sludge I, is an important condition for attaining high PA_{spec} values. This conclusion was confirmed by the observation that PA_{spec} of the flocculated biomass fraction of high-loaded starch sludge I was severalfold higher than that of the suspended fraction of the same sludge, in contrast to DA_{spec} which, on the average, was about equal in both fractions (Table 7.5). The high PA_{spec} of sludge flocs apparently results from relatively high rates of death and/or autolysis of cells in flocs due to relatively unfavourable growth conditions, rather than from long mean cell residence times (7.3).

Numbers of viable bacterial cells in starch sludge I amounted to about 130 × 10^7 bacteria/mg dry biomass at SL = 2.4 (t_s = 1 day) as contrasted to about 6 × 10^7 bacteria/mg at SL = 0.075 (t_s = c. 60 days); these viable counts were assessed to correspond with 90 and 4.5%, respectively, of the bacterial cell mass (Table 5.6). The graph of the relationship between the viable fraction and the net specific growth rate observed at the different SL values tended to go through the origin (Fig. 5.6). The latter relation allowed the calculation of t_d^* (mean doubling time of viable cells) of 0.65 day at t_s = 1 day and of an average maximum t_d^* of roughly 3 days. The latter is the actual one in sludges with a t_s of more than about 10 days. The results suggest that very slow growing or dormant bacteria do not emerge in very low-loaded starch sludges I. The bacteria apparently are obliged either to multiply or to die and cannot maintain themselves for more than 3 days without growth and division. The viable fraction of low-loaded starch sludge II seemed to be higher than that of comparable sludge I, indicating higher maximum t_d^* values. As t_s values of less than about 3 days do not occur in sludges of most of the sewage purification plants, specific growth rates of viable cells in these sludges may be assessed to vary maximally with a factor of about 2 above the minimum growth rate over the whole range of loadings applied. The parameter which reacts sharply to variation of SL, is the viability.

From the relation between the growth yield of starch sludge I and t_s, a maximum growth yield of 0.49 g dry biomass per g glucose equivalent and a biomass turnover coefficient of 0.05 day^{-1} were calculated (Table 5.7; Figs. 5.7 and 5.8). In very low-loaded sludge ($Y = 0.20$ g biomass per g GE) the value of the turnover coefficient was smaller, probably mainly due to the great refractoriness of dead cell material. Autolytic degradation of the cell wall of dead cells was apparently the rate-limiting step in the extracellular turnover of the protein of these dead cells in sludge I. Heterobacteriolysis was not demonstrated in sludge.

The standard amylase activity of starch sludges I decreased rapidly during starvation of these sludges (Figs. 5.9 and 5.10). Proteolytic enzymes only were responsible for the (initial) inactivation of the (cell-surface-bound) amylases (Figs. 5.10 and 5.12). This inactivation was prevented by saturating the amylases with starch (Fig. 5.10C). However, the very rapid and apparently irreversible inactivation of enzymes like Fungamyl 1600 (an α-amylase) and lysozyme, when added to activated sludge, was not attributed to proteolysis but to adsorption by the sludge (Figs. 7.3, 7.4 and 7.5; Table 7.6). During the enzymic degradation of amylases in starch sludge I, both amylases and proteinases probably remain cell-wall-bound (5.5.2). The (initial) rate of inactivation of amylases in starving starch sludges I with different amylase activities obeyed first order kinetics according to the equation $-\mathrm{d(DA)/dt} = k_i\,\mathrm{(DA)}$ (Figs. 5.12, 5.13 and 5.14A and B). This is in agreement with the high $K_{1/2}$ value of proteolytic enzymes of activated sludge (Fig. 6.11) and the very small amounts of cell surface-bound amylases in starch sludge, viz. roughly 0.006% of the biomass of the viable amylolytic bacteria which produced the enzymes (4.6.3). In fact, actual degradation rates of surface-bound amylases in situ might approximate 1/10,000 of the potential rates (6.6). The specific amylase inactivation rate (k_i) was proportional to $\mathrm{PA_{spec}}$ (Fig. 5.14A). Under reactor conditions, at 20°C, the average relation was defined as $k_i = 1.25\,\mathrm{PA_{spec}^{30^\circ C}}$ over the whole range of $\mathrm{PA_{spec}}$ values (0.038 to 0.87) found in the SL range of 0.075 to 2.4. The values of k_i were much higher than those of a^*, i.e. the decay rates of amylase were much higher than those of the entire biomass.

As the initial amylase inactivation rates as observed in starch sludge I under starvation conditions will also be valid under conditions of continuous operation (5.5.1), the following model was proposed: gross rate of amylase synthesis = rate of amylase wastage + rate of proteolytic inactivation of amylase = $(\mu_{net} + k_i)\mathrm{D\tilde{A}_{spec}}$ (Table 5.8). The relatively high turnover rate of amylases in low-loaded starch sludges is illustrated by the following calculation. The daily net production of amylases at SL = 0.075 was only roughly 1/550 of that at SL = 2.4, whereas the daily gross production at the former loading was almost 1/30 of that at the latter, i.e. roughly proportional to SL. Consequently, imaginary $D\tilde{A}_{spec}$ values ($k_i = 0$ and $a^* = 0$) were roughly equal for all SL values applied (Table 5.8; Fig. 4.8A). The decrease of $D\tilde{A}_{spec}$ actually observed with decreasing SL (Fig. 4.8A) is explained only by the much higher decay rate of amylases of dead cells compared to that of biomass. Starch sludge II is less proteolytic than comparable sludge I and obviously is considerably less dynamic, at least with respect to amylase degradation (Fig. 5.9B).

Starch sludges I and II of comparable SL or t_s generally differed in many respects. Sludges I grew mainly in flocs and contained many bacterial species, usually predominated by a few morphologically distinct flavobacteria. These sludges were highly proteolytic, contained a large proportion of proteolytic bacteria, especially at low SL, and showed a high amylase degradation rate; they had a large fraction of dead cells and showed a relatively low growth yield at low SL. Sludges II consisted mainly of dispersed or loosely aggregated bacterial cells, were predominated by one or a few bacterial species, once even for 500 days by a *Nocardia*-like bacterium (Plates 4.9, 4.10 and 4.11), showed a low PA_{spec}, contained a relatively small fraction of proteolytic bacteria, showed a low amylase inactivation rate, probably a large viable fraction, and a high growth yield.

It is concluded that the retention (feedback) of flocculated biomass is a condition necessary for the predominance of the flocculated bacteria over dispersed cells. The relatively adverse growth conditions within the flocs, creating different ecological niches, are the cause of most of the differences observed between sludges I and II.

ALGEMENE SAMENVATTING

Een onderzoek werd uitgevoerd naar de enzymatische afbraak van biopolymeren door aktief slib. Hiervoor werd zetmeel als modelsubstraat gekozen. Het werd kontinu in verschillende hoeveelheden toegediend zodat slibbelastingen werden verkregen die de belastingen van rioolwaterzuiveringsinstallaties overlapten. De aanpassing van aktief slib aan de belasting met zetmeel als enige koolstof- en energiebron werd nagegaan voor zover het substraatverwijdering, groei, amylase-aktiviteit en aantallen en typen van amylolytische bakteriën betrof. Bij dit onderzoek werd aandacht besteed aan de aard van de enzymen die verantwoordelijk zijn voor de zetmeelafbraak, terwijl werd nagegaan waar deze enzymen voorkomen in het slib. Tevens werden het mechanisme en de kinetiek van de amylolyse bestudeerd. De produktie en de afbraak van de biomassa en van de amylasen, inclusief de kinetiek van de afbraak van deze enzymen, werden bestudeerd in relatie tot de belasting van het slib met zetmeel en tot de proteolytische aktiviteit van het slib. De invloed van de retentie van de gevlokte biomassa op de eigenschappen van het slib werd nagegaan in twee reaktorsystemen, het ene met retentie van de gevlokte biomassa in de reaktor (slib I = aktief slib), het andere zonder retentie (slib II).

Zetmeel, toegediend als enige organische verbinding in hoeveelheden overeenkomende met 0,075–2,4 g COD/g slib.dag, werd nagenoeg volledig afgebroken door zowel slib van het type I als van type II, beide aangepast aan zetmeelvoeding (Tabel 4.3). Onmiddellijke en nagenoeg volledige verwijdering van het polymeer en andere koolhydraten door slib I gegroeid op glucose of maltose werd waargenomen wanneer de vóór de zetmeeltoevoeging in het slib I aanwezige amylolytische aktiviteit hoger was dan een bepaalde kritische waarde. Lag de aktiviteit beneden deze grenswaarde, dan werd een 'lag'-periode waargenomen wat betreft de toename van de biomassa en van de amylolytische aktiviteit (Fig. 4.3). Onder zulke omstandigheden werd zetmeel gedurende een periode van maximaal een week onvolledig verwijderd (Fig. 4.3E).

Het belangrijkste mechanisme bij de extracellulaire afbraak van zetmeel door slibbakteriën was waarschijnlijk de gekombineerde werking van α-amylasen en enzymen die de vertakking van zetmeel verbreken. De zetmeelhydrolyserende enzymen katalyseren ook de afbraak van dextrinen. Enzymen die lagere oligosacchariden, zoals maltotriose, specifiek hydrolyseren werden niet duidelijk aangetoond in zetmeelslib of in ultrasonicaat van zulk slib (3.2).

De gemiddelde amylolytische aktiviteit, berekend per (levende) amylolytische bakterie in zetmeelslib I, gegroeid bij pH 7,0, was ruwweg konstant over de slibbelasting-reeks 0,3–2,4 en was ongeveer gelijk aan de amylolytische aktiviteit van de amylolytische bakteriën in maltose- en glucoseslib I (SL = 0,3). Alleen bij een slibbelasting met zetmeel van 0,075 was deze aktiviteit hoger, waarschijnlijk als gevolg van aktieve amylasen gebonden aan dode cellen en celresten die het grootste deel van de biomassa van laagbelast slib uitmaken (Tabellen 4.5 en 4.6; Fig. 4.9).

Enzymen die makromolekulaire substraten zoals eiwit en zetmeel hydrolyseren, waren volledig gebonden aan de biomassa van het slib. Alleen in hoogbelast laboratoriumslib werd 0,5–3% van de aktiviteit in de slib-vrije vloeistof aangetroffen (Tabel 7.1). Dit resultaat is in overeenstemming met de waarneming dat dominerende amylolytische bakteriën geïsoleerd uit kontinu belast zetmeelslib I (SL = 0,3) geen amylasen uitscheidden in de media van 'batch'kulturen (Tabel 8.5). Het gebonden zijn van enzymen aan slib voorkomt het kontinue uitspoelen van deze enzymen uit de slibtanks. De proteïnasen zijn aan de buitenkant van de celwand van de slibbakteriën gelegen; ze zijn vrij bereikbaar voor makromolekulaire substraten evenals dit het geval is met ongeveer 50% van de amylasen. De reaktiesnelheden van de zetmeel- en caseïnehydrolyse, gemeten met de standaardmethoden, werden niet in belangrijke mate beïnvloed door het gebonden zijn van de enzymen aan de bakteriën en door diffusie van het substraat wegens het voorkomen van de bakteriën in slibvlokken (7.2).

De overige 50% van de amylasen bevindt zich waarschijnlijk in de periplasmatische ruimte van de celwand van de Gram-negatieve bakteriën in aktief slib (Hoofdstuk 8) d.w.z. buiten de cytoplasmatische membraan maar binnen de buitenmembraan van de celwand. Laatstgenoemde membraan funktioneert als een molekulaire zeef die geen enzymen of zetmeelmolekulen maar wel lagere oligosacchariden doorlaat die dan door de periplasmatische amylasen verder kunnen worden afgebroken. Deze amylasen zijn beschermd tegen proteolytische aantasting (7.2).

De relatie tussen de snelheid van de dextrinogene reaktie van amylasen uit aktief slib en de zetmeelkoncentratie voldeed slechts zelden strikt aan de Michaelis-Menten kinetiek (Fig. 6.4). In bijna alle andere gevallen (bij SL = 0,075–2,4) werd een kromlijnig verband gevonden in de Lineweaver-Burk grafiek (Fign. 6.5 en 6.6), hetgeen wijst op tamelijk grote verschillen tussen de $K_{1/2}$-waarden van de in slib aanwezige amylasen (6.1). De $K_{1/2}$-waarden van de amylasen met de grootste substraataffiniteit waren ongekend laag, nl. ongeveer 15–20 mg zetmeel/l (vergelijk gegevens van Tabel 6.1 en die van Fig. 6.7). De $K_{1/2}$-waarden werden niet in belangrijke mate beïnvloed door SL (0,075–2,4), door de tijdsduur van de slibbelasting met zetmeel, en door het gebonden zijn van de amylasen aan het slib (6.2; 6.3). Adsorptie van zetmeel aan zetmeelslib werd weliswaar waargenomen (Fign. 6.1 en 6.2), maar initieerde amylolyse op het oppervlak van de celwand niet en speelt waarschijnlijk een onbelangrijke rol in kontinu met zetmeel belast aktief slib (5.2, 6.3).

Vergelijking van de ademhalingssnelheden van slib gegroeid op zetmeel en voorzien van glucose of zetmeel gaf sterke aanwijzingen dat dextrinen en niet glucose eindprodukten zijn van de extracellulaire zetmeelhydrolyse (Fig. 6.10). Dezelfde konklusie werd getrokken uit het feit dat de verhouding tussen de kritische (potentiële) en feitelijke amylase-aktiviteit slechts ongeveer 1,2 was (Fig. 4.3E; 6.5).

Aannemende dat de dextrinen die de buitenste membraan van Gram-negatieve (amylolytische) bakteriën passeren, gemiddeld uit 5 glucose-eenheden bestonden, dat de kinetische parameters van slibamylasen dezelfde waren bij zetmeel en bij dextrinen met een DP van 6 of hoger als substraat, en dat $K_{1/2}$ en K_{diss} elkaar benaderden (6.5), konden de volgende konklusies met betrekking tot kontinue zetmeelbelasting van aktief slib worden getrokken (Tabel 6.4; sektie 6.5). a) Onvoldoende zetmeelafbraak vond slechts plaats indien meer dan ongeveer $\frac{1}{6}$ deel van de amylasen bezet was met substraat. Alle amylolytische aktiviteiten gemeten bij SL = 0,075 en 0,24 waren veel hoger dan de kritische waarden, maar die bij SL = 1,2 en 2,4 waren zo nu en dan lager dan de kritische aktiviteiten (Fign. 4.6A en 4.7). b) Gemiddeld was ongeveer 2 % (bij SL = 0,075) tot 5,5 % (bij SL = 2,4) van de aan het celoppervlak gebonden amylasen bezet met substraat. c) De gemiddelde koncentraties van hogere dextrinen (met een DP van ongeveer 6 of hoger) varieerden van 0,4–0,5 (bij SL = 0,075) tot 1,2–1,5 (bij SL = 2,4) mg glucose-equivalenten/liter slib-vrije vloeistof (vergelijk Tabel 4.3). d) De gemiddelde verblijftijden van deze dextrinen liepen uiteen van ongeveer 4 min (SL = 0,075) tot 0,4 min (SL = 2,4). e) De gemiddelde koncentraties van het totaal aan overgebleven substraat (lagere en hogere dextrinen) bedroegen waarschijnlijk 5–

10 % of minder van de COD in de slib-vrije vloeistof, geldend voor de gehele reeks van toegepaste slibbelastingen. De rest, overeenkomende met enkele procenten van de COD van het influent, bestond waarschijnlijk uit moeilijk afbreekbare bakteriële produkten die het zuiveringsproces limiteerden (Tabel 4.3).

De aanpassing van op glucose of maltose gegroeid slib I aan belasting met zetmeel was een gevolg van een populatieverschuiving en niet van induktie van enzymsynthese door de bestaande microflora. Indien de amylolytische aktiviteit van het op glucose of maltose gegroeide slib groter was dan de kritische waarde, namen de amylolytische bakteriën toe van 5 % van het totale aantal (levensvatbare) bakteriën tot bijna 100 % binnen 4 dagen (Fign. 4.3B en 4.4). DA_{spec} van dit slib bereikte na 3–5 dagen de waarde van op zetmeel gegroeid slib (Fign. 4.3 en 4.6A). Indien de amylolytische aktiviteit van het op glucose of maltose voorgekweekte slib lager was dan de kritische waarde, werd de toeneming van de amylolytische bakteriën tot bijna 100% in 7–10 dagen bereikt. Deze vertraagde toeneming was vermoedelijk een gevolg van de gelijktijdige groei van niet-amylolytische bakteriën ten koste van opgehoopte dextrinen (Fign. 4.3E en 4.5). Bij kontinue belasting met zetmeel (SL = 0,3–2,4) bleef het aandeel van de amylolytische bakteriën 80–90% van het totale aantal (levensvatbare) bakteriën. Bij SL = 0,075 was slechts ongeveer 60% van het totale aantal bakteriën amylolytisch, terwijl ongeveer $\frac{1}{3}$ van de bakteriën blijkbaar alléén substraten gebruikte die afkomstig waren van dode cellen (geheel cryptische groei). Bij volledig verbruik van zetmeel was dus nauwelijks sprake van uitscheiding van afbraakprodukten van zetmeel door amylolytische bakteriën (Tabellen 4.5 en 8.4).

Zetmeelslib I bereikte geen 'steady state' voor zover dit betrof samenstelling van de bakterie-populatie (8.4), het dominerende regulatiemechanisme van de amylasesynthese (8.5) en, afhankelijk van SL, amylolytische aktiviteit (Fig. 4.6A). Verscheidene morfologisch verschillende flavobakteriën volgden elkaar gewoonlijk op als meest voorkomende organismen (8.4). Het regulatiemechanisme betreffende de synthese van amylasen was in reinkulturen van deze bakteriën niet erg strikt (8.4). De amylasesynthese was bij de meeste geïsoleerde bakterietypen blijkbaar gedeeltelijk konstitutief. Proeven met zetmeelslib I wezen er echter op dat het dominerende regulatiemechanisme van de amylasesynthese kan variëren gedurende de tijd dat zetmeelbelasting van het slib plaats vond (8.5). Hieruit werd geconkludeerd dat het regulatiemechanisme van de amylasesynthese geen dominerende rol speelde bij de kompetitie tussen amylolytische bakteriën in aktief slib.

Slib I dat gedurende een lange tijd geen eiwit had ontvangen en gevoed werd met zetmeel en ammoniumsulfaat als enige koolstof- en stikstofverbindingen (C/N = 5), vertoonde een hoge proteolytische aktiviteit die gemiddeld met 50 % afnam bij toenemende belasting met zetmeel (Fig. 5.2), terwijl de gemiddelde amylolytische aktiviteit dan meer dan tienvoudig toenam (Tabel 5.2). Daarom kunnen PA_{spec}, tenminste in aktief-slib-systemen die niet met eiwit worden gevoed, en DA_{spec} beschouwd worden als intrinsieke resp. extrinsieke parameters (5.2.1). Proteolytische bakteriën maakten bij een lage slibbelasting (SL =

0,075 en 0,3) ongeveer 50% van alle (levensvatbare) bakteriën uit; bij SL = 2,4 was dit 20% (Tabel 5.5A; Fig. 5.3). Bij SL = 0,3 was ongeveer de helft van de amylolytische bakteriën ook proteolytisch terwijl de meerderheid van de eiwitsplitsers ook zetmeel konden afbreken (gedeeltelijk cryptische groei). De gemiddelde proteolytische aktiviteit, berekend per (levende) proteolytische bakterie, nam sterk toe bij afnemende SL (Fig. 5.4), waarschijnlijk als gevolg van de grote stabiliteit van proteïnasen (vergeleken met die van amylasen) in samenhang met grote aantallen dode cellen bij lage slibbelasting. Gekonkludeerd werd dat afbraak van cellen en hergebruik van vrijgekomen celmateriaal ('turnover') een belangrijke rol spelen bij de stofwisseling van slib I. PA_{spec} kan worden beschouwd als een maatstaf voor de intensiteit van de extracellulaire 'turnover' van celeiwit. In rioolwaterzuiveringsinstallaties dient een belangrijk deel van de proteolytische enzymen van het aktieve slib blijkbaar ook voor 'turnover'. Dit werd gekonkludeerd uit het feit dat laagbelast Bennekom-slib dezelfde PA_{spec} bezat als hoogbelast Zeist-slib, terwijl DA_{spec} van het eerstgenoemde slib slechts half zo groot was als die van het hoogbelaste slib (Tabel 5.3). De ongunstige werking van de lage temperatuur op PA_{spec} tijdens de wintermaanden resulteerde niet of nauwelijks in de vorming van grotere hoeveelheden proteolytische enzymen, in tegenstelling tot de kompensatie voor het lagetemperatuureffekt op DA_{spec} door de vorming van grotere hoeveelheden amylasen (Fig. 4.12).

Zetmeelslib II, eveneens gevoed met alleen zetmeel en ammoniumsulfaat als C- en N-bron, en hoofdzakelijk bestaande uit bakteriën die in suspensie groeiden, vertoonde een lage PA_{spec} en bevatte ongeveer 15% proteolytische bakteriën. Beide parameters werden nauwelijks beïnvloed door de belasting met zetmeel en door de verblijftijd van de cellen in de reaktor (Fign. 5.2 en 5.3; Tabel 5.5B). Dit wijst erop dat PA_{spec} niet kan worden verhoogd door alleen de slibleeftijd te verhogen, maar dat retentie van de slibvlokken in de reaktor, zoals die plaats vindt bij slib I, een belangrijke voorwaarde is om hoge PA_{spec}-waarden te verkrijgen. Deze konklusie werd bevestigd door de waarneming dat PA_{spec} van de in vlokken voorkomende biomassa van hoogbelast zetmeelslib I verscheidene keren hoger was dan de aktiviteit van de in suspensie groeiende biomassa van hetzelfde slib, in tegenstelling tot DA_{spec} die, gemiddeld, in beide frakties ongeveer gelijk was (Tabel 7.5). De hoge PA_{spec} van slibvlokken is blijkbaar een gevolg van relatief hoge snelheden van afsterving en/of autolyse van cellen in vlokken als gevolg van relatief ongunstige groei-omstandigheden en niet van de lange verblijftijden van de cellen (7.3).

Zetmeelslib I bevatte ongeveer 130 × 10^7 levensvatbare bakteriën/mg droge biomassa bij SL = 2,4 (t_s = 1 dag) in tegenstelling tot ongeveer 6 × 10^7 bakteriën/mg bij SL = 0,075 (t_s = ± 60 dagen). Geschat werd dat deze cijfers overeenkwamen met resp. 90 en 4,5% van de bakteriële celmassa (Tabel 5.6). De grafiek die het verband weergeeft tussen de fraktie levensvatbare bakteriën en de netto specifieke groeisnelheid bij verschillende slibbelastingen tendeerde door de oorsprong te gaan (Fig. 5.6). Laatstgenoemd verband maakte het mogelijk de gemiddelde verdubbelingstijd van levensvatbare cellen (t_d^*) te berekenen: 0,65 dag bij $t_s = 1$ dag, en een gemiddelde maximum t_d^*: ongeveer 3 dagen. De laatste is de werkelijke waarde in slib met een t_s van meer dan ongeveer 10 dagen. Deze uitkomsten doen vermoeden dat zeer langzaam groeiende of rustende bakteriën in zeer laag belast slib I niet voorkomen. De daarin voorkomende bakteriën zijn blijkbaar genoodzaakt of te vermenigvuldigen of te sterven; ze zijn niet in staat langer dan 3 dagen in leven te blijven zonder te groeien en te delen. Het aandeel van levensvatbare bakteriën in laagbelast zetmeelslib II leek groter te zijn dan dat van vergelijkbaar slib I, hetgeen wijst op hogere maximum t_d^*-waarden. Aangezien t_s-waarden lager dan ongeveer 3 dagen niet voorkomen in slib van de meeste rioolwaterzuiveringsinstallaties, kan worden aangenomen dat de specifieke groeisnelheden van levensvatbare cellen in het aktieve slib van deze installaties maximaal variëren met een faktor van ongeveer 2 boven de minimum-waarden. De parameter die scherp reageert op de variatie van SL is de levensvatbaarheid.

Uit het verband tussen celopbrengst van zetmeelslib I en t_s werden een maximale opbrengstkoëfficiënt (g droge biomassa/g glucose-equivalent) van 0,49 en een 'turnover'-koëfficiënt voor biomassa van 0,05 dag^{-1} berekend (Tabel 5.7; Fign. 5.7 en 5.8). In zeer laag belast slib ($Y = 0{,}20$ g biomassa per g GE) was de waarde van de 'turnover'-koëfficiënt kleiner, vermoedelijk in hoofdzaak te danken aan de geringe afbreekbaarheid van dood celmateriaal. Afbraak door autolyse van de celwand van dode bakteriën was blijkbaar de snelheidsbeperkende stap bij de extracellulaire 'turnover' van het eiwit van deze dode cellen in slib I. Heterobakteriolyse werd in aktief slib niet aangetoond.

De standaardamylase-aktiviteit van zetmeelslib I daalde snel bij uithongering van het slib (Fign. 5.9 en 5.10). Alléén proteolytische enzymen waren verantwoordelijk voor de (initiële) inaktivering van de op het celoppervlak gebonden amylasen (Fign. 5.10 en 5.12). Deze inaktivering werd voorkomen door de amylasen te verzadigen met zetmeel (Fig. 5.10C). De zeer snelle en blijkbaar irreversibele inaktivering van enzymen zoals Fungamyl 1600 (een α-amylase) en lysozym, na toevoeging aan aktief slib, werd niet toegeschreven aan proteolyse maar aan adsorptie aan het slib (Fign. 7.3, 7.4 en 7.5; Tabel 7.6). Bij de enzymatische afbraak van amylasen in zetmeelslib I blijven de amylasen zowel als de proteïnasen vermoedelijk gebonden aan de celwand (5.5.2). De (initiële) snelheid van inaktivering van amylasen in aan uithongering blootgestelde slibmonsters van het type I met verschillende amylase-aktiviteiten volgde een reaktie van de eerste orde: $-d(DA)/dt = k_i(DA)$ (Fign. 5.12, 5.13 en 5.14A en B). Dit is in overeenstemming met de hoge $K_{1/2}$-waarde van proteolytische enzymen van aktief slib (Fig. 6.11) en de zeer kleine hoeveelheden van aan het celoppervlak gebonden amylasen in zetmeelslib nl. ruwweg 0,006% van de biomassa van de levensvatbare amylolytische bakteriën die de enzymen produceerden (4.6.3). In feite zouden de werkelijke afbraaksnelheden van aan het celoppervlak gebonden amylasen in situ ruwweg 1/10.000 kunnen zijn van de potentiële snelheden. De specifieke amylase-inaktiveringssnelheid (k_i) was evenredig met PA_{spec} (Fig. 5.14A). Onder reaktoromstandigheden, bij 20°C, werd het (gemiddelde) verband als volgt gedefinieerd: $k_i = 1.25\, PA_{spec}^{30°C}$ geldend voor de gehele reeks van PA_{spec}-waarden (0,038–0,87) die gevonden waren bij verschillende belastingen met zetmeel (SL van 0,075–2,4). De waarden voor k_i waren veel hoger dan die voor a^*, d.w.z. dat de afbraaksnelheden voor amylase veel hoger waren dan die voor de gehele biomassa.

Aangezien de initiële amylase-inaktiveringssnelheden, zoals waargenomen in zetmeelslib I bij uithongering, ook zullen gelden bij kontinue belasting (5.5.1), werd het volgende model voorgesteld: brutosnelheid van amylasesynthese = snelheid van amylase-eliminering (door afvoer van overmaat slib) + snelheid van proteolytische inaktivering van amylase = $(\mu_{net} + k_i)\mathrm{D\tilde{A}_{spec}}$ (Tabel 5.8). De relatief hoge 'turnover'-snelheid voor amylasen in laagbelast zetmeelslib wordt geïllustreerd door de volgende berekening. De dagelijkse nettoproduktie van amylasen bij SL = 0,075 was ruwweg slechts 1/550 van die bij SL = 2,4, terwijl de dagelijkse brutoproduktie in het laagbelaste aktieve slib bijna 1/30 was van die in het hoogbelaste slib, d.w.z. ongeveer evenredig met SL. Hieruit volgt dat de imaginaire $\mathrm{D\tilde{A}_{spec}}$-waarden (bij $k_i = 0$ en $a^* = 0$) ruwweg gelijk waren voor alle toegepaste SL-waarden (Tabel 5.8; Fig. 4.8A). De daling van $\mathrm{D\tilde{A}_{spec}}$ die werd waargenomen bij dalende slibbelasting (Fig. 4.8A) kan slechts worden verklaard door de veel hogere snelheid van afbraak van amylasen van dode cellen dan die van de totale biomassa van deze cellen. Zetmeelslib II is minder proteolytisch dan vergelijkbaar slib I en is blijkbaar aanzienlijk minder dynamisch, tenminste voor zover het de amylase-afbraak betreft (Fig. 5.9B).

Zetmeelslib I en II met vergelijkbare SL en t_s verschilden in het algemeen in vele opzichten. Slib I groeide hoofdzakelijk in vlokken en bevatte vele bakteriesoorten waarvan een paar morfologisch verschillende flavobakteriën gewoonlijk het meest voorkwamen. Dit slib was sterk proteolytisch, bezat een hoog percentage proteolytische bakteriën, in het bijzonder bij een lage SL, en vertoonde een hoge snelheid van afbraak van amylasen; het bezat een grote fraktie dode cellen en vertoonde een relatief lage opbrengstkoëfficiënt bij een lage SL. Slib II bestond voornamelijk uit afzonderlijke of uit los geaggregeerde bakteriën waarvan één soort of een paar soorten domineerden, een *Nocardia*-achtige bakterie zelfs gedurende 500 dagen (Foto's 4.9, 4.10, 4.11). Dit slib vertoonde een lage $\mathrm{PA_{spec}}$, bevatte een betrekkelijk kleine fraktie proteolytische bakteriën en vertoonde een lage snelheid van inaktivering van amylasen. Het bezat waarschijnlijk een grote fraktie levensvatbare bakteriën en vertoonde een hoge opbrengstkoëfficiënt.

Gekonkludeerd wordt dat de retentie ('feedback') van gevlokte biomassa in de reaktor een voorwaarde is voor het domineren van de in vlokken voorkomende bakteriën over de in gedispergeerde toestand aanwezige cellen. De relatief ongunstige groei-omstandigheden in de vlokken die resulteren in verschillende oecologische nissen, vormen de oorzaak van de meeste waargenomen verschillen tussen slib I en slib II.

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LIST OF ABBREVIATIONS AND SYMBOLS

Abbreviation or symbol	Description	Unit
a	endogenous consumption coefficient of biomass in growing pure cultures (Herbert, 1958); specific maintenance rate ($= m\ Y_{max}$) (Marr et al., 1963); units of biomass utilized for maintenance per unit of biomass per unit of time	day^{-1}
a^*	biomass (micro-organism) turnover (decay) coefficient of sludge, including maintenance, decay by cell death and lysis, predator activities, cryptic growth etc.; units of biomass per unit of biomass per unit of time	day^{-1}
BOD	biochemical oxygen demand; units mass of oxygen consumed by micro-organisms (usually during 5 days) for biochemical oxidation of substrate	g
C/N ratio	mass ratio carbon to nitrogen	
COD	chemical oxygen demand; units mass of oxygen required for chemical oxidation of substrate	g
D	hydraulic dilution rate, hydraulic (space) loading; units volume of inflowing medium per unit volume of culture liquid in the reactor per unit of time	day^{-1}
d	(MPN-based) estimate of δ	ml^{-1}, mg^{-1}
$\mathrm{DA}_{(spec)}$	(specific) dextrinizing activity; units mass of starch (equivalents) hydrolysed per unit of time per unit of volume or per unit of biomass (SS)	g.h^{-1} per l or per g
DE	dextrose equivalents; a figure after DE indicates reducing carbohydrates, in a mixture of dextrins, as percentage of total carbohydrates	
$\overline{\mathrm{DP}}$	average degree of polymerization (ratio of total glucose equivalents to reducing glucose equivalents)	
dry wt	dry weight (105°C)	g
E	extinction	
f	viable fraction of (bacterial) population, i.e. the fraction of cells that is able to multiply	

	under (defined) favourable conditions of growth	
GE	glucose equivalents (anhydrous glucose)	
g	generation time of cells	h
k_i	specific (amylolytic) enzyme inactivation rate; units of (amylolytic) enzyme activity per unit of enzyme activity per unit of time	day^{-1}
K_{diss}	dissociation constant of enzyme-substrate complex	
K_m	Michaelis (substrate, saturation) constant; substrate concentration required for half-maximal rate of an enzyme reaction	g.l^{-1}
$K_{1/2}$	half-rate constant; substrate concentration required for half-maximal rate of a process determined analogously to K_m	g.l^{-1}
K_s	concentration of growth-limiting substrate required for half-maximal specific growth rate	g.l^{-1}
L-B(-plot)	Lineweaver-Burk(-plot)	
M	molar	mole.l^{-1}
m	maintenance coefficient (for growing pure cultures); units mass of energy source consumed for maintenance per unit of biomass per unit of time	g.g^{-1}.h^{-1}
(ML)SS	(mixed-liquor) suspended solids, i.e. units of dry matter of sludge	g
(ML)VSS	(mixed-liquor) volatile suspended solids, i.e. weight loss of MLSS by incineration	g
MPN	most probable number	
mol.wt	molecular weight	
n	number of estimates	
PA$_{(spec)}$	(specific) proteolytic activity; units mass of casein hydrolysed per unit of time per unit of volume or per unit of biomass (SS)	g.h^{-1} per l or per g
PE	population equivalent; the average daily production of oxygen-consuming substances (equivalent to 180 g COD) per inhabitant	
Q	flow rate of liquid through reactor; units volume of inflowing medium in the reactor per unit of time	l.day^{-1}
Q_{10}	quotient of a reaction constant at a temperature of T + 10 and this constant at temperature T	
q	specific rate of substrate uptake; units mass of substrate transported into cells per unit of biomass per unit of time	g.g^{-1}.day^{-1}

q_{max}	maximum specific rate of substrate uptake, viz. at saturating concentrations of substrate	$g.g^{-1}.day^{-1}$
r	sample correlation coefficient	
S	substrate concentration	$g.l^{-1}$
s	sample standard deviation	
$SA_{(spec)}$	(specific) saccharifying activity; units mass of glucose equivalents formed per unit of time per unit of volume or per unit of biomass (SS)	$g.h^{-1}$ per l or per g
SE	starch (-extinction) equivalents (iodine reaction)	
SL	sludge loading or food-to-micro-organism ratio; units of substrate COD supplied per unit of biomass (SS) per unit of time	$g.g^{-1}.day^{-1}$
T	temperature	°C
t	time	day, h or min
t_d	doubling time of whole culture, time in which the biomass doubles	day
t_d^*	doubling time of viable cells	day
t_h	mean hydraulic retention (residence, replacement) time in reactor	day or h
tris	tris-(hydroxymethyl)-aminomethane	
t_s	sludge age, mean cell residence (replacement) time, biological solids retention time, reciprocal value of μ_{net}	day
V	working volume of reactor	l
v	reaction rate at a given substrate concentration S	$mg.h^{-1}$ per l or per g
VL	organic (space) loading; units of substrate COD supplied per unit of reactor working volume per unit of time	$g.l^{-1}.day^{-1}$
V_{max}	maximum reaction rate at saturating substrate concentration	$mg.h^{-1}$ per l or per g
X	microbial mass concentration; units of biomass in dry mass per unit volume of culture	$g.l^{-1}$
X_1	biomass concentration in reactor	$g.l^{-1}$
X_2	biomass concentration in the mixture of removed sludge and (sponge) effluent	$g.l^{-1}$
Y_{max}	maximum (true) yield coefficient; units of biomass theoretically formed per unit mass of energy source consumed, if no energy were required for maintenance or, in the case of activated sludge, in addition no decay, predation and reutilization of sludge material occurred	$g.g^{-1}$

$Y_{(obs)}$	(observed) growth yield coefficient; units of biomass formed per unit mass of limiting substrate consumed	$g.g^{-1}$
α	index of viability, $(f + 1)/2$; probability that a newly formed cell will be viable	
δ	density of (viable) bacteria per ml or per mg	ml^{-1}, mg^{-1}
$\mu_{(net)}$	(net) specific growth rate of biomass, specific sludge wastage rate; units of biomass formed per unit of biomass per unit of time	day^{-1}
μ_{tot}	total specific growth rate; sum of μ and a (or of μ and a^*); hypothetic growth rate if no energy were required for maintenance (or for maintenance and cell decay)	day^{-1}
μ_{max}	maximum total specific growth rate, viz. at saturating concentrations of the growth-limiting substrate	day^{-1}
μ^*	specific growth rate of viable organisms	day^{-1}

superscript $\bar{}$ denotes arithmetical mean
superscript $\tilde{}$ denotes logarithmic mean

CURRICULUM VITAE

De auteur is op 24 januari 1946 geboren te Berg en Dal. Hij bezocht het Gymnasium α van het St.-Canisius College te Nijmegen van 1958 tot 1964. Na het behalen van het diploma volgde een studie aan de Landbouwhogeschool te Wageningen, richting Cultuurtechniek, specialisatie Waterzuivering. Deze studie werd in 1972 afgerond met het behalen van het ingenieursdiploma. Het vakkenpakket in de ingenieursfase bestond uit waterzuivering (hoofdvak), mikrobiologie (verzwaard vak) en biochemie. Van 1973 tot 1978 werd het promotie-onderzoek verricht in het Laboratorium voor Microbiologie van de Landbouwhogeschool. Sinds 1979 is de auteur als wetenschappelijk medewerker verbonden aan de afdeling Water en Bodem van het Instituut voor Milieuhygiëne en Gezondheidstechniek, TNO, te Delft.