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BACTERIOLOGICAL STUDIES ON DAIRY WASTE ACTIVATED SLUDGE

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CHAPTER 1

INTRODUCTION

Aerobic biological treatment is the most effective method for the mineralization of municipal sewage and industrial waste water. Properly applied, this method could result in water that almost satisfies drinking water requirements. Trickling filters and the activated sludge process are the two principal applications. The latter was developed in England by ARDERN and LOCKETT (1914). Since then it has become one of the most versatile of the aerobic biological treatment processes applied.

Activated sludge is a flocculated mass, for a large part made up of aerobic microorganisms. Bacteria are the main constituents of the activated sludge flocs, but also protozoa and rotifers are always present.

Although the activated sludge process is extensively used and numerous technical applications have been described, the knowledge and the literature concerning the fundamental aspects of this purification method are relatively scarce and of recent date. However, a growing interest in and the need for treatment of various industrial wastes have stimulated more fundamental research in this field and have introduced the activated sludge into many a microbiological laboratory.

At one time the general conception was that the purifying effect of activated sludge should depend on the activity of one or a few special types of bacteria. These bacteria should also be essential for the formation and the structure of the activated sludge because of their floc-forming ability. In agreement with this conception a bacterium was isolated that appeared to be active in both respects (BUTTERFIELD, 1935; BUTTERFIELD et al., 1937). Afterwards, however, more bacteria with floc-forming and purifying capacities were found to occur (MCKINNEY and HORWOOD, 1952; MCKINNEY and WEICHLEIN, 1953). According to more recent opinions, activated sludge is considered to consist of a number of predominating types of bacteria, which, under prevailing conditions, exert their activity partly simultaneously or partly in succession. The bacterial composition of the activated sludge floc is mainly determined by the chemical composition of the waste water. However, to be included into the activated sludge flora, the bacteria should have the ability to settle in the floc.

In view of this concept, experiments were designed to obtain a better understanding of the bacteriological formation of activated sludge from dairy waste water, and of the behaviour of this activated sludge under varying conditions. Dairy waste water was chosen as the subject for this study on account of its important contribution to the total industrial waste discharged in the Netherlands. Moreover, it offers the possibility to prepare a sterile artificial dairy waste of a constant and representative composition.

Part of the laboratory experiments were compared with the starting procedure in an oxidation ditch near the dairy plant in De Wilp (province of Groningen).

CHAPTER 2

EXPERIMENTAL METHODS

2.1. BACTERIOLOGICAL METHODS

2.1.1. *Total viable count*

2.1.1.1. Plate count

Plate counts were performed on tryptone glucose extract agar-50 % (TGEA-50 %), supplemented with 0.5 ml of skim milk per petri dish prior to pouring plates.

Preliminary experiments with nutrient agar, artificial dairy waste agar, TGEA, TGEA-50 % and TGEA-10 % showed the TGEA-50 % to be preferable for plate count and isolation procedures. Colony numbers and colours developed better on TGEA-50 % than on nutrient agar and artificial dairy waste agar. The strains growing rapidly on TGEA developed noticeably slower on TGEA-50 % and therefore gave smaller colonies, but on TGEA-10 % growth was over-retarded. Overgrowing of small colonies by rapidly growing ones occurred much less frequently on TGEA-50 % than on TGEA. Consequently, total counts were highest on TGEA-50 %.

The composition of the TGEA-50 % was achieved by preparing TGE-broth (Oxoid) of half the concentration prescribed and supplementing this with 1.5 % agar; pH: 7.0. Colonies were counted after 3 days' incubation at 25 °C.

2.1.1.2. Spot plate count

This method is a modification of the previous one. A short description of the technique is found in MCKINNEY's 'Microbiology for Sanitary Engineers' (1962). Five 0.02 ml spots of a suitable dilution were pipetted on an agar plate using a microscrew attachment (Agla-micrometer syringe outfit; the syringe being sterilized in alcohol, rinsed with sterile water and thereafter with part of the sample). The agar plates were dried by storage at 25 °C for 3 days prior to use. The spots were counted after 20 hours' incubation at 25 °C under a Wild-M5 stereomicroscope.

This method was especially used for bacterial counts in experiments with pure cultures or mixtures of bacteria which could be easily recognized by their colony type. With 5 spots on a single plate, counts in fivefold were obtained more quickly and with less expenditure of materials than with the common plate count method. For isolation purposes the conventional plate count procedure was found to be preferable.

2.1.2. *Hugh and Leifson test*

The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates in various Gram-negative bacteria has been studied by HUGH and LEIFSON (1953). Their method, in combination with other tests, gives valuable information for taxonomic examinations in general.

Before applying this test to the strains isolated from the activated sludge, representative strains of many genera of bacteria from the laboratory collection had been subjected to this

test. The results proved to be in accordance with the corresponding characteristics listed in SKERMAN's 'Guide' (1959) or in BERGEY's 'Manual' (1957).

The tests were performed in small 10 × 120 mm test tubes and were further comparable to the method described by the cited authors.

2.1.3. *Replica method*

Strains isolated were tested for casein hydrolysis, for catalase reaction on TGEA skim milk plates, and for *Arthrobacter* characteristics on this medium and on casein agar, with the replica method of MALING (1960).

2.1.4. *Staining methods*

2.1.4.1. Gram's stain

The Gram's stain was carried out according to HUCKER's modification described in the 'Manual of Microbiological Methods' (1957).

2.1.4.2. Flagella stain

Of the three methods tested – the CONN and WOLFE (1938), the LEIFSON (1951) and the BAILEY (1953) methods – the latter was found to give the best and the most consistent results. This method was carried out in conformation with the recommendations of FISHER and COHN (1942) for bacteria on which flagella are difficult to stain, as is frequently the case with water microorganisms. The slides were examined under a Wild-M20 phase contrast microscope. Preparations were made of actively growing – approximately 20 hours old – cultures.

2.1.5. *Media*

2.1.5.1. Tryptone glucose extract agar (TGEA) + skim milk

This medium contains: beef extract, 3 g; tryptone, 5 g; glucose, 1 g; agar, 15 g; tap water, 1000 ml; pH: 7.0. To each petri dish 0.5 ml of sterile skim milk is added prior to the pouring of the plates.

2.1.5.2. Casein agar

This medium consists of: casein, 1 g; glucose, 1 g; K_2HPO_4, 1 g; $Ca(HPO_4)_2$, 0.25 g; $MgSO_4.7H_20$, 0.25 g; $(NH_4)_2SO_4$, 0.25 g; yeast extract, 0.7 g; Davis agar, 10 g; soil extract, 500 ml; tap water, 500 ml; pH: 7.0.

2.1.5.3. Hugh and Leifson medium

This medium is composed of: glucose or lactose, 10 g; pepton, 2 g; NaCl, 5 g; K_2HPO_4, 0.3 g; bromothymol blue, 0.03 g; agar, 3 g; tap water, 1000 ml; pH: 7.1. Anaerobic tubes were closed by a vaspar seal.

All the media were autoclaved for 15 minutes at 120°C.

2.2. Chemical and biochemical methods

2.2.1. *Biochemical oxygen demand*

The biochemical oxygen demand (BOD) has been determined according to the procedure of the 'American Standard Methods' (1955). Dissolved oxygen was determined by the Winkler method.

2.2.2. *Chemical oxygen demand*

2.2.2.1. Modified dichromate method

The chemical oxygen demand (COD) was mainly estimated according to the procedure described in the 'Industrial Waste Guide' (1953). This procedure was adapted to a semi-micromethod by consistently using one-fifth of the prescribed volumes of reagents and samples. The results obtained with this semi-micromethod were in accordance with those of the original and the official dichromate reflux method of the 'American Standard Methods' (1955).

2.2.2.2. Dichromate reflux method

In a small part of the experiments a modified dichromate reflux method was employed (Anonymus, 1960). To each 5 ml of sample contained in a 250 ml round-bottom flask, 10 ml of a 0.125 N potassium dichromate solution, 15 ml of concentrated sulphuric acid, 100 mg of silver sulphate and a few pumice granules were added. The mixture was refluxed for 15 minutes, using a Dimroth's condenser. An equal volume of distilled water was then added. The solution was titrated at 40° to 50°C with a 0.125 N ferrous ammonium sulphate solution, using three drops of a ferroin indicator (1.485 g of 1,10-phenantroline and 0.695 g of ferrous sulphate in 100 ml of water). The endpoint was reached when the colour changed from blue-green to wine-red.

2.2.3. *Determination of carbohydrates*

2.2.3.1. Anthrone colorimetric method

This method was carried out according to TREVELYAN and HARRISON (1952). A reference standard was run with every sample tested. Determinations were performed in triplicate.

2.2.3.2. Phenol-sulphuric acid colorimetric method

The prescription of WHISTLER and WOLFROM (1962) was followed. A reference standard was run with every sample simultaneously. Determinations were performed in triplicate.

For both determination methods a Beckman spectrophotometer was used.

2.2.4. *Determination of nitrogen compounds*

2.2.4.1. Modified micro-Kjeldahl method (total nitrogen)

Duplicate 1 ml samples were gently heated to dryness, supplied with concentrated H_2SO_4, and digested for approximately 15 minutes. A few drops

of a 30% H_2O_2 solution were added until the mixture remained clear. Heating was continued for another 5 minutes. Ammonia was distilled with a Markham steam distillation apparatus into a 2% H_3BO_3 solution containing methyl-red-bromocresol-green indicator, and titrated with 0.002 N H_2SO_4.

2.2.4.2. Folin-Ciocalteu colorimetric method (proteins)

Duplicate samples were treated according to the method described by COLOWICK and KAPLAN (1957). A freshly prepared reference standard was run simultaneously. This standard had previously been assayed according to method 2.2.4.1. Optical density was determined with a Beckman spectrophotometer.

2.2.5. *Determinations of acids*

2.2.5.1. Total volatile fatty acids

The total amount of volatile fatty acids was determined according to FRIEDEMANN (1938) by steam distillation and redistillation of the samples. The distillate was titrated with a 0.01 N NaOH solution, using phenolphtalein as the indicator.

2.2.5.2. Lactic acid (qualitatively)

Qualitative determinations of lactic acid were performed with *p*-hydroxydiphenyl and sulphuric acid (FEIGL, 1960).

2.2.5.3. Filter paper partition chromatography

Qualitative analysis of organic acids in an ether extract of a dilute sample was carried out by one or two-dimensional paper chromatography. Ederol-202 filter paper was used exclusively. Before bringing the paper into contact with the solvent, the spots containing the acids were exposed for 2 minutes to the vapour of concentrated ammonia for their conversion into the corresponding salts.

For analysis of volatile fatty acids, a mixture of ethanol and ammonia was used as a solvent in the proportion 100 to 1. After drying, the papers were sprayed with a bromophenol-blue solution (40 mg of bromophenol blue per 100 ml of 10% ethanol containing 200 mg of citric acid). For Rf reference the sample was always run with one or more of the following acids: formic, acetic, propionic and butyric acids.

For analysis of keto, hydroxy and dicarboxylic acids, two-dimensional chromatograms were used. The solvent mixtures were: ethanol, ammonia and water (80:4:16) for one dimension, and after drying: butanol, formic acid and water (77:12:11) for the second dimension. The papers were sprayed with an indicator solution containing: anilin, butanol, ethanol and a solution of 7.5% glucose in water (20:365:175:100). The colour developed after drying the paper for 15 minutes or more at 105°C. For Rf reference a separate chromatogram was run with a mixture of the following acids: citric, α-ketoglutaric, succinic, fumaric, malic, oxalacetic, lactic, gluconic and glutaric acids.

2.2.6. *Formol titration*

Formol titrations were performed according to HOWARD BROWN (1923). The endpoints of the titrations were determined potentiometrically.

2.2.7. *Determination of ammonia and nitrate*

For the determination of ammonia as well as for nitrate, the micro-diffusion technique of CONWAY (1950) was used.

2.3. ELECTROCHEMICAL METHODS

2.3.1. *Recording of dissolved oxygen*

Dissolved oxygen (DO) can be determined continuously by electrochemical methods. Several devices have been described in the literature. Since no ready-made apparatus was directly available, a simple device was developed (ADAMSE, 1965). In view of descriptions by TÖDT (1953, 1958), AMBÜHL (1955) and BRIGGS et al. (1958), an electrode system based upon the galvanic cell principle and consisting of a mercury cathode and a zinc anode, the latter immersed in a saturated KCl solution, was chosen (Fig. 1). The mercury surface of the cathode is kept clean by a quickly operating stirrer. Both the electrodes are connected with a microammeter which has an output for connection with a recorder. This design permits measurements of DO in the laboratory apparatus over a long period. Microammeter readings are expressed as ppm O_2 with the aid of a calibration graph. A linear relationship exists between readings in μA and ppm O_2 values obtained titrimetrically (Winkler method). It is necessary to make the conductivity of the samples approximately 3000 μmho by adding KCl. (Distilled water supplemented with 0.16% KCl has a conductivity of approximately 3000 μmho). The influence of temperature on the measurements is eliminated by maintaining a constant temperature throughout the experiments.

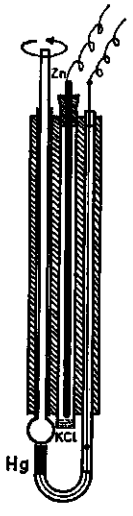

FIG. 1. Galvanic-cell electrode combination.

2.3.2. *Application*

Recording of the DO content in an aerated activated sludge suspension was carried out in the assembly illustrated in fig. 2. This consists of an aerated vessel of a special construction, adapted to experiments with activated sludge. The sinter for the aeration has been built into the stopcock at the bottom of the vessel, so that it can be turned away when aeration must be stopped. This prevents the liquid from flowing back into the compressed air line. Once the sludge has settled, the effluent can be drawn off by a side tube, usually closed by a screw clip on a short piece of rubber tubing connected with this side tube. On both sides of the vessel there are tubes for introducing pH electrodes into the liquid. The oxygen electrodes are introduced through a stopper at the top of the vessel, which also has a substrate inlet and an air outlet. The DO content of the liquid is measured continuously and registered by a recorder which, at the same time, may also register the pH during the experiment. The aeration rate is determined by a calibrated rotameter type of flowmeter. Before entering the vessel the air is saturated with water.

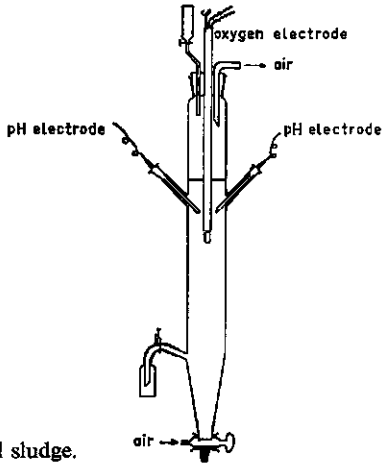

FIG. 2. Aerated flask for experiments with activated sludge.

CHAPTER 3

FORMATION OF DAIRY WASTE ACTIVATED SLUDGE

3.1. Introduction

An activated sludge procedure according to the oxidation ditch principle (Pasveer, 1959) can be started by using either activated sludge from an established aerobic installation as the inoculation material, or by building up the necessary bacterial floc material step by step on the spot. The former method saves time but defects in the sludge may be transferred from one installation to the other. Such defects are generally difficult to eliminate. The second method is often the only possibility when local activated sludge is unavailable. This procedure was adopted when the R.A.A.D.[1] started an oxidation ditch near the dairy plant of De Wilp, a small village in the northern part of the country.

The author compared the development of the activated sludge flora in a small scale apparatus in the laboratory of Microbiology at Wageningen with that in the oxidation ditch in de De Wilp. This parallel experiment in the laboratory was carried out in order to investigate whether the activated sludge built up in the laboratory was bacteriologically equal to that obtained in practice.

In the laboratory apparatus an artificial dairy waste water of a chemical composition similar to that of the mean waste of the dairy plant in De Wilp was used as the substrate, and a suspension of soil and manure in sewage – without settled solids – served as the natural inoculation material.

The second and main purpose of the investigations was to study the microbial succession during the development of the activated sludge flora.

3.2. Literature

Since the activated sludge process was developed some fifty years ago, it has been questioned which microorganisms participate in this aeration process of waste treatment.

Activated sludge is a flocculated mass of mainly bacteria and protozoa. The bacteria are considered to be the most important group, forming a heterogeneous flora. The bacterial composition of the floc depends upon the chemical characteristics of the waste.

The flora of activated sludge has been studied by several bacteriologists. Most have attempted to classify the predominant bacteria of this sludge according to the physiological activities of these microorganisms, others have tried to isolate particular species or physiological groups of bacteria in which they were interested.

One of the first reports on microorganisms in activated sludge came from Johnson (1914), who observed the presence of zoogloeal bacteria and protozoa.

[1] Government Agricultural Waste Water Institute, Arnhem, Netherlands.

RUSSEL and BARTOW (1916) and KAMM (1917) described species of nitrifying and non-nitrifying bacteria isolated from activated sludge. HOTCHKISS (1923) studied bacteria of the sulphur and nitrogen cycles and typical proteolytic microorganisms. Filamentous forms – chlamydobacteria – among large masses of zoogloea-forming bacteria were observed by BUSWELL and LONG (1923) upon microscopical examination of activated sludge. Intestinal bacteria were reported as being predominant in a sewage treatment plant sludge by GAUB (1924). HARRIS, COCKBURN and ANDERSON (1927) found 61% of *Bacterium aerogenes* type and 38% of *Proteus* type of bacteria in the activated sludge from a sewage treatment plant. This, combined with the proteolytic character of many species led to the conclusion, that the intestinal group of bacteria was important in sewage purification. BUSWELL (1928, 1931), however, was convinced that zoogloeal bacteria and protozoa were of primary importance in activated sludge. This opinion was confirmed by TAYLOR (1930) who stated that the bulk of the activated sludge was composed of a jelly-like mass in which bacteria were present in large numbers. This was thougt to be a typical zoogloea formation. BUTTERFIELD (1935) was the first to obtain a zoogloea-forming bacterium from activated sludge in pure culture. This microorganism, identified as a variety of *Zoogloea ramigera*, produced flocs resembling activated sludge. The supposed importance of this bacterium in the purification process was confirmed later by experiments of BUTTERFIELD et al. (1937). HEUKELEKIAN and LITTMAN (1939) examined zoogloea-forming bacteria from 15 different sludges. All of these bacteria resembled *Zoogloea ramigera* as described by BUTTERFIELD (1935). It seemed that *Zoogloea ramigera*, because of its ability to form flocs and mineralizing nutrient substrates, should be considered as the principal microorganisms in activated sludge.

However, it was questioned how far the different results of many isolations had been due to the methods or materials used. ALLEN (1944) subjected domestic sewage sludge to homogenization before making bacteriological examinations. The disintegration of the floc enabled the isolation of bacteria from its interior and to distinguish them from the smaller number of bacteria in the interstitial liquid, which would have predominated if homogenization had not been applied. Without this disruption of the flocs, the relatively few colonies of true floc-forming bacteria would have been derived from clumps consisting of several hundreds of individuals. The bacterial counts were increased from 10- to 100-fold by employing this method. ALLEN (1944) carried out experiments to investigate which bacteriological alterations would occur when domestic sewage was aerated continuously for a long period, resulting in the formation of a small quantity of activated sludge. After an aeration period of 14 days, the sludge was allowed to settle, the supernatant drawn off and a fresh portion of sewage supplied. A second period of aeration lasted 17 days. At intervals during these aeration periods, samples of the suspension were withdrawn and examined for the presence of certain groups of bacteria. It was shown that acid-forming microorganisms – tested in litmus dextrose milk synthetic sewage – were always present in comparatively large numbers, but they were usually

not predominant. Bacteria present in the highest dilutions in this medium often showed growth but no acid production. Aerobic spore-forming bacteria were found in only small numbers. Spore-forming anaerobes, coliform bacteria, moulds and yeasts also formed a small proportion of the total count. Isolations made by ALLEN (1944) from the activated sludge produced in the laboratory on domestic sewage showed that the predominant flora of the sludge had a marked tendency to change from a non-proteolytic into a proteolytic type as the sludge aged from the initial to a several weeks old stage. The majority of 48 isolated strains consisted of Gram-negative rods, showing no acid production from carbohydrates, while many had decidedly proteolytic characteristics. Most of them belonged to the genera *Achromobacter, Chromobacterium* (*Flavobacterium*) and *Pseudomonas*.

These results led to the assumption that bacteria other than *Zoogloea ramigera* may also be able to form flocs when aerated in a suitable nutrient substrate. This assumption was supported by experiments of MCKINNEY and HORWOOD (1952) and MCKINNEY and WEICHLEIN (1953). From these and further experiments MCKINNEY (1955, 1957) concluded that all bacteria under certain environmental conditions may develop the ability to flocculate. The latter would primarily be determined by the relative surface charges and energy levels of the bacteria. When the floc has started to form, some bacteria die and lyse. According to MCKINNEY the insoluble polysaccharides would remain in the floc and would entrap the less active bacteria.

The present conception – MCKINNEY (1962) – is that several types of bacteria can make up activated sludge and that the nature of the organic compounds in the waste supplied determines which bacteria will predominate. Thus the opinion that exclusively *Zoogloea ramigera* plays an important role in the activated sludge flora has been changed.

The fact that floc formation is apparently not a special property of any particular group of bacteria explains the ease with which activated sludge is formed in various industrial wastes. A proteinous waste will favour the development of *Alcaligenes, Flavobacterium* and *Bacillus* species, while a waste containing carbohydrates or hydrocarbons tends to favour *Pseudomonas* as well.

LEVINE and SOPPELAND (1926) are considered to be the first workers having made a systematic study of the bacteria active on creamery waste (PORGES, 1960). They found that anaerobiosis favoured the growth of acid-producing bacteria, whereas under aerobic conditions proteolytic forms predominated. When creamery wastes were treated on a trickling filter, acid-producing and acid-decomposing bacteria were shown to develop; however, other types of microorganisms also subsisted (LEVINE and WATKINS, 1932).

A considerable amount of research on aerobic dairy waste treatment has been carried out by PORGES and collaborators. JASEWICZ and PORGES (1956; see also PORGES, 1960) isolated and studied bacteria present during rapid biochemical oxidation of dairy waste. They used a laboratory scale aerator that had been operated for 6 months. It contained 18 litres of a well aerated and agitated sludge skim milk mixture. The supernantant solution was removed

daily after settling of the sludge, and the sludge suspension was fed with 18 grams of dried skim milk. Dilutions were made 3 days after the last skim milk addition, when the bacteria were in the phase of endogenous metabolism. These dilutions were plated in duplicate on a nutrient agar containing 0.1% of skim milk. After 3 days' incubation at 30°C the microorganisms forming colonies on the 10^{-8} dilution plates were isolated, purified and studied morphologically and physiologically. The daily addition of dried skim milk was resumed and several weeks later a sample was removed 4 hours after feeding. The bacteria were then in the assimilative phase. Plate isolations and identification tests were made as before. The results obtained are summarized in table 1.

TABLE 1. Predominant bacteria in skim milk sludges[1].

Genera	In % of total numbers isolated	
	Assimilative phase	Endogenous phase
Achromobacter	–	2
Alcaligenes	–	26
Bacillus	31	8
Bacterium (B. linens)	43 (40)	–
Corynebacterium	6	–
Flavobacterium	8	34
Microbacterium	6	–
Micrococcus	2	14
Pseudomonas	4	16

[1] JASEWICZ and PORGES (1956); PORGES (1960).

3.3. EXPERIMENTAL

3.3.1. *Design of the experiment*

3.3.1.1. Oxidation ditch

The oxidation ditch of the dairy plant in De Wilp (Fig. 3) is a modification of the oxidation ditch described by PASVEER (1959). It has the capacity for a daily treatment of 70 m^3 of dairy waste with a mean BOD of 750 ppm. As the total volume of the ditch is 200 m^3, the mean load amounts to 250 ppm of BOD per day. This is discharged for 8 hours, mainly in the morning. The mean detention time is 3 days. The chemical composition of the waste is determined by the products made at the plant, e.g. butter, cheese and whey powder (Hatmaker).

The ditch functions automatically. When the contents reach a fixed highest level, a contact electrode system stops the inner brush, as a result of which aeration and agitation of the interior section cease. The activated sludge in this section is allowed to settle during a certain period of time (here 1 hour). By opening of a magnetic valve the effluent is drawn off, until the contents of the ditch have reached a fixed minimum level. The electrode system then resumes aeration and agitation in the interior section by slipping in the magnetic clutch

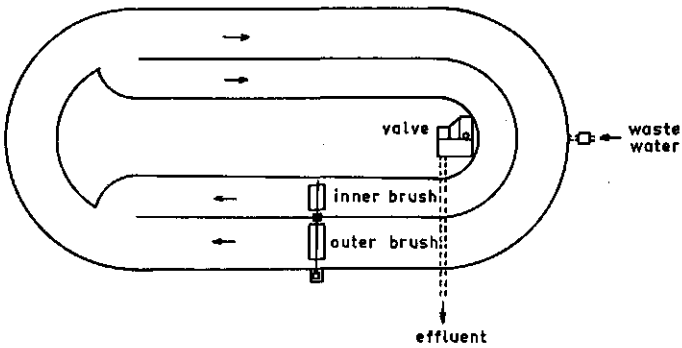

FIG. 3. Schematic diagram of the oxidation ditch in De Wilp. (R.A.A.D.)

that operates the brush. This process is repeated as long as waste water is being discharged by the dairy plant.

3.3.1.2. Laboratory apparatus

The laboratory apparatus consists of a series of Kluyver aeration flasks each containing 1.5 litres of liquid. Artifical dairy waste used in a number of these flasks was prepared from a powder mixture consisting of 3 parts of whey powder (Hatmaker) and 1 part of skim milk powder (spray). A solution of 1 gram of powder mixture per litre has a BOD of approximately 660 ppm (COD of about 1000 ppm). The composition of this artificial dairy waste was equivalent to that of the mean waste of the dairy plant in De Wilp as analysed by the R.A.A.D. laboratory.

3.3.1.3. Design

The experiment was set up as follows:

A. oxidation ditch: direct bacteriological analysis of the activated sludge samples.

B. laboratory apparatus: bacteriological analysis of samples from the aeration flasks containing:

Ia and Ib: natural inoculation material (see under 3.3.2.2.) and artificial dairy waste;

IIa and IIb: activated sludge from De Wilp and artificial dairy waste;

IIIa and IIIb: activated sludge from De Wilp and dairy waste from De Wilp;

IVa and IVb: the first days' contents of the oxidation ditch in De Wilp and dairy waste from De Wilp.

(a and b were duplicate flasks).

3.3.2. *Formation of activated sludge*

3.3.2.1. Oxidation ditch

Activated sludge formation in the oxidation ditch was induced by aeration of a portion of dairy waste added to groundwater. Seeding was considered unnecessary since the waste itself contained many bacteria and the ditch would

be heavily contaminated by soil bacteria. Flocculation was promoted by addition of approximately 5 ppm of $FeSO_4$. The operation was planned by the R.A.A.D. expert. After about 50 days, the amount of activated sludge formed had attained the planned capacity for treating 250 ppm of BOD. The initial load approximated 20 ppm of BOD. Thereafter a regular increase of the load followed until the optimal stage was reached (see under 3.3.2.2.).

3.3.2.2. Laboratory apparatus

The activated sludge formation in the aeration flasks was started as follows: each flask of series I, II and III was supplied with:

i. natural inoculation material consisting of 25 ml of supernatant – after settling – of a suspension of soil and manure in municipal sewage, and approximately 5 ppm of $FeSO_4$ (flasks Ia and Ib),
or: 1 ml of the one week old activated sludge from De Wilp (flasks IIa, IIb, IIIa and IIIb);
ii. substrate giving an ultimate BOD of approximately 20 ppm, consisting of artificial dairy waste (flasks Ia, Ib, IIa and IIb),
or: dairy waste from De Wilp (flasks IIIa and IIIb);
iii. sufficient tap water to make up the final volume of liquid in each of these flasks to 1.5 litre.

The flasks IVa and IVb received 1.5 litre of the contents of the oxidation ditch in De Wilp collected on the first day shortly after the dairy waste and $FeSO_4$ had been dosed. These flasks were supplied with dairy waste from De Wilp as the substrate.

A heavy aeration was applied, so that shortage of oxygen was not to be expected. Parallel with the manipulations in De Wilp, drawing off of supernatant after settling, and dosing of substrate took place in the laboratory apparatus as plotted in fig. 4. The volumes were kept constant at 1.5 litre. The flasks were placed in a cool and dark room. There was a gradual rise of the temperature from initially 15°C to finally 20°C. (The temperature

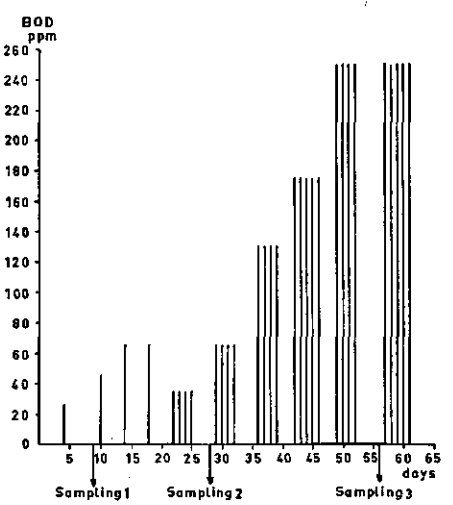

FIG. 4. Schematic representation of dairy waste supply to the oxidation ditch as well as to the laboratory apparatus.

in the oxidation ditch in De Wilp rose gradually from 8 °C to 12 °C in the same period of time.)

After approximately 60 days when the stage of full operation had been reached, purification characters of the activated sludge grown in the laboratory apparatus (flasks Ia and Ib) were tested by estimation of the sludge volume index, the relative stability and the BOD of the effluent after supplying 0.5 g per litre of powder mixture a day previously. The results were: (see 'American Standard Methods', 1955)

sludge volume index:	30.2 ml/g
relative stability: more than	90 %
BOD-reduction:	90.9 %

These data show that a reasonable purification had been obtained. In subsequent experiments the COD test was used for testing the purification effect of the activated sludge. The COD-reduction data were usually approximately 98 %.

3.3.3. *Sampling*

Samples of 10 ml were taken at 9, 28 and 56 days after the start of the experiment. The regular transport of activated sludge samples and dairy waste from De Wilp to the laboratory in Wageningen was organized in cooperation with the R.A.A.D. During the transport the bottles were kept in an ice box. On arrival the activated sludge samples were treated according to the procedure described in the next paragraph. Simultaneously, samples were taken from each flask of the laboratory equipment and treated in the same way.

3.3.4. *Isolation procedure*

Each activated sludge sample was disintegrated for 2 minutes with a Desaga vibro stirrer in order to liberate bacteria from the interior of the floc. The importance of this treatment has been demonstrated by ALLEN (1944).

Preliminary experiments showed that the effect of disintegrating floc material by means of a Desaga vibro stirrer was somewhat less pronounced than the disintegrating effect of a cream maker (homogenizer, 'Bel'), as used by ALLEN. This is demonstrated in table 2.

TABLE 2.

Total count on TGEA-50 % + skim milk	
Cream maker (4 times)	5.5×10^7
Vibro stirrer (2 min)	1.0×10^7

The smaller disintegrating effect is compensated to some extent by the easier sterilization of the vibro stirrer (with alcohol, which subsequently is removed by burning).

Dilution series were set up in sterile water and 0.1 ml portions of these dilutions were streaked in duplicate on TGEA-50 % + skim milk by means of a thin bent glass rod. The plates were incubated for 3 to 4 days at 25 °C.

To illustrate that the strains isolated represented the true activated sludge bacteria, the following experiment was carried out. Dilutions of activated sludge were streaked on TGEA-

50 % + skim milk. After 3 days' incubation at 25 °C a suspension was made of all the bacteria of a plate containing approximately one hundred colonies. Half a litre of a ten times diluted sterile artificial dairy waste was inoculated with this suspension and then aerated. Within one week's incubation sludge formation had started. This sludge was fed once a week, starting with 10 and finishing with 50 ml of a ten times concentrated artificial dairy waste solution. After 4 weeks the sludge was allowed to settle. This took half an hour, the supernatant being clear. The colour and the microscopical appearance of this sludge were identical to those of the original activated sludge, except that the protozoa were absent. From this result it was concluded that the isolated strains were characteristic of the dairy waste activated sludge flora.

Since upon microscopical examination no difference in microbial flora of duplicate plates obtained from the flasks a and b was found to occur, the most regularly occupied plates were chosen for isolation of bacterial strains. The total number of colonies of the selected plates varied between 100 and 500. Sections were taken in such a way that they contained an average number of 50 colonies. The bacterial strains isolated were transferred to TGEA slants and tested for purity by a passage on TGEA + skim milk plates. Subsequently each pure culture was again transferred to a TGEA slant and stored at 5 °C until identification.

3.3.5. *Identification procedure*

The isolated strains were tested for the following morphological and physiological characteristics:
colony type and colour on TGEA + skim milk;
cell form, dimensions, motility, Gram's reaction, spore formation, flagellation; oxygen requirement, catalase activity, reaction in the glucose and lactose media of the Hugh and Leifson test (HUGH and LEIFSON, 1953), decomposition of casein (clearification zone in TGEA + skim milk plates) and gelatin liquefaction (gelatin stab method).
The incubation temperature was 25 °C.

Identification and classification of the cultures into families, and sometimes tribes or genera, were achieved by comparing the results of the tests with the properties listed in SKERMAN's 'Guide to the Identification of the Genera of Bacteria' (1959). The relative low number of isolated yeasts and moulds were not identified.

3.3.6. *Results*

The numbers of bacteria belonging to the various groups were expressed as percentages of the total number of strains isolated and tested per sample (Table 3). Approximately 50 strains per plate were tested.

It appears that there was a striking correlation between A (oxidation ditch) and the flasks B. I, B. II, B. III and B. IV (laboratory apparatus) as to the major groups of bacteria. This indicates that, in spite of the differences in experimental conditions (size of installations, conditions at the start of the experiment and, to some extent, the temperature) the development of the activated sludge flora in both types of experimental design was almost equal. From this it can be

TABLE 3. Microbial composition, in % of the total number of strains tested, at different sampling times, of developing activated sludge from the oxidation ditch A and the laboratory apparatus B.

Organisms isolated	A			B												
				I °)			II °)			III °)			IV °)			
	1[1])	2[2])	3[3])	1	2	3	1	2	3	1	2	3	1	2	3	
Pseudomonadaceae	30.5	20.0	15.4	51.8	31.0	4.6	29.6	15.4	2.8	44.1	38.1	2.9	50.0	25.0	8.5	
Chromobacterium	19.1	–	–	–	–	–	–	–	–	–	2.4	–	2.9	2.5	–	
Achromobacteraceae	41.5	22.5	23.1	41.3	59.5	15.9	34.1	41.0	8.3	39.7	21.4	22.8	8.8	27.5	42.8	
Enterobacteriaceae	–	2.5	–	–	–	9.1	–	7.7	2.8	–	–	2.9	–	2.5	2.9	
Corynebacteriaceae	4.5	40.0	61.5	6.9	4.7	54.5	13.6	28.2	80.5	2.3	28.6	60.0	23.6	42.5	40.0	
Brevibacterium	–	2.5	–	–	–	4.6	–	–	–	–	–	–	–	–	–	
Bacillus	2.2	–	–	–	–	–	2.3	–	2.8	–	–	5.7	–	–	2.9	
Streptococceae	2.2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	
Micrococcaceae	–	–	–	–	–	–	–	–	2.8	–	–	–	14.7	–	2.9	
Yeasts	–	12.5	–	–	2.4	6.8	15.9	5.1	–	4.6	7.1	5.7	–	–	–	
Moulds	–	–	–	–	2.4	4.5	4.5	2.6	–	9.3	2.4	–	–	–	–	

°) Flasks containing:

I: Natural inoculation material + artificial dairy waste;
II: Activated sludge from De Wilp + artificial dairy waste;
III: Activated sludge from De Wilp + dairy waste from De Wilp;
IV: First day's contents of the oxidation ditch in De Wilp + dairy waste from De Wilp.

[1]), [2]) and [3]): Samples taken 9, 28 and 56 days, respectively, after starting the experiment.

concluded that the bacterial composition of the activated sludge depended on the chemical composition of the dairy waste.

The major groups of bacteria in this experiment were found to consist of representatives of the *Pseudomonadaceae*, the *Achromobacteraceae* and the *Corynebacteriaceae* (*Brevibacterium* included). Other types of microorganisms never formed a high percentage of the total number of isolates, except *Chromobacterium* which occurred in appreciable numbers in the first sample of the oxidation ditch A.

If a comparison is made of the composition of the activated sludge flora at different sampling times, it appears that initially the majority of the isolated strains belonged to the *Pseudomonadaceae* and *Achromobacteraceae*. After one month, and more clearly after two months the *Achromobacteraceae* and still more the *Pseudomonadaceae* had decreased in numbers, whereas the *Corynebacteriaceae* had become the predominant group.

3.3.7. *Reproducibility of the classification*

To ascertain the reproducibility of the results, treatment I of the abovementioned experiment was repeated one year later. The procedure employed was the same as before. The results of this second analysis, given in table 4, and based upon the isolation and examination of approximately 300 strains of bacteria and a few yeasts and moulds are in good agreement with those of the preceding year.

TABLE 4. Microbial composition of developing activated sludge from the laboratory experiment. Replication of the experiment carried out a year previously (see table 3: B. I). Data are given in % of the total number of strains tested; a and b are duplicate flasks. Samples were taken 9 (1), 28 (2) and 56 (3) days after the start of the experiment.

Organisms isolated	a			b		
	1	2	3	1	2	3
Pseudomonadaceae	34.9	18.7	6.1	38.8	4.0	4.0
Chromobacterium	–	–	–	2.0	–	–
Achromobacteraceae	41.8	50.0	20.5	38.8	36.0	10.0
Enterobacteriaceae	2.0	–	–	2.0	2.0	–
Corynebacteriaceae	8.3	22.9	59.3	18.4	58.0	74.0
Brevibacterium	2.0	2.1	2.0	–	–	–
Micrococcaceae	–	2.1	2.0	–	–	–
Caulobacter	–	–	2.0	–	–	4.0
Yeasts	–	–	6.1	–	–	8.0
Moulds	2.0	4.2	2.0	–	–	–

3.3.8. *Analysis of the bacterial flora before and 4 hours after feeding*

This experiment was performed to investigate if a difference exists between the bacterial composition of the sludge flora of the endogenous phase and that of the assimilative phase, as claimed by JASEWICZ and PORGES (1956).

The experimental procedure was the same as in previous experiments. The substrate used was artificial dairy waste, consisting of 1 gram of powder mixture per litre of sludge suspension. The results, based upon approximately 400 strains tested, are given in table 5. It will be seen that no significant difference in bacterial flora occurred between the endogenous and assimilative phases.

TABEL 5. Microbial composition, in % of the total number of strains tested, of the activated sludge in the endogenous and in the assimilative phase, respectively; a and b are duplicate flasks; samples were taken 28 (1) and 56 (2) days after the start of the experiment.

Organisms isolated	1				2			
	a		b		a		b	
	End.	Ass.	End.	Ass.	End.	Ass.	End.	Ass.
Pseudomonadaceae	18.7	26.0	4.0	10.0	6.1	4.1	4.0	–
Achromobacteraceae	50.0	54.0	36.0	12.0	20.5	10.0	10.0	–
Enterobacteriaceae	–	4.0	2.0	2.0	–	–	–	–
Corynebacteriaceae	22.9	12.0	58.0	70.0	59.3	83.8	74.0	79.5
Brevibacterium	2.1	2.0	–	–	2.0	–	–	2.1
Mycobacteriaceae	–	–	–	2.0	–	–	–	2.0
Bacillus	–	–	–	–	–	–	–	2.1
Micrococcaceae	2.1	–	–	–	2.0	2.1	–	–
Sarcina	–	–	–	–	–	–	–	2.0
Caulobacter	–	–	–	–	2.0	–	4.0	2.1
Yeasts	–	2.0	–	2.0	6.1	–	8.0	8.2
Mould	4.2	–	–	2.0	2.0	–	–	2.0

3.3.9. *Examination of the isolated coryneform bacteria for* Arthrobacter *characteristics*

Microscopical examination of the isolated coryneform bacteria did suggest that many of the tested strains might belong to the genus *Arthrobacter*. This was checked by cultivating them for 16 hours on a rich (tryptone glucose extract agar + skim milk) and for 5 days on a poor (casein agar) medium. When an isolate mainly produced rods on the rich and spheres on the poor agar, it was assumed to belong to the *Arthrobacter*-like bacteria. It appeared that 85% of the tested strains of coryneform bacteria should be classified as *Arthrobacter*-like microorganisms.

3.3.10. *Physiological characteristics of the isolated bacteria*

The results of the physiological tests employed in the identification procedure are given in table 6. The data obtained from the Hugh and Leifson test are given in combination with the results of the protein decomposition tests.

TABLE 6. Percentage of bacteria giving an acid, alkaline or neutral reaction on glucose only (G), lactose only (L) or on glucose as well as on lactose (G + L), respectively, when tested according to the Hugh and Leifson technique. Each distribution is given for microorganisms decomposing gelatin only (Gel), casein only (Cas), gelatin as wel as casein (Gel + Cas), respectively, or being non-proteolytic.

Protein decomposition [1]		Hugh and Leifson technique (aerobic)									Tot.
		Acid reaction when tested on:				Alkaline reaction when tested on:				Neutral	
		G	G+L	L	Tot. acid	G	G+L	L	Tot. alk.	G+L	
Sample 1. [2]		(1)	(2)	(3)	(1,2,3)	(4)	(5)	(6)	(4,5,6)	(7)	(1,2,3,4, 5,6,7)
Gelatin	(1)	2.5	5.6	1.0	9.1	–	0.5	2.0	2.5	3.0	14.6
Gel + Cas	(2)	9.1	4.0	0.5	13.6	0.5	4.0	5.6	10.1	13.6	37.3
Casein	(3)	1.0	1.0	–	2.0	–	–	1.0	1.0	3.5	6.5
Gel and/or Cas	(1,2,3)	12.6	10.6	1.5	24.7	0.5	4.5	8.6	13.6	20.1	58.4
No Gel nor Cas	(4)	6.6	5.6	–	12.2	0.5	7.6	6.6	14.7	14.7	41.6
Total	(1,2,3,4)	19,2	16.2	1.5	36.9	1.0	12.1	15.2	28.3	34.8	100
Sample 2.											
Gelatin	(1)	1.6	2.1	–	3.7	–	–	1.6	1.6	19.6	24.9
Gel + Cas	(2)	3.2	1.6	0.5	5.3	–	–	3.7	3.7	21.1	30.1
Casein	(3)	–	1.6	0.5	2.1	–	–	–	–	4.3	6.4
Gel and/or Cas	(1,2,3)	4.8	5.3	1.0	11.1	–	–	5.3	5.3	45.0	61.4
No Gel nor Cas	(4)	2.6	9.0	0.5	12.1	–	6.9	1.6	8.5	18.0	38.6
Total	(1,2,3,4)	7.4	14.3	1.5	23.2	–	6.9	6.9	13.8	63.0	100
Sample 3.											
Gelatin	(1)	2.6	–	–	2.6	–	0.5	1.6	2.1	10.6	15.3
Gel + Cas	(2)	1.1	2.1	1.1	4.3	0.6	–	0.5	1.1	21.7	27.1
Casein	(3)	0.5	–	–	0.5	–	–	0.5	0.5	1.1	2.1
Gel and/or Cas	(1,2,3)	4.2	2.1	1.1	7.4	0.6	0.5	2.6	3.7	33.4	44.5
No Gel nor Cas	(4)	4.3	7.9	1.0	13.2	0.5	4.3	3.7	8.5	33.8	55.5
Total	(1,2,3,4)	8.5	10.0	2.1	20.6	1.1	4.8	6.3	12.2	67.2	100

[1] Decomposition of gelatin: liquefaction of gelatin; decomposition of casein: halo formation in TGEA + skim milk.
[2] Samples were taken 9 (1), 28 (2) and 56 (3) days after the start of the experiment.

In the successive samples (1, 2 and 3) a decrease of the percentage of acid-forming bacteria was observed (36.9 – 23.2 – 20.6), particularly on the glucose medium (19.2 – 7.4 – 8.5) (see table 6). A similar decrease of alkali-producing bacteria was found to occur (28.3 – 13.8 – 12.2). Consequently bacteria giving no acid or alkaline reaction in the Hugh and Leifson test, increased considerably in the course of the activated sludge formation (34.8 – 63.0 – 67.2). Absence of a pH-shift in the Hugh and Leifson test may be due to a complete respiration of the carbohydrates, or to the production of acid and alkali in equivalent amounts.

The relative numbers of non-proteolytic bacteria increased slightly with ageing of the activated sludge (41.6 – 38.6 – 55.5) (see table 6). This is in contrast to the observations of ALLEN (1944) who found an increase of proteolytic bacteria with ageing domestic activated sludge.

The relative decrease of proteolytic strains upon ageing of the dairy waste activated sludge presumably depended on the replacement of bacteria of the *Pseudomonadaceae* and *Achromobacteraceae* by *Arthrobacter*-like microorganisms. The physiological characteristics of the isolated strains are summarized in tabel 7.

A survey of the principal reactions is given in table 8.

TABLE 7. Some mean characteristics of the isolates classified into the three major groups of bacteria, expressed as % of the total numbers of strains per group tested.

Microorganisms:	Positive reaction on:		No proteolytic activity	Reaction on the media of Hugh and Leifson:		
	Gelatin	Casein		Acid	Neutral	Alkaline
Pseudomonadaceae	49	36	48	54	25	21
Achromobacteraceae	60	43	37	26	61	13
Coryneform bacteria	28	20	72	10	87	3

TABLE 8. Survey of the principle reactions given by bacteria isolated from dairy waste activated sludge in 3 different stages of development. Samples taken 9 (1), 28 (2) and 56 (3) days, respectively, after starting the experiments.

Characters	% of tested bacteria		
	1	2	3
Hugh and Leifson test:			
acid	36.9	23.2	20.6
alkaline	28.3	13.8	12.2
neutral	34.8	63.0	67.2
Gelatin liquefaction and/or casein hydrolysis	58.4	61.4	44.5
Gram's reaction:			
negative	80.1	66.0	27.3
positive or variable	19.9	34.0	72.7
Motility	50.0	33.3	27.2

Activated sludge from dairy waste is characterized by an orange-brown colour which is apparently due to the presence of a particular type of microorganism. In the course of the present investigations it was attempted to discover which bacteria present in the dairy waste activated sludge are responsible for this orange-brown colour.

Of the 1200 bacterial strains isolated and tested in the preceding experiments, approximately 10% gave ochreous, orange or brown colonies on TGEA + skim milk. Of these coloured strains 78.5% belonged to the *Achromobacteraceae* (mostly *Flavobacterium*), 12.5% to the *Pseudomonadaceae* and only 9% to the coryneform bacteria. The majority of the *Arthrobacter*-like bacteria were cream-coloured or white. From this it may be assumed that the orange-brown colour of dairy waste activated sludge was due to the group of the *Achromobacteraceae* and more particularly to the Flavobacteria, and not to the predominant group of coryneform bacteria.

3.4. Discussion

The results of the reported experiments point to a distinct similarity in bacteriological composition of the activated sludge formed in a newly established oxidation ditch fed with dairy waste water and that developed in the laboratory apparatus under comparable nutritional conditions. These results justify the conclusion that the described equipment for laboratory scale studies is adequate to study certain aspects of the purification of dairy waste water.

When the strains isolated from each sample are classified into 3 major groups, viz. *Pseudomonadaceae*, *Achromobacteraceae* and coryneform bacteria, and a rest group, it will be seen that alterations of the bacterial flora in the laboratory flasks and in the oxidation ditch in De Wilp proceeded along the same lines (Fig. 5). In the initial phase – first sampling – *Pseudomonadaceae* and *Achromobacteraceae* were present in predominant numbers, each of these groups occurring in approximately equal proportions. In this stage representatives of the coryneform bacteria were found in considerably lower numbers. At the time of the second sampling a decrease of the *Pseudomonadaceae* in favour of an increase of the coryneform bacteria had taken place. Finally – at the third sampling – the sequence of groups had altered largely in favour of the coryneform bacteria. The *Pseudomonadaceae* at this stage showed the main decline but the same was true, though on a smaller scale, of the *Achromobacteraceae*.

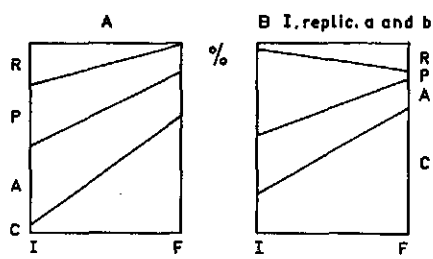

FIG. 5. Comparison of the initial and final composition of the activated sludge flora of the oxidation ditch A and that of the average values of the laboratory experiment B. I (see table 3) and the replications a and b (see table 4) expressed in % of the total numbers of isolated strains tested. R = restgroup; P = *Pseudomonadaceae*; A = *Achromobacteraceae*; C = coryneform bacteria; I = initial compostion; F = final composition.

A closer examination of the bacterial strains showed that the coryneform bacteria isolated comprised 85% of *Arthrobacter*-like bacteria.

The observed alteration in bacteriological composition of the activated sludge runs parallel with a decrease of the percentage of motile strains. It is in agreement with a shift within the tested strains from predominantly Gram-negative to predominantly Gram-positive or Gram-variable.

The results obtained in these experiments led to the conclusion that in stabilized activated sludge derived from dairy waste water as used in the present study, *Arthrobacter*-like bacteria play a predominant role.

The adaptation of the bacterial flora to the dairy waste, as demonstrated by the alterations of the major groups of bacteria, is a slow process.

The presence of *Pseudomonadaceae* and *Achromobacteraceae* in activated sludge, adapted to municipal sewage, has already been observed (ALLEN, 1944; MCKINNEY, 1962). VAN GILS (1964) found the *Achromobacteraceae* to be predominant, while DIAS and BHAT (1964) observed predominant numbers of *Zoogloea* and *Comamonas*, and ANDERSON and MCCOY (1963) a majority of *Pseudomonas* species. JASEWICZ and PORGES (1956) and PORGES (1960) described the presence of *Pseudomonadaceae* and *Achromobacteraceae* in activated sludge from a dairy waste, when the sludge was in its endogenous phase. Isolations made when the sludge was in its assimilative phase – several weeks later – showed a predominance of *Bacillus* and *Bacterium* species. The latter were largely classified as *Bacterium* (= *Brevibacterium*) *linens*.

These results were not confirmed by the present experiments. Samples taken from the sludge (a) in its endogenous phase, and (b) 4 hours after substrate had been supplied, showed no significant difference in the composition of the bacterial flora of the sludge before and after feeding. Bacilli were never found to be present in appreciable numbers, although no special precautions were taken to exclude air infection; however, the flasks did have a cover.

The large amount of *Arthrobacter*-like bacteria found in the dairy waste activated sludge of the present investigations, compared with the rather high percentage of *Bacterium* (=*Brevibacterium*) *linens* mentioned by JASEWICZ and PORGES (1956) might suggest that a different name is given to a similar type of bacterium. This hypothesis may be supported by the recent finding that *Brevibacterium linens* is a coryneform bacterium of the *Arthrobacter* type (MULDER et al., 1966). It must be stressed, however, that *Brevibacterium linens* forms orange-coloured colonies on most media, whereas *Arthrobacter*-like bacteria isolated in the present investigations formed cream-coloured or white colonies. From the description of JASEWICZ and PORGES it cannot be concluded that their *Bacterium linens* had orange-coloured colonies and had the morphology of an *Arthrobacter*.

3.5. SUMMARY

A comparison of the bacterial flora of dairy waste activated sludge developing from the initial to the optimal stage in an oxidation ditch and in a laboratory

apparatus, showed a close similarity between both systems, so that the latter may be used for representative experiments.

Adaptation of the bacterial flora to the supplied waste was found to be a slow process. The composition of the flora altered with time until ultimately it consisted predominantly of coryneform (*Arthrobacter*-like) bacteria. Next to these, considerable amounts of *Achromobacteraceae* were found. *Pseudomonadaceae*, initially present in large numbers, decreased to the least important of the three major groups of bacteria in the ultimate flora.

The alteration of the bacterial groups coincided with an alteration of the physiological characteristics of the total activated sludge flora. In the final stage two thirds of the isolated bacteria gave a neutral reaction on Hugh and Leifson media and more than half were non-proteolytic. This result is in agreement with the predominating position held by the group of coryneform (particularly *Arthrobacter*-like) bacteria.

The brown colour of dairy waste activated sludge is attributed to Flavobacteria.

CHAPTER 4

SOME CHARACTERISTICS OF REPRESENTATIVES OF THE MAJOR GROUPS OF BACTERIA OF DAIRY WASTE ACTIVATED SLUDGE

4.1. INTRODUCTION

The purpose of the experiments described in this chapter was to study factors thought to be responsible for the alteration of the microflora of developing dairy waste activated sludge. The experiments were carried out with representative strains of the three major groups of bacteria found in this type of activated sludge, viz. an *Arthrobacter*, an *Achromobacter* and a *Pseudomonas* species.

It can be assumed that the substrate used is one of the primary factors affecting the microbial flora of the sludge. Therefore growth rates, yields, oxygen uptake rates and growth in mixed cultures of the three representatives were studied in relation to the characteristics of the substrate.

4.2. GROWTH RATES IN PURE CULTURES

For this examination the bacteria were grown in aeration flasks containing 700 ml of heavily aerated artificial dairy waste water at 25°C. The artificial dairy waste water consisted of 1 gram of powder mixture per litre similar to that used in preceding experiments (see section 3.3.1.2.). The growth rates were derived from total viable cell counts on TGEA made at regular intervals. The plates were incubated for 3 days at 25°C. Counts were made in triplicate. Pure cultures of an *Arthrobacter*, an *Achromobacter* and a *Pseudomonas* species, 24 hours old, grown on TGEA slopes, were used for inoculation. The bacterial suspensions were standardized nephelometrically.

The mean growth rates were calculated from the exponential phase of the growth curves, as plotted in fig. 6. It will be seen that *Arthrobacter* was growing faster than the *Pseudomonas* and *Achromobacter* species, their respective mean generation times in this particular medium being 2.5, 3.0 and 3.3 hours.

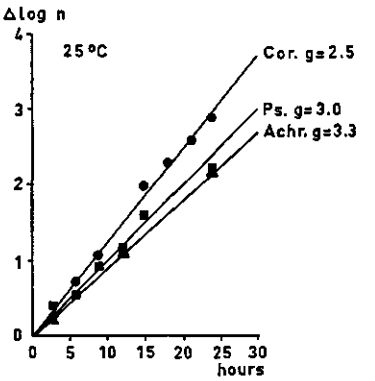

FIG. 6. Growth rates in pure cultures of a coryneform (*Arthrobacter*-like) bacterium (Cor.), an *Achromobacter* species (*Achr.*) and a *Pseudomonas* species (Ps). and their respective generation times, when grown on artificial dairy waste water at 25°C.

4.3. Relative growth in mixed culture

The relative growth of the three types of bacteria was determined as in previous experiments at 25°C in aeration flasks with artificial dairy waste as the substrate. Concentrated suspensions of the three bacteria of almost equal densities were used for inoculation purposes. Samples were taken at the start of the experiment and after 8, 24, 32 and 48 hours' incubation at 25°C. Dilution series were made for plate counts. For this procedure the spot plate count method was used; counts were made using a Wild-M5 stereomicroscope. Colonies were differentiated according to their morphological characters on this particular medium.

The results of this experiment, plotted in fig. 7, show that during the first 8 hours the *Achromobacter* species grew more rapidly than the *Pseudomonas* and the *Arthrobacter* species. From the 8th to the 24th hour both the *Achromobacter* and the *Pseudomonas* increased only slightly, and after 24 hours there was a pronounced drop in their numbers. This was in sharp contrast to the *Arthrobacter* species which continued to increase in number until the 40th hour, when it outnumbered both other species to a large extent. This different behaviour may partly have been due to the transformation of the *Arthrobacter* species from the rod form into cocci, a phenomenon frequently occurring when one or more components of the medium become limiting. This transformation brings about an increase of the number of viable cells without a simultaneous increase of cell dry weight.

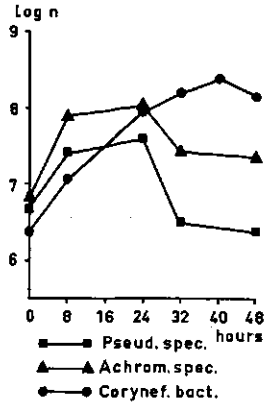

Fig. 7. Relative growth in mixed culture of an *Arthrobacter* (coryneform bacterium), an *Achromobacter* and a *Pseudomonas* species. Substrate: artificial dairy waste water; temperature 25°C.

The following experiment carried out with a pure culture of *Arthrobacter* may illustrate this conception. In this case numbers of viable cells and dry weights in mg both per ml of suspension were determined and plotted (Fig. 8).

It will be seen that the maximum value of cell dry weight was reached earlier than that of the viable cell counts. As a result of this the number of viable cells was still increasing at a time that cell dry weight was decreasing. The conversion of rods into cocci was also observed microscopically.

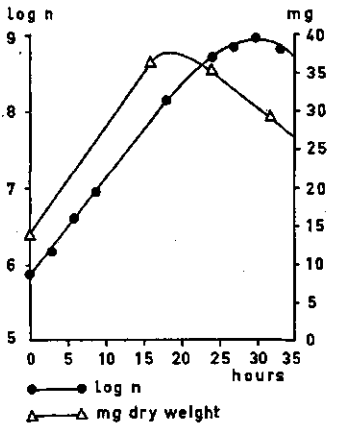

FIG. 8. Growth curve and the corresponding yields of cell dry weight of *Arthrobacter*, when grown in artificial dairy waste at 25°C.

The differences between the growth curves of the three types of bacteria when grown in mixed and in pure cultures, respectively, may be explained by the existence of mutual influences among the three species.

4.4. ACCUMULATION OF POLYSACCHARIDES

It is known that many bacteria of the *Arthrobacter* type possess the ability to synthesize and accumulate large amounts of polysaccharides (MULDER et al., 1962; ZEVENHUIZEN, 1966). These polysaccharides can partly be used as a supplementary source of energy after depletion of the exogenous substrate, and this may contribute to the survival of the microorganisms. This accumulation of polysaccharides, therefore, may be of interest when considering the relation between groups of bacteria in a mixed population such as activated sludge.

Other storage compounds may be accumulated instead of or in addition to polysaccharides. Synthesis and accumulation of poly-β-hydroxybutyrate by *Pseudomonas* species has been described, although this ability appeared to be restricted mainly to plant and maybe animal pathogens (MORRIS and MORRIS, 1959; HAYWARD et al., 1959). VAN GILS (1964) in experiments with activated sludge found the formation of poly-β-hydroxybutyrate to be of minor importance. The same was found for lipids. PORGES, JASEWICZ and HOOVER (1955) found polysaccharides to be the storage compounds in activated sludge bacteria grown in dairy waste water.

In the present experiments only accumulation of polysaccharides was examined. These compounds were estimated as total carbohydrates according to the method of WHISTLER and WOLFROM (1962). Experiments were carried out with the three above-mentioned strains representing the major groups of bacteria found in dairy waste activated sludge. A solution of 1% of lactose and 0.4% of casitone was used as a substrate with a moderately high C/N ratio, which may favour synthesis and accumulation of polysaccharides. The experiments were performed in 300-ml flasks containing 100 ml of medium. The flasks were inocul-

ated with dilute suspensions of the respective bacteria and placed on a rotary shaker at 25°C. After 48 hours of incubation the contents of the flasks were separated by centrifugation and analysed for excess of lactose in the supernatant. The sediment was washed twice and used for determination of dry weight and lactose content of the cellular material.

The results of this experiment (Table 9) show that the total carbohydrate content of *Arthrobacter* was markedly higher than that of both other types of bacteria. This was undoubtedly due to the pronounced ability of synthesizing and accumulating polysaccharides by *Arthrobacter* types as compared with the *Achromobacter* and *Pseudomonas* species.

TABLE 9. Accumulation of polysaccharides (in quadruplicate).

Microorganism:	mg/ml dry weight produced:	Total carbohydrates in % of dry weight:
Arthrobacter sp.	10.17	49.3
	10.71	50.4
	9.85	52.0
	8.65	55.1
Achromobacter sp.	2.11	8.1
	2.64	9.8
	1.71	8.3
	1.58	6.1
Pseudomonas sp.	3.62	13.3
	3.55	13.0
	2.49	12.6
	2.19	13.1

4.5. GROWTH YIELDS

Growth yields of the three types of bacteria were determined by growing them separately in aeration flasks containing artificial dairy waste water (1 gram of powder mixture per litre) as the substrate. The incubation temperature was 25°C. Washed cells, 24 hours old, were used as inoculation material. Samples were taken at the start of the experiment and after 24 hours. The samples were separated by centrifugation and the supernatants used for COD determinations according to the reflux method. The sediments were washed twice and then cell dry weights were determined. The growth yields were calculated as the production of cell dry weight per unit of COD consumed (see table 10).

The efficiency of converting the artificial dairy waste into cell material, as is demonstrated by this growth yield, was slightly higher with *Arthrobacter*. Growth rate, the increase of cell dry weight in 24 hours, was appreciable higher in the case of *Arthrobacter*, indicating a much higher cell activity. This is in agreement with the results of section 4.2. (Fig. 6).

TABLE 10. Growth yields of three representative bacteria of dairy waste activated sludge with artificial dairy waste as the substrate.

Microorganisms:	Dry cell yield in mg/l:	COD consumed in mg/l:	Growth yield:
	(A)	(B)	(A)/(B)
Arthrobacter species	157	358	0.44
	173	361	0.48
Achromobacter species	101	282	0.36
	105	262	0.40
Pseudomonas species	102	257	0.40
	76	181	0.42

4.6. OXYGEN UPTAKE RATES

Oxygen uptake rates of the three bacterial types were determined in a series of Warburg experiments. The microorganisms were precultivated on TGEA slopes for 48 hours at 25 °C, harvested, washed with distilled water, and suspended in a buffer solution, containing approximately 200 mg of NH_4Cl per litre. The suspensions were aerated for 15 hours, approximating the conditions to which bacteria of activated sludge were subjected when applying shock-loading procedures (see chapters 6 and 7). Immediately before use in the Warburg experiments the cells were washed once more and subsequently suspended in distilled water. For these experiments 0.5 ml of cell suspension (cell dry weights derived from extinction values) was mixed with 0.5 ml of a

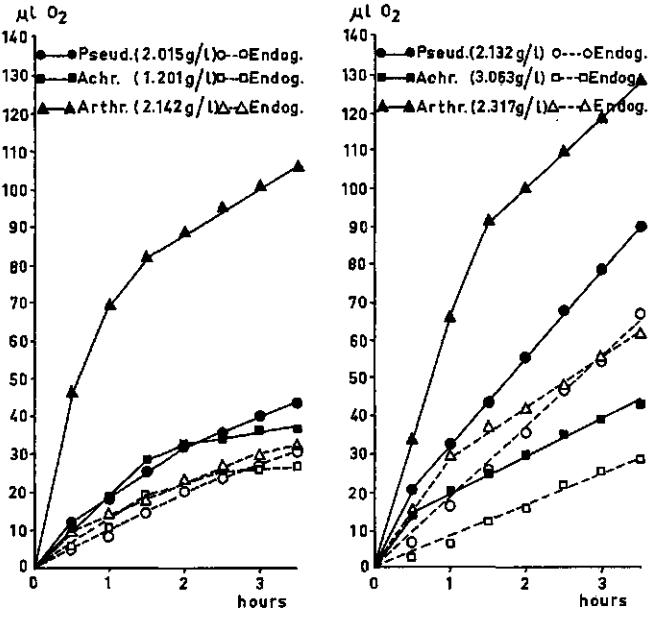

FIG. 9. Oxygen uptake rates at 25 °C of washed suspensions of an *Arthrobacter*, an *Achromobacter* and a *Pseudomonas* species, respectively, after addition of artificial dairy waste. The bacterial suspension had been submitted to a preceding aeration for 15 hours in the presence of NH_4Cl (in duplicate).

buffer solution, containing $Na_2HPO_4.12H_2O$, 2.5 g; KH_2PO_4, 0.4 g; $MgSO_4.7H_2O$, 0.05 g; $FeSO_4.7H_2O$, 0.01 g; $CaCl_2.2H_2O$, 0.005 g, and traces of $MnCl_2.4H_2O$ and $Na_2MoO_4.2H_2O$ in 1000 ml of distilled water. Artificial dairy waste (2.4 g of powder mixture per litre) was used as the substrate, 0.3 ml being added per vessel. The inner well was supplied with 0.2 ml of a 20% KOH solution. The experiments were carried out at 25°C in a Braun-P85 Warburg apparatus, operating at maximum amplitude and at second maximum speed.

The results of these experiments (Fig. 9) show that after the addition of substrate remarkably lower values for oxygen uptake rate were obtained with the *Pseudomonas* and *Achromobacter* species than with the *Arthrobacter* species. The latter, after the period without food supply, had maintained a higher activity. This undoubtedly was due to accumulation of large amounts of polysaccharides (see table 8), subsequently serving as an endogenous source of energy supply.

4.7. Discussion

The experiments described in this chapter deal with a number of physiological characteristics of three strains of bacteria representing the three major groups of microorganisms in the dairy waste activated sludge, viz. an *Arthrobacter*, a *Pseudomonas* and an *Achromobacter* species. Although different strains of these three major groups may possess deviating characteristics, the deviations between different strains belonging to the same group are assumed to be much less pronounced than those between strains belonging to different groups. Evidence as to support this assumption may be derived from the extensive investigations on *Arthrobacter* of Mulder and Antheunisse (1963) and Mulder et al. (1966), who found much conformity in major characteristics such as synthesis and accumulation of polysaccharides, utilization of inorganic nitrogen compounds, etc., between different strains of *Arthrobacter*. As to the *Pseudomonas* and *Achromobacter* strains conformity within the groups is slight, although deviations between the groups are more pronounced (Shewan, Hobbs and Hodgkiss, 1960), especially when selectivity of the nutritional conditions are taken into account.

The results of the experiments with pure cultures of the representative strains of the three groups of bacteria, as well as those with mixed cultures of these strains, generally are in good agreement with the observed predominant position held in the activated sludge by bacteria of the *Arthrobacter* type. This holds for growth rate (when compared as pure culture with the strains of the *Pseudomonas* and *Achromobacter* types) as well as for survival when cultivated in a mixed culture with the two other bacteria.

The favourable position of the *Arthrobacter* type of microorganism in aged mixed cultures may partly be attributed to its conversion from the rod into the coccus form. This phenomenon, however, can only to some extent explain the predominant position the *Arthrobacter* species finally held in the mixed culture and in the dairy waste activated sludge. It therefore must be assumed

that two further characters found to distinguish *Arthrobacter* from *Pseudomonas* and *Achromobacter*, viz. the ability to synthesize and to accumulate large amounts of polysaccharides, and the somewhat higher growth yield values of the *Arthrobacter* type are more responsible for its predominance. The high content of storage compounds of *Arthrobacter*, as contrasted to that of the other two bacteria, may enable the former to maintain a higher metabolic activity for a considerable amount of time after the exogenous substrate has been consumed. As a result of this *Arthrobacter* is expected to resume a ready uptake and utilization of nutrients when after a period of interruption a new amount of substrate is supplied.

4.8. Summary

Representative species of the three major groups of bacteria in dairy waste activated sludge were taken from the isolates collected from this type of sludge. These included one strain of the *Arthrobacter* type, one of the *Pseudomonas* and one of the *Achromobacter* group. The three strains were examined for a number of properties which were thought to be important in determining their relative positions in the sludge.

This examination revealed that the *Arthrobacter* species was the most active and efficient of the three types, having the shortest generation time in pure culture. In a mixed culture of the three bacteria, *Arthrobacter* grew somewhat more slowly but it continued its growth for a considerably longer period so that after 40 hours' incubation it largely outnumbered both other species. This was supposed to be due partly to the conversion of rods into cocci, and partly to the ready accumulation of polysaccharides which were available as a source of energy when the original exogenous substrate had been consumed. The other two species showed hardly any accumulation of polysaccharides.

The growth yield, calculated as mg cell dry weight produced per mg of COD removed, was slightly higher in the case of the *Arthrobacter* species.

Endogenous respiration after an aeration period without substrate supplied, was slightly higher in *Arthrobacter* as compared with the other two species, but the oxygen uptake rate was found to be much higher in the case of *Arthrobacter* upon the addition of artificial dairy waste.

These results are in agreement with the ultimate predominant position of the group of *Arthrobacter*-like bacteria in the dairy waste activated sludge.

CHAPTER 5

RESPONSE OF pH AND DISSOLVED OXYGEN OF AN ACTIVATED SLUDGE SUSPENSION TO DIFFERENT EXPERIMENTAL CONDITIONS

5.1. Introductory experiments in batches and in a continuously fed cascade apparatus

In the following experiments dairy waste activated sludge was used from a stock culture at 25 °C that was supplied daily with artificial dairy waste consisting of a mixed skim milk powder whey powder solution (see section 3.3.1.2.) as the substrate. The pH of the solution had been adjusted to pH 7. Before the substrate was added to the activated sludge suspension (1 gram of powder mixture to 4 – 5 grams of sludge dry weight per litre) the flocs were allowed to settle for 30 minutes and then the effluent was drawn off. The bacterial composition of this sludge was similar to that of the stabilized activated sludge described in the preceding chapter.

In long-term experiments with activated sludge in batches (fill and draw system) supplied daily with concentrated artificial dairy waste, the pH of the suspension was found to be rather constant at values between 7 and 8, so that no pH corrections had to be made. The measurements of the pH were carried out daily at a fixed time. These observations were in contrast with experiments in a continuously fed cascade apparatus (Fig. 10), in which clear fluctuations of the pH in the respective flasks were noticed.

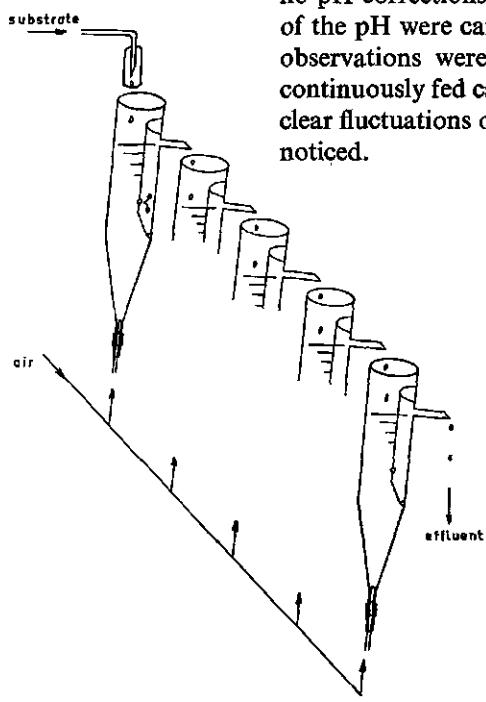

Fig. 10. Cascade apparatus consisting of 5 aeration flasks, each of them containing a settling compartment with an overflow for the effluent.

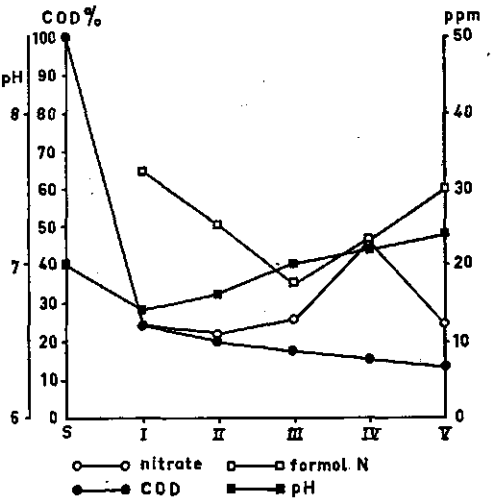

FIG. 11. Analyses of the effluent of the respective flasks of the continuously fed cascade apparatus. S = substrate (influent); I, II, III, IV and V are the respective flasks (effluent).

The cascade apparatus consisted of a series of 500 ml aeration flasks containing an activated sludge suspension (approximately 0.4 gram of sludge dry weight per litre per flask). Every flask contained a sedimentation section with an overflow for retaining the sludge as the supernatant dropped into a subsequent flask. The apparatus was originally designed for differentiating the bacterial processes engaged in the decomposition of the artificial dairy waste, localizing the different types of microorganisms in different flasks. The separation of microorganisms was difficult to attain, since overflowing of the activated sludge could not be prevented, yet a certain differentiation of the processes taking part in the decomposition of the artificial dairy waste was obtained. The results of an experiment in which the artificial dairy waste was fed continuously at a rate of 75 ml per hour are plotted in fig. 11.

It will be seen that the pH value of flask I was lowest, presumbly due to the formation of organic acids. The rise of the pH in the subsequent flasks may be attributed (a) to the oxidation of organic acids and (b) to the deamination of amino acids. However, ammonia was detected only in traces, apparently owing to its rapid nitrification. The rise of amino acid nitrogen (formol titration values of protein-free effluents) in flask IV and V presumably was due to protein decomposition. This presumption is supported by the results of subsequent experiments in which the protein fraction of the dairy waste was found to be decomposed at a markedly lower rate than the carbohydrate fraction.

5.2. THE COURSE OF pH AND DISSOLVED OXYGEN IN BATCH EXPERIMENTS

Experiments in which the course of the pH and of dissolved oxygen (DO) were measured at intervals of 0.5, 1.0 or 5 minutes using a pH meter and an oxygen electrode equipment are recorded below. In subsequent experiments a recorder was used for continuous registration of both values. A special aeration

flask developed for this type of experiments has been described in section 2.3.2. The measurements were continued until the values were almost constant. The aeration flask was placed in an incubator at 25°C.

An activated sludge suspension was supplied with an amount of artificial dairy waste (see 3.3.1.2.) up to 1 gram of powder mixture (COD approximately 1000 ppm) per 4 to 5 grams of sludge dry weight per litre of suspension. Aeration was kept constant at a rate of 20 litres of air per hour. Fig. 12 illustrates the results of such an experiment. The curves of this graph show a rapid removal of substrate accompanied by the production of acid intermediates. The sharpest decrease of DO occurred immediately after feeding the sludge. During this phase the demand for oxygen temporarily surpassed the amount absorbed. When the rate of COD reduction decreased, the pH curve indicated a turning point. As is demonstrated more accurately in experiments with less substrate supplied (Fig. 20), the DO curve too has a turning point after an equal period of time. After the greater part of the substrate has been removed from the liquid phase, the demand for oxygen decreases and after a certain lapse of time the oxygen supply exceeds the oxygen demand again, as is shown by an increase of the DO content of the sludge suspension. The course of the pH suggests a gradual oxidation of the acid fraction of the suspension. The course of the DO curve is comparable with that of the oxygen-sag curve in stream pollution research (KLEIN, 1957).

If the decrease of the pH retards the rate of the sludge metabolism, then neutralization of the acid intermediates would eliminate this effect. Therefore a similar experiment was carried out at a constant pH of 7.5, controlled by an automatic titrator with a 1% NaOH solution. The results plotted in fig. 13, show a noticeable effect. After a rapid fall of the DO, its reappearance in the liquid phase proceeded more quickly than in the former experiment, indicating that in the neutralized medium the maximal oxygen demand occurred at an

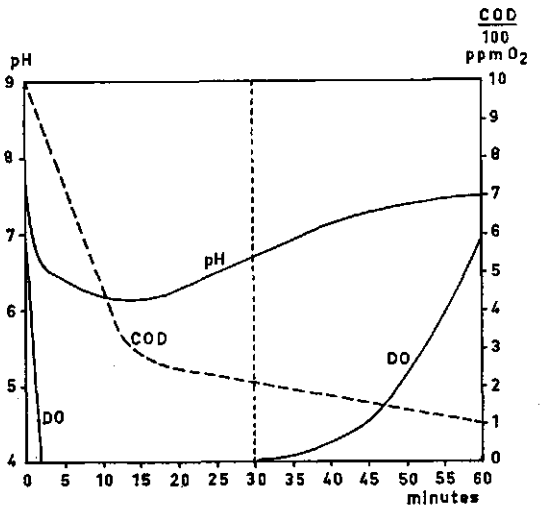

FIG. 12. Dissolved oxygen, pH and COD curves of an activated sludge suspension (4 to 5 grams of sludge dry weight per litre) at 25°C after addition of artificial dairy waste (1 gram of powder mixture per litre).

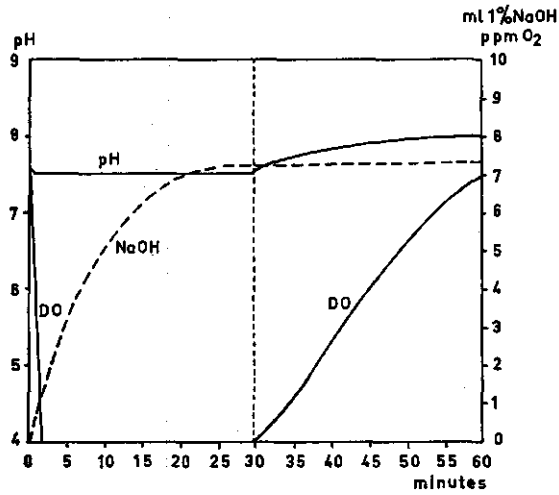

FIG. 13. Dissolved oxygen, pH and NaOH consumption curves of an activated sludge suspension at 25°C after addition of artificial dairy waste. The pH was kept constant at 7.5 (addition of 1% NaOH) with an automatic titrator.

earlier stage owing to a higher rate of decomposition by the sludge microorganisms. The ultimate pH of the suspension reached a markedly higher level, apparently due to oxidation of the neutralized intermediates and the subsequent liberation of part of the alkali used.

5.3. EFFECT OF VARYING AERATION RATES

In the preceding experiments the activated sludge suspensions were aerated by forcing compressed air through a porous sinter plate, giving a constant stream of finely dispersed bubbles with an aeration rate of 20 litres of air per hour.

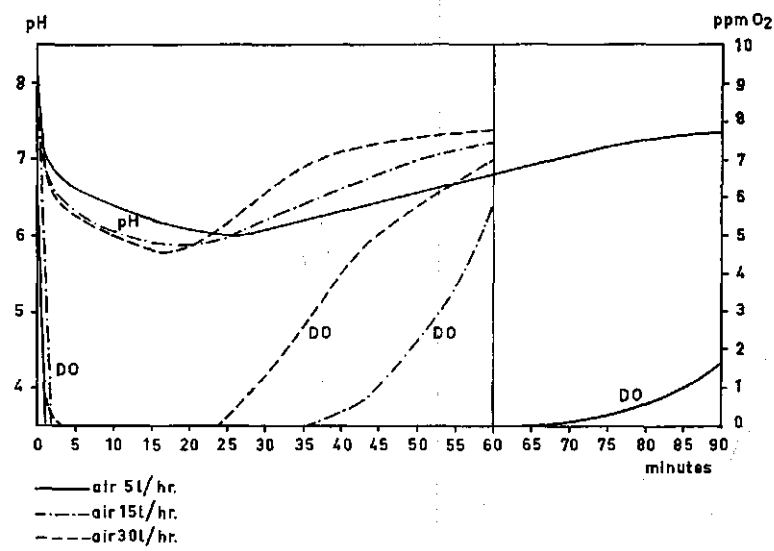

FIG. 14. Dissolved oxygen and pH curves of activated sludge suspensions at 25°C and at varying aeration rates. Substrate: artificial dairy waste.

The aeration rate was measured with a rotameter. In the experiments described in this section the aeration rate was varied. The experimental conditions were the same as before. The substrate was a powder mixture of 1 gram per litre. Oxygen deficiency was readily observed in the DO curve as well as in the pH curve (Fig. 14). The decline and especially the rise of the pH curve thereafter was retarded when less oxygen was supplied. The minimal value of the curve was attained later and it was somewhat higher than with a more liberal oxygen supply. When the aeration rate was lowered DO reappeared into the liquid at a later stage.

5.4. EFFECT OF THE VARYING pH LEVELS OF THE SUSPENSION

The effect of pH on DO was studied in a series of experiments in nutrient solutions automatically maintained at varying pH levels with an automatic titrator, using 1% NaOH or 1% HCl. An aeration rate of 20 litres of air per hour was applied. The sludge dry weight amounted to approximately 5 grams per litre of suspension.

In the first experiments artificial dairy waste (1 gram of powder mixture per litre) was fed to the activated sludge suspension. The course of the DO curve was determined at pH 8.0 (NaOH), 7.5 (NaOH), 6.0 (HCl) and 5.0 (HCl), respectively, (Fig. 15). In all cases a rapid initial oxygen consumption occurred as a result of which the DO content of the liquid fell to zero within a few minutes after the addition of the substrate. The period of time during which the DO content of the liquid remained at the zero level was apparently related to the pH of the suspension, the recovery of the DO curve starting sooner when the pH was lower. This would suggest that the amount of oxygen consumed was larger at higher pH values, a conclusion which is in agreement with the fact

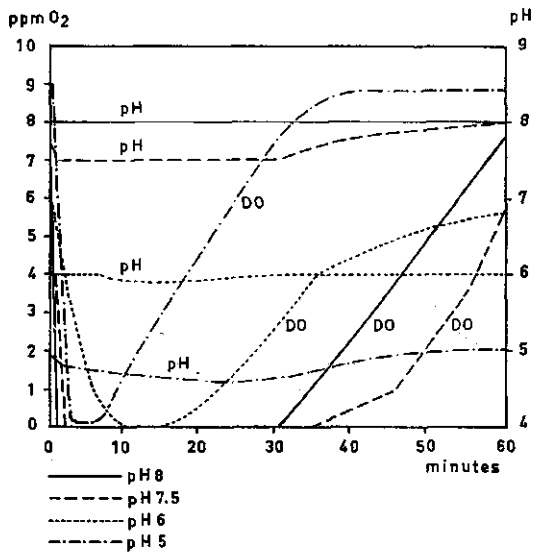

FIG. 15. Dissolved oxygen curves of activated sludge suspensions at 25°C and at varying controlled pH values. Substrate: artificial dairy waste.

that many bacteria grow less rapidly at pH 5 or 6 than at neutral or slightly alkaline reactions.

To prevent DO values from falling to zero, the following experiment was carried out with only 0.5 gram of lactose per litre as the substrate. The pH was controlled at 6.0 (HCl, NaOH), 7.0 (NaOH, HCl) and 8.0 (NaOH, HCl), respectively.

The DO curves of this experiment are shown in fig. 16. It appears that in the absence of proteins hardly any difference in the course of the DO curves occurred. The dissimilation of lactose was practically the same at pH 6.0, 7.0 and 8.0. Entirely different results were obtained at pH 5.0, 4.5 and 4.0 (Fig. 17). In addition to the DO curves the corresponding lactose content of the liquid phase and the total carbohydrates of the activated sludge were determined (Fig. 18). The curves of fig. 18 clearly demonstrate that lactose uptake from the liquid

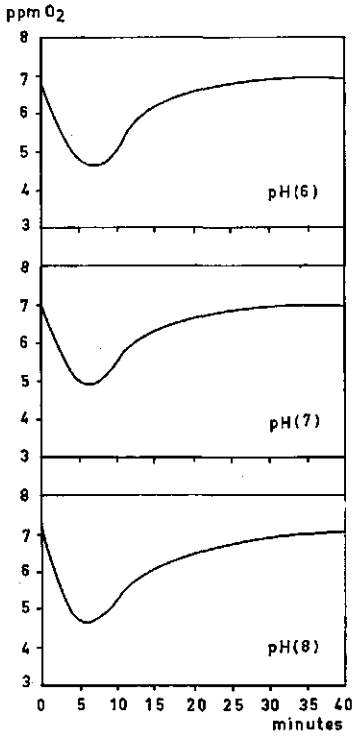

FIG. 16. Dissolved oxygen curves of activated sludge suspensions at 25°C and at varying constant pH values. Substrate: lactose, 0.5 gram per litre.

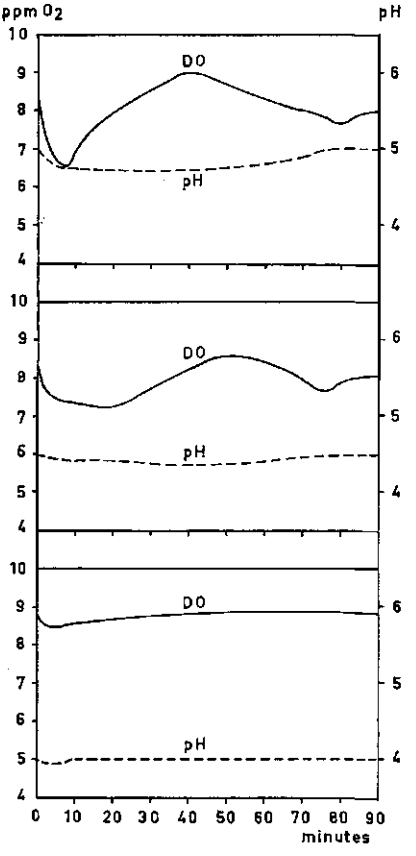

FIG. 17. Dissolved oxygen curves of activated sludge suspensions at 25°C and at varying low controlled pH values. Substrate: lactose, approximately 0.5 gram per litre.

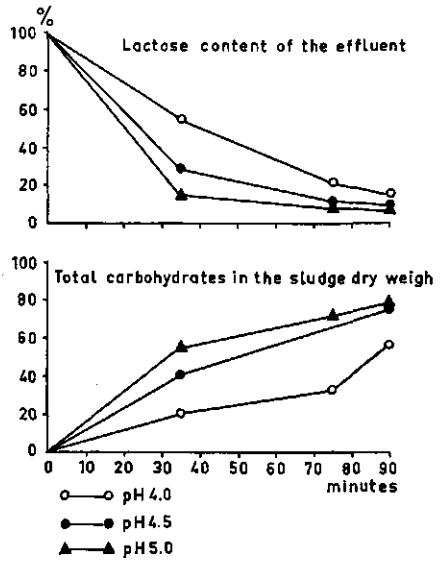

FIG. 18. Lactose uptake and polysaccharide accumulation by activated sludge suspensions at 25°C and at varying low controlled pH values. Substrate: lactose, approximately 0.5 gram per litre (see figure 17).

and carbohydrate accumulation in the activated sludge decreased considerably when the pH was lower than 5.0. This indicates that the microbial activity had decreased. A similar reduced activity below pH 5.0 was noticed in the DO curves (Fig. 17). After the primary decline of the DO curve, followed by a recovery, a secondary decrease of DO occurred. For this phenomenon, observed at pH 5.0 and 4.5 only, no explanation can be given.

From the preceding experiments it can be concluded that dissimilation of lactose is not affected by fluctuations of the pH, unless it drops below 5.0. Fluctuations of the DO curve when the pH of the sludge suspension is above pH 5, as observed with artificial dairy waste as the substrate, are apparently caused by an effect of pH on the decomposition of its protein fraction. This is in agreement with the fact that the majority of protein-decomposing enzymes of activated sludge bacteria have their optimal activity at pH 7 or higher. Thus when artificial dairy waste (powder mixture of 1 gram per litre) is fed to activated sludge, the ready dissimilation of the lactose causes a decrease of the pH, resulting in a retarded protein decomposition. When the acid intermediates of the lactose dissimilation are oxidized, the pH rises and the conditions become more favourable for decomposition of the protein fraction.

5.5. EFFECT OF VARYING AMOUNTS OF ARTIFICIAL DAIRY WASTE

To elucidate the effect of substrate concentration on pH of an activated sludge suspension, the following experiment was carried out. Quantities of 1, 2 and 3 grams per litre of powder mixture were added to an activated sludge suspension under conditions similar to those of the preceding experiments. DO was not determined. The results obtained (Fig. 19) show that the amount of

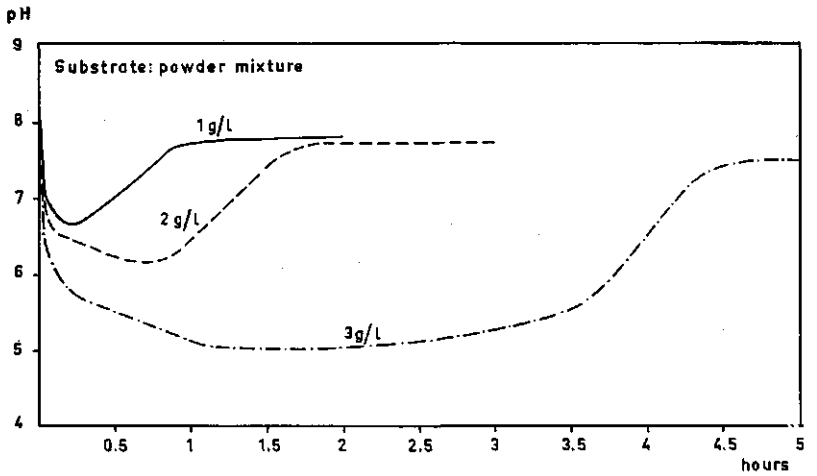

FIG. 19. The influence of varying amounts of substrate (artificial dairy waste, consisting of a powder mixture) on the course of the pH of an activated sludge suspension at 25°C.

substrate supplied determined the decrease of the pH and, moreover, the time of recovery. The larger the amount of substrate, the lower the pH dropped and the longer acid conditions persisted. The periods of time during which acid conditions existed were disproportional to the amounts of substrate supplied. This led to the conclusion that the activity of the activated sludge flora was repressed by the prolonged acid conditions.

In a subsequent experiment both the pH curves and the DO curves were recorded. Lower concentrations of substrate were used, viz. 1.0, 0.75, 0.6 and 0.4 gram per litre of powder mixture. The results are given in fig. 20. The pH curves show a similar trend to that in the preceding experiments, although with smaller amounts of substrate the accumulation of acid intermediates tends to

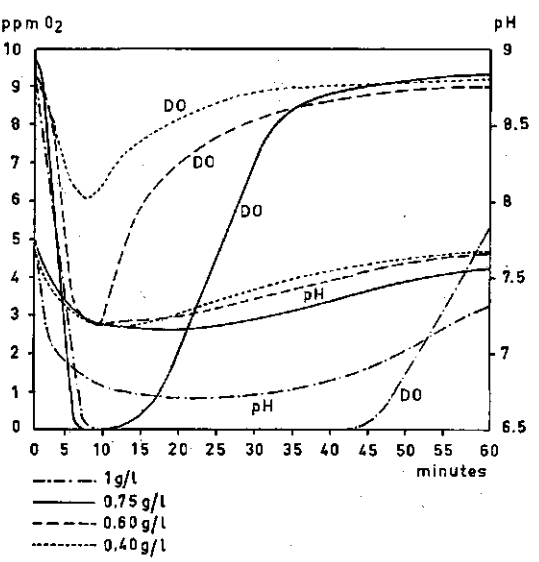

FIG. 20. Dissolved oxygen and pH curves of activated sludge suspensions at 25°C after addition of varying amounts of artificial dairy waste (powder mixture).

a minimum. The influence of small concentrations of substrate was more readily demonstrated in the course of the DO curves, which did not reach zero values at concentrations below 0.75. Recovery of the DO content started more readily with the lower concentrations of substrate. However, the rate of recovery was accordingly lower, so that the ends of the recovery periods nearly coincided.

5.6. Effect of varying fractions of artificial dairy waste

As previously reported, the decrease of the DO content of the sludge suspension immediately after feeding was accompanied by a sharp drop of the pH, presumably due to an incomplete oxidation of the carbohydrate fraction. To check this assumption, different fractions of the substrate were compared in a series of experiments performed in the aeration flask (Fig. 2).

In these experiments a suspension containing approximately 4 to 5 grams of activated sludge (dry weight) per litre was supplied with either: a. lactose, equivalent to the lactose content of the artificial dairy waste (680 mg/l); b. casein, equivalent to the casein content of the artificial dairy waste (190 mg/l) or c. a combination of a and b. These experiments were compared with a similar one in which artificial dairy waste consisting of 1 gram of powder mixture per litre was supplied.

The automatically recorded pH and DO curves are given in fig. 21. A sharp decrease of pH and DO was found in the case of substrates containing lactose (a, c and the control with the usual powder mixture). When casein was given alone, the effect on pH and DO was delayed and much less pronounced. This indicates that lactose is more readily attacked than casein. The effect of casein on pH and DO in the presence of lactose was much more pronounced

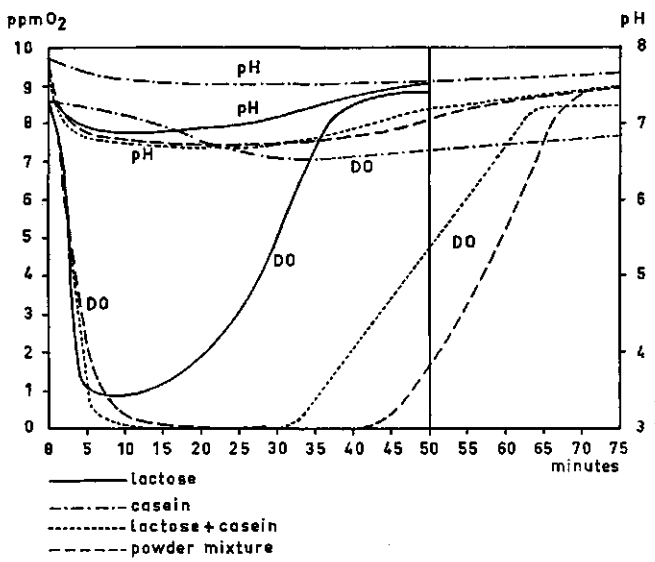

FIG. 21. Dissolved oxygen and pH curves of activated sludge suspensions at 25°C after addition of lactose, casein, lactose + casein, and powder mixture, respectively, as the substrate.

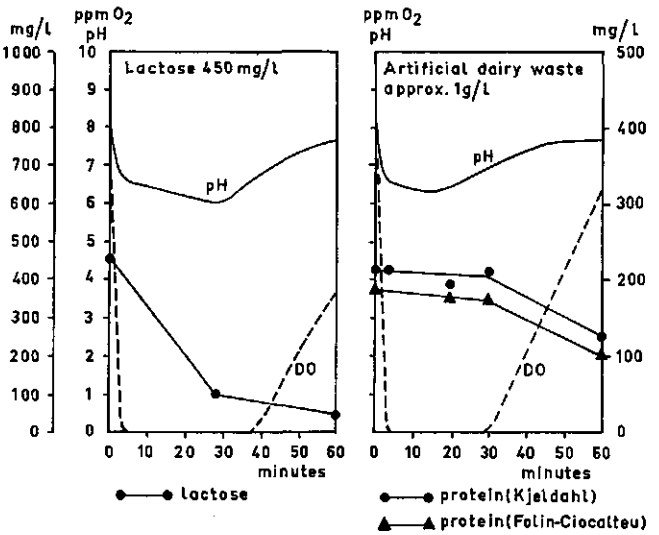

FIG. 22. Decomposition of carbohydrate and protein by activated sludge at 25°C after addition of lactose and artificial dairy waste, respectively, as determined by the anthrone method (lactose) and the Kjeldahl and Folin-Ciocalteu procedures (protein).

than in its absence. This could indicate a stimulating effect of lactose on protein decomposition.

Similar experiments were performed to determine the removal of lactose and protein, respectively, from the suspension. A suspension fed with only lactose showed a ready uptake of this substrate, whereas when supplied with artificial dairy waste, the decrease of nitrogen-containing compounds largely occurred when lactose of this substrate had been consumed. The latter is indicated by the rise of DO as well as of pH (Fig. 22).

From these experiments it can be concluded that the oxidation of the carbohydrate fraction proceeds so rapidly that much oxygen is required in a relatively short period of time. The decomposition of the protein fraction proceeds more slowly so that the demand for oxygen is spread over a longer period of time. In the former case lack of oxygen may readily occur, even when the aeration is excessive. In this stage acids are accumulated. On the other hand, with only casein as the substrate acute oxygen deficiency is not likely to occur under the conditions of this experiment.

5.7. DISCUSSION

The experiments described in this chapter indicate a parallelism between the pH and the DO content of an activated sludge suspension fed with dairy waste. This was demonstrated in experiments in batches, the operation of which is more or less comparable with many oxidation ditches. During the breakdown of artificial dairy waste, the dissimilation of its carbohydrate fraction, almost exclusively lactose, required in a rather short period of time more oxygen than could be supplied. This may have been due to a limited oxygen absorption rate. It is known that the oxygen demand is dependent both on the quantity and the

activity of activated sludge present and on the amount of substrate dosed. With large amounts the DO content of the liquid rapidly falls to zero. As a result of this the oxidation of the substrate may be limited. The fact that the DO values are approaching zero does not imply that anaerobic conditions should occur since the oxygenation rate is high. Therefore the production of acid intermediates (drop of pH of the suspension) presumably is due to the activity of aerobic microorganisms. This is corroborated by the fact that in the present experiments acid production started immediately after feeding when DO was still high. Furthermore, that it occurred when only small amounts of substrate were supplied and the minimal DO values stayed well above zero.

Acid intermediates accumulate because formation of acids proceeds at a higher rate than their oxidation to CO_2 and H_2O. It may be expected that under conditions of limited oxygen supply the oxidation of the acid intermediates is more retarded than their formation, so that the accumulation is accentuated. When the supply of lactose is exhausted, a ready oxidation of the acid intermediates takes place, resulting in a rise of pH. In the course of this phase, DO reappears.

The protein compounds of the dairy waste, mainly casein, neither exhausted the DO content of the liquid, nor affected the pH to a large extent. The minimum of the DO curve in the case of casein occurred at a later moment compared with that of lactose only. This suggests that the dissimilation of the carbohydrates in the dairy waste proceeds more readily than the dissimilation of its protein fraction. Moreover, this may be accentuated by the fact that the activity of many proteolytic enzymes is depressed by a low pH. Consequently, in dairy waste water the dissimilation products derived from carbohydrate and protein breakdown are not simultaneously available for the growth of the activated sludge bacteria. This may be of consequence for processes of cell synthesis and polysaccharide formation, as far as these processes are dependent on a certain actual C/N ratio at a particular moment, which may differ from the potential C/N ratio of the substrate supplied.

In order to show the interaction of COD, DO and pH, schematical curves were drawn from the above experiments with 1 gram of artificial dairy waste powder mixture and 4 to 5 grams of sludge dry weight per litre (Fig. 23). Four phases during the decomposition of the substrate can be distinguished.

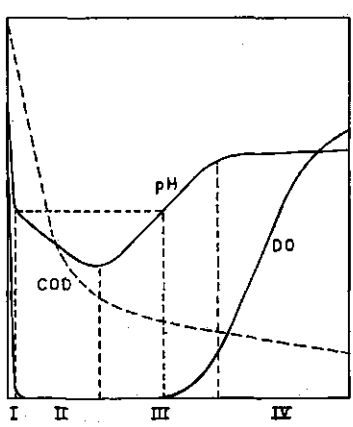

FIG. 23. Schematical curves demonstrating the course of dissolved oxygen, pH and COD, when artificial dairy waste (1 gram of powder mixture per litre) was supplied to activated sludge (4 to 5 grams of sludge dry weight per litre).

I. After the addition of the substrate a very high oxygen uptake rate coincides with the production of acid intermediates from the carbohydrate fraction, resulting in a rapid drop of the pH. The COD of the effluent also decreases rapidly. A similar effect of carbohydrate breakdown on pH is mentioned by McKINNEY (1962).

II. Owing to the fact that the oxygen supply is exceeded by the oxygen demand, the DO content of the liquid reaches its lowest point. The decline of the pH curve is less pronounced, because either the formation of acid intermediates proceeds less readily, or oxidation of these acids has begun. The COD curve tends to its minimum.

III. The pH curve is rising, suggesting that acid intermediates are disappearing. The oxygen demand is decreasing and soon DO is reappearing. The readily assimilable substrate has almost entirely been removed as is demonstrated by the low remaining COD decrease of the effluent.

IV. The rise of the pH has almost stopped. The demand for oxygen has markedly decreased as is demonstrated by a rapid increase of the DO content of the liquid. The dissimilation of the carbohydrate fraction and the intermediates is complete; the dissimilation of the protein fraction still proceeds.

5.8. SUMMARY

In experiments in batches with well aerated activated sludge, oxygen demand immediately after feeding an artificial dairy waste was found to exceed the oxygen supply. This caused a decrease of the DO content of the liquid which, under the prevailing conditions depending on the amount of substrate supplied, reached a zero value. Simultaneously the COD of the effluent decreased rapidly. During the dissimilation of the substrate, acid intermediates of the carbohydrate breakdown were excreted into the suspension, causing a sharp drop of pH. This suggests that the oxidation of the carbohydrate fraction of the substrate was limited, even when adequate amounts of oxygen were supplied. The sharp fall of the pH proceeded more slowly when the DO content of the liquid approached zero, indicating that the acid formation was due to the activity of aerobic microorganisms. After a minimum value had been reached the pH curve rose again, and soon afterwards also DO reappeared.

From the results obtained it was concluded that the dissimilation of the protein fraction of the artificial dairy waste proceeded at a markedly lower rate than the dissimilation of its carbohydrate fraction. The latter was less affected by low pH values than the former. Decreasing the aeration rate diminished the sludge activity, whereas an increase stimulated the activity until the absorption rate of the oxygen became limiting. Even with small quantities of artificial dairy waste a drop of the pH occurred. With larger amounts of substrate the pH remained accordingly longer at a minimum level. A similar influence of the amounts of substrate was found to exist on the course of the DO curve.

CHAPTER 6

EXPERIMENTS ON ACTIVATED SLUDGE METABOLISM

6.1. INTRODUCTION

In the preceding chapter it was shown that immediately after feeding a carbohydrate-containing substrate to a dairy waste activated sludge a temporary drop of the pH occurred. This was thought to be caused by the transitory appearance in the sludge suspension of acid intermediates of the carbohydrate breakdown.

To check the validity of this assumption, a series of experiments were carried out, to be described in the present chapter. The occurrence of acid intermediates was studied under conditions of varying oxygen demand. The dissimilation rates of the main acid intermediates found after chromatographical identification were determined by pH controlled suppletion of those acids. The same method was used for studying the dissimilation rate of the artificial dairy waste under conditions of varying pH of suspension and substrate.

Moreover, the accumulation of storage compounds and the oxygen uptake rate (OUR) were studied in relation to the dissimilatory activity of the activated sludge.

6.2. ACID PRODUCTION SHORTLY AFTER FEEDING

When an activated sludge suspension was supplied with a certain amount of artificial dairy waste a decrease of the pH was observed. This phenomenon gave rise to the assumption that acid intermediates were formed. In the following experiments this assumption was tested.

6.2.1. *Separation of effluent and activated sludge*

An activated sludge suspension was separated by centrifugation, or more quickly by passing it through a fine porous suction filter. It appeared that the pH of the effluent was slightly higher than that of the original suspension, whereas that of the concentrated activated sludge mass was considerably

TABLE 11. Comparison of the pH of the activated sludge suspension, and that of the effluent and the sludge, after separation, before and after feeding different substrates.

Substrate	pH before feeding			pH after feeding			
	Susp.	Effl.	Sl.	Susp.	Effl.	Sl.	
Artif. dairy waste	7.70	7.80	7.20	6.60	7.05	5.35	(s.f.)
Artif. dairy waste	7.00	7.70	6.60	6.15	7.00	5.60	(p.f.)
Lactose	7.20	7.45	7.15	6.40	6.90	5.35	(s.f.)
Alanine	7.20	7.45	7.25	7.20	7.35	6.80	(s.f.)

(s.f. = suction filter; p.f. = pressure filter)

lower. When the separation was carried out 5 minutes after supplying artificial dairy waste or lactose to the activated sludge suspension, the difference was even more pronounced. Replacement of the suction filter by a pressure filter (Seitz filter) to avoid loss of carbon dioxide made no significant difference (Table 11). When an amino acid, e.g. alanine, was added, only a slight effect or no on the pH was noticed.

6.2.2. *Effect of washing on the pH of effluent (washing water) and sludge*

In a subsequent experiment one litre of an activated sludge suspension containing approximately 100 ml of settled sludge was passed through the suction filter. The pH values of the effluent and of the sludge were measured (8.24 and 8.12, respectively) and then both components recombined and 1 gram of powder mixture added. After violent aeration for 1 minute to ensure good mixing, the effluent and sludge were once more separated and the pH values measured (6.15 and 5.35, respectively). Subsequently the sludge was made up to 1 litre with distilled water (pH 6.75). After aeration for 1 minute the suspension was again filtered. Measurements of the pH gave values of 5.51 (effluent) and 5.35 (sludge).

In similar experiments the washings with distilled water were repeated with the sludge mass left on the filter. The pH values are shown in table 12. From the data it will be seen that even after 8 washings acidification of the washing water had occurred. It may be concluded that very shortly after the addition of a carbohydrate-containing substrate, acid intermediates are produced by the activated sludge. This acid production continues after withdrawal of the remaining substrate and the acid products until the stock of the absorbed substrate becomes exhausted.

TABLE 12. Effect of washing-out of acid intermediates on the pH of effluent (washing water) and sludge.

Treatment	pH			
	I		II	
	Effl.	Sl.	Effl.	Sl.
Before feeding	6.72	6.51	7.21	–
Within 5 minutes after feeding	6.09	5.00	6.10	–
Immediately after washing: 1[1])	5.20	–	5.70	–
2	5.10	–	5.59	–
3	5.00	–	5.53	–
4	5.02	–	5.59	–
5	5.12	–	5.79	–
6	5.16	–		
7	5.35	5.29		
8	5.65	–		

I and II are duplicate experiments.
[1]) Number of washing

6.2.3. *Examination of the effluent for organic acids*

To investigate the cause of the acidification, an examination was made by paper chromatography of the organic acids present in the effluent of activated sludge after feeding. For this purpose samples of 100 ml of a concentrated sludge suspension were supplied with 60 ml of a concentrated artificial dairy waste or with a solution of lactose equivalent to the amount present in artificial dairy waste. After a 15 minutes' aeration period the suspension was filtered quickly and the filtrate neutralized with NaOH. To stop biochemical activities the filtrate was heated at 75°C for 5 minutes and subsequently cooled. For the determination of non-volatile acids one half of the liquid was submitted directly to ether perforation. The volatile acid fraction was also concentrated by ether perforation after steam distillation and redistillation. The concentrated fractions were used for chromatographical identification.

Acetic acid was shown to be the main constituent of the acid intermediates. In addition appreciable amounts of lactic acid were found, accompanied by traces of α-ketoglutaric, succinic, malic and fumaric acids.

Accumulated acid intermediates derived from artificial dairy waste may occur in considerable amounts. This can be easily calculated from the amounts of alkali required for neu ralization of the acids. In the experiment recorded in fig. 13, for instance, approximately 7.25 ml of a 1% NaOH solution was consumed. This amount is equivalent to 110 mg of acetic acid per 690 ml of sludge suspension, or with 160 mg per litre. About 1 gram of artificial dairy waste powder mixture (containing 680 mg of lactose) was used per litre of suspension. This means that the amount of acid intermediates expressed as acetic acid, approximated to 23% of the lactose supplied.

In subsequent experiments acid production by activated sludge was examined at two pH values (4.0 and 8.0) and in the presence or absence of DO. The latter was effected by applying different aeration rates. The results of these experiments (Table 13) clearly show that in the presence of DO acetic acid was formed almost exclusively, whereas under conditions of DO depletion lactic acid accumulated. Variation of the pH had no effect on the composition of the acid fraction.

TABLE 13. The major components of acid intermediates found by chromatographical analyses.

Substrate	pH[1])	Dissolved oxygen	Acetic acid	Lactic acid[2])
Artificial dairy waste	7.5	n.e.[3]	present	present
Lactose	7.7	n.e.[3]	present	present
Lactose	|4.0|	present	present	traces
Lactose	|8.0|	present	present	absent
Lactose	|4.0|	absent	traces	present
Lactose	|8.0|	absent	traces	present

[1]) At the start of the experiment; |pH|: the pH was automatically controlled at the stated value;
[2]) The presence of lactic acid was confirmed by a spot test according to FEIGL (1960);
[3]) n.e. = not estimated.

In the experiments in which both acetic and lactic acid were found to be present, the pH was not controlled and the aeration rate unchanged. The samples were taken when the pH reached a minimum value. The simultaneous occurrence of both acids was thought to be due to the swift reverse of the ratio oxygen demand/oxygen supply. If so it might be possible to avoid overlapping of acetic and lactic acid forming phases by taking samples prior to the alteration of the oxygen demand of the activated sludge suspension. This was done in the following experiment.

Sixty ml of a 0.8% lactose solution was added to 630 ml of an activated sludge suspension which was aerated in an aeration flask. The DO content of the liquid was recorded continuously. A pH of 8 was maintained by addition of NaOH and the temperature was kept at 25°C. Five-ml samples were taken at 2, 15 and 20 minutes after the start of the experiment, when DO in the liquid was still available, was nil and had reappeared, respectively. The samples were withdrawn by means of a syringe and were forced through a Swinney-adapter, containing an asbestos filter, and connected to the syringe. These filtered samples were submitted to the spot test for lactate. The results are given in table 14. It will be seen that lactic acid is produced only when DO is depleted. It quickly disappeared when DO reappeared. In the beginning of this experiment and presumably in the third phase, when no lactic acid was detected, acetic acid will have been formed, since during the entire experimental period the presence of acid intermediates was demonstrated (Fig. 13, NaOH curve). This result is in accordance with the results of the preceding chromatographical assays.

TABLE 14. The appearance of lactic acid as an acid intermediate (spot tests).

Substrate	pH[1])	Time[2])	DO[3])	Lactic acid
Lactose	|8.0|	2	present	absent
		15	absent	present
		20	present	absent

[1]) In brackets denotes that the pH was kept constant;
[2]) Minutes;
[3]) DO = dissolved oxygen.

6.3. DISSIMILATION RATES OF LACTIC AND ACETIC ACIDS, AND OF LACTOSE

The transitory accumulation of acid intermediates in an activated sludge suspension shortly after feeding, disappearing upon continued aeration, may enable to determine the oxidation rates, or more generally, the dissimilation rates of both acids. Before feeding, the pH of an activated sludge suspension usually was between 7 and 8. Using an automatic titrator the pH was lowered slightly by titration with one of the acids to be tested. During the subsequent dissimilation of the acid the pH rose so that an additional amount of acid from the titrator was introduced. In this way the dissimilation rate of the added

acid may be measured. For the successive experiments, portions of activated sludge from the same stock suspension were used (sludge dry weight approximately 5 grams per litre). The automatic titrator was adjusted at pH 7.0. Usually 1N solutions were applied for the titrations. The amounts of acid dosed in this way were measured at short intervals by burette readings for 2 to 3 hours. The mean dissimilation rates in m.moles per litre of activated sludge suspension per hour, calculated from the experimental data, are given in table 15.

TABLE 15. Mean dissimilation rates of acetic and lactic acids (m.moles per litre per hour; temperature 25°C; sludge dry weight approximately 5 gram per litre of activated sludge suspension.

Experiment	Acetic acid	Lactic acid
I	–	3.4
II	2.2	4.4
III	2.1	2.7
IV	3.3	2.9
V	2.0	2.4
Mean	2.4	3.2

The mean dissimilation rate of lactic acid appeared to be higher than that of acetic acid. The rapid dissimilation of lactic acid is in agreement with the observation that this acid disappears rapidly from the liquid when DO reappears (see table 14).

In a similar experiment, performed with a 10% lactose solution as the titrator liquid, a dissimilation rate of approximately 2.2 m.moles per litre of suspension per hour was found. The calculation of the dissimilation rate of lactose depends on the fact that a considerable amount of acid intermediates is formed very shortly after the addition of the carbohydrate to the activated sludge suspension. Furthermore on the assumption that during the experimental period a constant percentage of the lactose is excreted as acid intermediates by the activated sludge bacteria. However, it should be kept in mind that only a minor part of the carbohydrate is converted into acid intermediates, whereas no information is available as to the fate of the major part. Under the conditions of the experiment described in this section, the apparent dissimilation rate of lactose, when expressed as m.moles per litre per hour (2.2) was almost equal to that of the mean value calculated for acetic acid (2.4, see table 15).

When the dissimilation rates are expressed in mg/l/hr, the following values are obtained: lactic acid, 288, acetic acid, 144, and lactose, 752. These figures show that under the conditions of the present experiment, lactic acid is dissimilated twice as fast as acetic acid, while the apparent dissimilation rate of lactose is approximately six times as fast as that of acetic acid.

6.4. Apparent dissimilation rate of the lactose fraction of artificial dairy waste at different pH values

In the previous experiments the dissimilation rates of lactose, lactic and acetic acids were determined at 25°C, with the automatic titrator adjusted at pH 7.0. In the following experiments the artificial dairy waste water itself was used as the titrator liquid. This heterogenous substrate may be employed for a limited period of time to determine the apparent dissimilation rate of its lactose fraction, since it also causes a temporary pH decline shortly after feeding. However, it should be kept in mind that this application is subject to the same restrictions as for lactose, the presence of protein being regarded as an interfering factor. The protein fraction of the artificial dairy waste is decomposed at a markedly lower rate and therefore in prolonged experiments proteins accumulate.

Samples of 690 ml of activated sludge suspensions, containing 5 gram of sludge dry weight per litre, were titrated with a tenfold concentration of artificial dairy waste (10 grams of powder mixture per litre), having a pH of 7.0. To study the effect of pH of the sludge suspension on the apparent dissimilation rate the automatic titrator was adjusted at pH 7.5, 7.0, 6.5, 6.0 and 5.2, respectively, for a series of successive experiments. The measurements were started when the pH of the suspension stabilized at the desired value and a constant supply of the titrator liquid was obtained. This occurred 1 to 2 hours after the adjustment of the titrator. The apparent mean dissimilation rates in ml per hour were derived from measurements of the volumes of titrator liquid over a period of one hour. The results are given in table 16 (I), and are plotted in fig. 24.

From the data it will be seen that under the described experimental conditions the apparent dissimilation rate is highest at pH 6.5. The DO content of the liquid during the measurements fluctuated between 1 and 2 mg per litre.

In the above experiments a titration liquid of pH 7.0 was used. This may

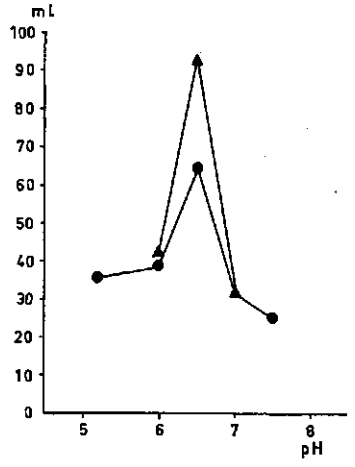

FIG. 24. The apparent mean dissimilation rates of the lactose fraction of artificial dairy waste at varying pH values of the suspension (circles, see table 16 (I)) and at varying pH values of both the suspension and the substrate (triangles, see table 16 (II)).

Table 16. The apparent mean dissimilation rates of the lactose fraction of artificial dairy waste at varying pH values of the suspension (I), and of the suspension as well as the substrate (II).

Experiment (I)[1]	pH				
	7.5	7.0	6.5	6.0	5.2
Apparent mean dissimilation rates (ml/hr)	24.6	31.2	64.0	38.4	35.4

Experiment (II)	pH		
	7.0	6.5	6.0
Apparent mean dissimilation rates (ml/hr)	31.2	94.2	42.0

[1]) (I) Activated sludge suspensions only adjusted to the various pH values; pH of the substrate: 7.0; (II) Both sludge suspension and substrate adjusted to the various pH values.

affect the apparent dissimilation rate in suspensions in which the pH differs from that of the titrator liquid. This was checked in the following experiments, designed as before, but now using a similar artificial dairy waste with a pH equal to that set on the automatic titrator. Successive experiments were carried out at pH 7.0, 6.5 and 6.0, respectively. The results are illustrated in table 16 (II) and in fig. 24.

Especially at pH 6.5, adjustment of the pH of the titrator liquid to the same value as the pH of the suspension had a markedly stimulating effect upon the apparent mean dissimilation rate of the lactose fraction of the artificial dairy waste.

6.5. The influence of different factors upon the dissimilatory activity of activated sludge

In general, the dissimilation products of a carbohydrate may be recovered as carbon dioxide, as structural and vital cell materials, as reserve materials and as acid or unknown intermediates. Depending upon different factors, the relative amounts of these dissimilation products may vary considerably. The effect of some of these factors upon substrate dissimilation by activated sludge was studied in a number of Warburg experiments, mostly with parallel experiments in the aeration flask under identical conditions. Some experiments were carried out in the aeration flask only.

In the preceding experiments no exact information had been obtained about the respired part of the dissimilated substrate. Therefore Warburg experiments with activated sludge were performed using lactose as the substrate, and NH_4Cl as the nitrogen source for obtaining maximal dissimilatory activity.

In a first set of experiments (Table 17.I) a sludge suspension cultivated on artificial dairy waste had previously (for 15 hours) been supplied with an

TABLE 17. The dissimilatory activity of activated sludge tested under varying conditions.

Cultural conditions and metabolic characteristics[1])	Experiments						
	I[2])			II			III
	(a)	(b)	(c)	(a)	(b)	(c)	
Cultural conditions:							
NH_4Cl (mg/l) during pretreatment	500	500	500	–	–	–	–
Lactose supplied (mg/l) during the experimental period	600	600	900	600	600	550	680[3])
Metabolic characteristics:							
Sludge dry weight (g/l)	–	5.6	6.3	1.9	4.2	5.5	2.4
Oxygen uptake rates (ml/l of suspension/hr)	45	36	37.5	74	150	–	–
Qo_2 (ml/g of sludge dry weight minus total carbohydrates/hr)	–	6.9	6.8	48.3	48.6	–	–
% of substrate respired	9.5	9.5	10.5	9.5	9.5	–	–
Initial amount of total carbohydrates of the sludge in % of sludge dry weight	–	7.3	11.4	19.5	26.7	19.8	22.9
Increase of total carbohydrates in % of substrate supplied	–	36.3	68.4	50.7	57.5	45.5	37.5

[1]) During the experimental period 500 mg of NH_4Cl per litre of suspension was added to the Warburg vessels;
[2]) I:NH_4Cl, II and III: no NH_4Cl during pretreatment.
[3]) Substrate was added as artificial dairy waste (1 gram of powder mixture per litre) containing approximately 680 mg of lactose and 190 mg of casein.

excessive amount of NH_4Cl, in order to eliminate as much as possible available storage products (mainly polysaccharides) of the sludge by promoting bacterial growth. Using this suspension, the experiments were performed with a. 600, b. 600 and c. 900 mg of lactose, respectively, added per litre of sludge suspension. Oxygen uptake, measured at 25°C, is plotted in fig. 25. From these curves oxygen uptake rates (ml per litre of sludge suspension per hour, and Qo_2 = ml

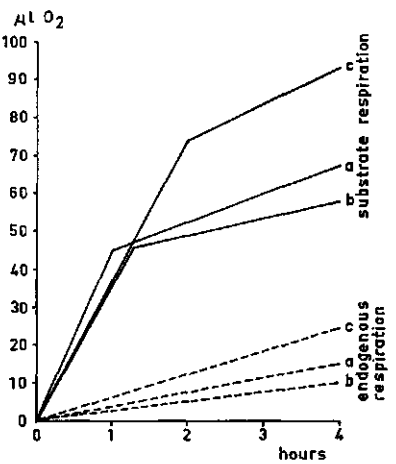

FIG. 25. Oxygen uptake (µl/hr) of varying activated sludge suspensions previously aerated with added NH_4Cl (low amount of total carbohydrates). Temperature 25°C (see also table 17. I).

per gram of sludge dry weight per hour) were calculated. The percentages of lactose respired, after complete consumption of this substrate, were derived from the total amounts of oxygen consumed up to the point where the rates of oxygen uptake decreased abruptly, indicating depletion of the substrate. The values found and additional data are shown in table 17. I.

The highest amount of lactose gave a somewhat lower oxygen uptake rate. Nevertheless, in all cases approximately 10% of the lactose was respired.

In a subsequent series of experiments (Table 17. II) special attention was paid to the effect of different amounts of activated sludge on the dissimilatory activity of the sludge bacteria. In addition the initial total carbohydrate content of the sludge differed because pretreatment with NH_4Cl was omitted. The experimental data, recorded in table 17. II and plotted in fig. 26, show that the oxygen uptake rate of lactose supplied sludges is proportional to the sludge concentration (II, (a) and (b)). Owing to this, almost equal Qo_2 values were found in these two experiments. Sludge grown without NH_4Cl during the pretreatment period had much higher values for oxygen uptake rate during the experimental period than that pretreated with this nitrogen compound (Qo_2 values of approximately 48 in II, (a) and (b) compared with 6.9 in I, (b)). Initial total carbohydrate content of the latter was much lower than that of the former, because during the pretreatment period the stored polysaccharides had been utilized owing to the presence of added NH_4Cl. The decreased content of polysaccharides was likely to be closely related to the decreased metabolic activity of the sludge. The data of table 17 and fig. 25 and 26 furthermore show that the percentages of the added carbohydrate respired are low and are independent of the sludge concentration and of its initial total carbohydrate content. In all the experiments of these series the values approximated to 10%.

The data of table 17. I and II were supplemented with those of an experiment performed in the aeration flask with artificial dairy waste as the substrate during the experimental period (III). The activated sludge used in this experiment had been pre-aerated without added NH_4Cl. For this reason the initial

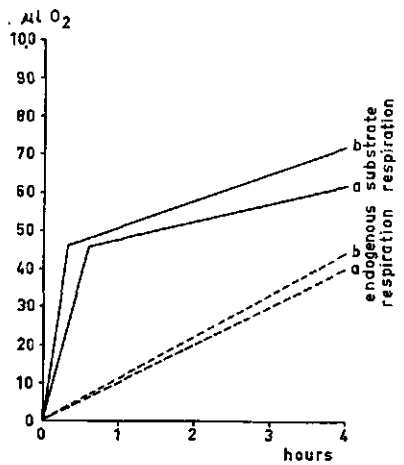

FIG. 26. Oxygen uptake (µl/hr) of varying activated sludge suspensions without an NH_4Cl pretreatment. Temperature 25°C (see also Table 17. II).

percentage of total carbohydrates in the sludge dry matter was almost equal to those of experiment II. With this substrate also a large percentage of the carbohydrate was converted into polysaccharides.

6.6. Discussion

An interesting phenomenon is the drop of the pH of a suspension of activated sludge immediately after feeding artificial dairy waste or lactose. The drop of the pH was found to be due to the formation and transitory accumulation of acid intermediates from lactose by the bacterial cells of the sludge.

There are a few records in literature of the formation of acids in activated sludge suspensions after the addition of a carbohydrate-containing substrate. Ingols and Heukelekian (1939), in experiments with activated sludge, reported the occurrence of an organic acid only under conditions of limited oxygen supply. They assumed this acid to be lactic acid, because of its frequent occurrence in anaerobic processes. Jenkins and Wilkinson (1940) in similar experiments recorded that lactose, when added to the activated sludge, was removed at a high rate during the first hour. With 2190 ppm of lactose used they observed a drop of the pH to 4-5, shortly after feeding. In the early part of the experiment small amounts of lactic acid were found, which after 24 hours had disappeared.

Although in the present investigation the drop of pH was accompanied by a decrease of the dissolved oxygen content of the sludge suspension, the formation of the acid intermediates was assumed to proceed under aerobic conditions. Under these circumstances acetic acid was the main organic acid formed and accumulated. When the oxygen demand continued to exceed the oxygen supply, dissolved oxygen values of zero were reached and then lactic acid was the main acid accumulating. In this case conditions of oxygen supply may have been more or less similar to those of Jenkins and Wilkinson, who also noted the formation of lactic acid in sludge suspensions upon the addition of lactose.

So far it is unknown whether the formation of relatively large amounts of acetic acid in well aerated activated sludge suspensions, supplied with lactose or lactose-containing dairy wastes, depended on the presence in the sludge of a well-defined type of bacterium or rather that the specific conditions in the sludge floc were responsible for it.

Although the accumulation of acetic acid, and under certain conditions that of lactic acid, indicate a readier formation than dissimilation of these acids, both compounds are easily dissimilated by the sludge microorganisms. This was studied in separate experiments, using the organic acids as titrator liquid. In these experiments lactic acid appeared to be metabolized more quickly than acetic acid. This may have been due to lactic acid entering more readily into the carboxylic acid cycle than acetic acid, which first has to be phosphorylized. Utilization of acetic acid in the glyoxylic acid system and of lactic acid in the carboxylic acid cycle may also have been responsible for the difference observed in dissimilation rates. Differences in adaptation velocity have presumably played

no role since dissimilation of both acids by activated sludge was found to proceed without any adaptation period. This is in agreement with the results obtained by VAN GILS (1964) with municipal sewage activated sludge.

That some types of microorganisms may be important in the transitory accumulation of acetic acid from a carbohydrate under aerobic conditions was shown recently by HADJIPETROU et al. (1964, 1965) in growth experiments with *Aerobacter aerogenes*. In spite of vigorous aeration of the glucose-containing nutrient medium, acetate accumulated in this medium.

That variation of the pH may affect the dissimilation rate was shown in an experiment with artificial dairy waste as the substrate. Apparent maximal dissimilation of the carbohydrate fraction of this substrate was obtained at pH 6.5. This activity was decreased more by higher than by lower pH values.

When a carbohydrate under aerobic conditions is subjected to the dissimilatory activity of activated sludge, it may be (a) respired to CO_2 and H_2O with liberation of chemical energy (ATP formation) or heat, (b) used for synthesis of structural and vital cell components, (c) converted into storage compounds e.g. polysaccharides, (d) converted into acids or other intermediate compounds, which sometimes may be excreted and accumulated in the medium in considerable amounts. The relative proportions of these different types of biochemical activities diverge widely depending on the type of microorganism as well as on certain growth conditions such as C/N ratio of the substrate, availability of oxygen, pH etc.

Since the mineralization of dairy waste by activated sludge depends to a large extent on the above-mentioned aspects of biochemical activity, elucidation of these aspects and particularly of the factors affecting them is of great importance.

To measure the activity of the activated sludge, oxygen uptake rates were determined in Warburg experiments. In parallel experiments sludge dry weight and total carbohydrate content of the sludge dry weight were estimated. From the oxygen uptake rate values, oxygen uptake rates per gram of sludge dry weights per hour (Qo_2 values) were calculated. These oxygen uptake rates were found to be closely related with the initial carbohydrate content of the sludge. Lowering this content by pretreatment of the sludge for 15 hours with an excess of NH_4Cl gave much lower oxygen uptake rates during the subsequent experimental period, both in the absence and presence of added lactose (endogenous and substrate respiration, respectively).

Approximately 10% of lactose was respired. This value was independent of the amount of lactose added, of the activated sludge concentration applied, of the initial total carbohydrate content of the sludge and of the oxygen uptake rate.

The fact that only 10% of the lactose added to activated sludge was oxidized to CO_2 and H_2O would indicate that a large part of the substrate was retained in the sludge as structural and vital cell material and as storage compounds. A further part will have been present as acid intermediates in the effluent. Approximately 50% of the lactose was found to be stored as polysaccharides

(Table 17). This percentage was lower when the sludge had been pretreated in the presence of NH_4Cl, except in the case when the sludge had been supplied with a higher amount of lactose. The reducing effect of NH_4Cl during pretreatment on the polysaccharide content of the sludge undoubtedly depended on the utilization of part of the polysaccharides for the synthesis of organic nitrogen compounds.

Accumulation of polysaccharides may also occur when there is a discrepancy between the actual C/N ratio and the potential C/N ratio of the substrate, as was shown when artificial dairy waste was used. Nitrogen supply to the cells was limited owing to the slow dissimilation rate of the protein fraction of the substrate as compared with that of its carbohydrate fraction. This may have resulted in an initial accumulation of polysaccharides. However, when the carbohydrate fraction of the substrate became exhausted, and protein decomposition was at its maximum, part of the accumulated polysaccharides may have been used in the synthesis of nitrogen-containing structural and vital cell constituents. This resulted in a decrease of the amount of initially accumulated polysaccharides.

As synthesis of nitrogen-containing cell material, using accumulated polysaccharides, proceeds at a low rate, ammonium ions from protein decomposition may appear in excess and can give rise to nitrification (HEUKELEKIAN and LITTMAN, 1939).

The influence of the C/N ratio of the substrate on polysaccharide synthesis by *Arthrobacter* has been recorded by MULDER et al. (1962) and ZEVENHUIZEN (1966). At a high C/N ratio of the substrate these bacteria were found capable of accumulating large amounts of polysaccharides. In chapter 3 it was shown that an important part of the dairy waste activated sludge flora consisted of *Arthrobacter*-like bacteria. Therefore it may be assumed that these bacteria have played an important part in the observed accumulation of total carbohydrates in the sludge dry matter. With lactose as the substrate up to 31.5% of total carbohydrates in the sludge dry matter was found. This is consistent with data reported by PORGES et al. (1955).

Polysaccharides stored by *Arthrobacter*, at least partly, may be utilized as a source of energy and for cell synthesis, when no exogenous substrates are available (ZEVENHUIZEN, 1966). However, its rate of utilization is much lower than that of supplied substrates. This may be seen from respiration experiments in which oxygen uptake rate dropped considerably when the exogenous substrate was depleted and the cells utilized reserve materials (Fig. 25, 26; VAN GILS, 1964).

6.7. SUMMARY

The drop of the pH (chapter 5) in the activated sludge suspension shortly after feeding, was found to be due to accumulation of acetic acid when DO was present, and of lactic acid when DO was absent. Both acids were dissimilated after the substrate had been exhausted. Lactic acid was dissimilated more

readily than acetic acid. The dissimilation rate of the latter was almost equal to that of lactose when calculated on a molar basis.

The apparent dissimilation rate of artificial dairy waste water was found to be optimal at a pH of 6.5 of the activated sludge suspension.

When lactose was added to activated sludge, approximately 10% was respired, up to 50% was accumulated as polysaccharides, and the remainder was partly used for the synthesis of structural and vital cell constituents, and partly accumulated as intermediates in the suspension. The percentage of supplied lactose respired was independent of the amount added, of the activated sludge concentration, of the total carbohydrate content of the sludge or of the oxygen uptake rate.

The total carbohydrate content of the sludge depends on the C/N ratio of the substrate supplied, a high ratio giving sludge with a high carbohydrate content. Supplying such a sludge with an excess of NH_4Cl and aerating for 15 hours gave a marked drop of the carbohydrate content, coinciding with a pronounced decrease of the oxygen uptake rate.

CHAPTER 7

BULKING OF ACTIVATED SLUDGE

7.1. INTRODUCTIONAL

The results of the experiments described in the previous chapters suggest that the composition of the activated sludge flora may be affected by alterations of the substrate supplied. This principle was thought to be applicable in changing the microbial composition of bulking activated sludge in order to improve the settling capacity of this sludge.

Bulking of activated sludge is characterized by a deterioration of the structure of the sludge flocs, resulting in poor settling properties. Owing to this the sludge volume index, i.e. the volume of settled sludge in proportion to sludge dry weight, is extremely high. The modified structure of the flocs generally depends on an excessive growth of filamentous microorganisms like *Sphaerotilus natans*.

According to HEUKELEKIAN (1941) bulking of activated sludge should result when conditions are not so unfavourable as to destroy the purification mechanism and yet sufficiently unfavourable as to bring a shift in the delicate biological balance. One of these unfavourable conditions should be an inadequate oxygen supply. If the oxygen supply in relation to the demand becomes inadequate, *Sphaerotilus* and other filamentous organisms attain the ascendency and the sludge becomes bulking. Biochemical activities of these organisms would bring about the purification in a way similar to the desirable sludge organims except with a lower and more efficient oxygen utilization at the lower tensions. In other words when the sludge is diffuse and filamentous it exposes more surface which might enable the sludge to obtain the limited amount of oxygen present in the medium immediately surrounding it. HEUKELEKIAN suggests that this may be confirmed by the fact that bulking sludge usually produces the most sparkling effluents.

RUCHHOFT and KACHMAR (1941) considered bulking to be a response of sludge organisms to a sudden disturbance in biological equilibrium, produced not only by variations of the rate of oxygen supply, but also by variations of the food. HEUKELEKIAN and INGOLS (1941), discussing the paper of RUCHHOFT and KACHMAR, stress the role of the oxygen supply pre-eminently as a primary factor. The adverse effect of available sugar is seen by these authors primarily as an increased demand for oxygen. LACKEY and WATTIE (1958) by supplying a normal activated sludge with heavy doses of disaccharides, frequently succeeded in obtaining a bulking sludge in which *Sphaerotilus* was very abundant. This is not in contradiction to the conception of HEUKELEKIAN and INGOLS (1941) concerning the effect of carbohydrates on the oxygen demand. HATTINGH (1963) found wide BOD/N and BOD/P ratios to favour growth of *Sphaerotilus*.

In a literature review on 'Slime Infestation' HARRISON and HEUKELEKIAN (1958) concluded that for luxurious growth, *Spaeratilus* requires organic nitro-

gen. This was confirmed by MULDER and VAN VEEN (1963; see also MULDER, 1964). These authors have made an extensive examination of the nutritional requirements of *Sphaerotilus natans*. Peptone and casamino acids without added vitamins were found to be excellent N sources. Good growth was also obtained with aspartic or glutamic acid but in this case vitamin B_{12} (cyanocobalamin) had to be supplemented. This vitamin was also required when ammonium nitrate was the nitrogen source, but in this case growth of *Spaerotilus natans* was much less abundant than with organic nitrogen. Vitamin B_{12} could be replaced by small amounts of L-methionine.

The ability of *Sphaerotilus* to grow under low oxygen tensions has been clearly shown by RUCHHOFT and KACHMAR (1941) and also recently by MULDER (1964). WUHRMANN (1946) stated that the oxygen requirement of this bacterium would depend upon the available carbon source.

In the present investigation bulking of activated sludge is considered from a point of view of population dynamics. It is not sufficient to know whether *Sphaerotilus* will or will not grow under certain conditions. It is of equal importance to know why *Sphaerotilus* grows more readily than the desirable microorganisms in bulking activated sludge.

When the activated sludge flora is fully adapted to a certain waste water, it has developed an optimal dissimilatory activity in this particular waste water. The activity of a mixed population, such as activated sludge, is the mean of the activities of different types of microorganisms forming the sludge flora. Usually the environmental conditions for the activity of the sludge are not optimal and often liable to variations. The latter particularly occur when shock-loading procedures are applied. This means that also the facilities for the sludge bacteria to obtain a maximal activity are continuously changing.

In the previous experiments with powder mixture or with its main constituent components supplied separately as the substrate, the dissimilatory activity of the activated sludge was found to be different with the carbohydrate and the protein fractions, respectively. As a result an important difference exists between the potential and the actual C/N ratios of the substrate during the overall period of substrate consumption. Especially those bacteria that accumulate storage compounds during the dissimilation of the carbohydrate fraction and which are able to utilize these reserve materials for subsequent growth when the protein dissimilation products become available, may obtain a predominant position in the activated sludge flora.

Bulking activated sludge in dairy waste water purification frequently occurs when the sludge has repeatedly been overloaded, or when the waste possesses a high C/N ratio, for example, when much whey is continuously being discharged.

One of the main microorganisms considered to cause bulking of dairy waste activated sludge is *Sphaerotilus natans*. This filamentous microorganism grows well in an environment with a relatively high C/N ratio (INGOLS and HEUKELEKIAN, 1939; HEUKELEKIAN and INGOLS, 1940; HATTINGH, 1963). This was confirmed by MULDER et al. (1962). They further found that *Sphaerotilus*

natans, when cultivated in a mineral salts' medium supplied with 5 grams of glucose and 1.5 gram of peptone, contained 29% of poly-β-hydroxybutyrate, 4% of lipids and 41% of polysaccharides. The nitrogen content of the cells was 4%.

In subsequent experiments MULDER (1964) compared the growth of *Sphaerotilus natans* with the growth of an *Arthrobacter globiformis* strain, isolated from activated sludge, in experiments in pure cultures which were continued for several days. Using glucose and peptone as the substrates, both growth rate and cell yield (dry weight) were higher with *Sphaerotilus* than with *Arthrobacter*. This held for aerated as well as for non-aerated cultures. However, in the latter case the difference between the two bacteria was much more pronounced, confirming the conception that *Sphaerotilus* is able to grow well at low oxygen tensions. The growth of *Arthrobacter* under such conditions was strongly depressed. A further striking difference between the two bacteria was found to exist in the decrease of the dry weight of cell mass, especially in aerated cultures, after the substrate had been utilized. Cell weight of *Sphaerotilus* decreased much more readily than that of *Arthrobacter*, presumably owing to a much higher rate of respiration of accumulated storage compounds (polysaccharides and poly-β-hydroxybutyrate).

The experiments recorded by MULDER (1964) were repeated in the present investigation using the same strain of *Sphaerotilus natans* and an *Arthrobacter* strain occurring in large numbers in the dairy waste activated sludge of the present investigation. Both bacteria were grown in a medium containing 0.1% of lactose and 0.05% of casamino acids. Yield curves, resembling those of the above experiments of MULDER (1964), are plotted in fig. 27.

The results of these experiments and those of investigations described in chapter 3 (*Arthrobacter*-like bacteria being the predominant major group of microorganisms in dairy waste activated sludge) and in chapter 5 (difference between dissimilation rates of carbohydrate and protein fractions of dairy waste) are considered to be of fundamental importance in explaining the phenomenon of bulking in dairy waste activated sludge.

When activated sludge containing *Sphaerotilus natans* and *Arthrobacter*-like bacteria is supplied with a moderate amount of dairy waste of a normal composition, it may be expected that synthesis of cellular material is curtailed owing to a deficiency of readily available nitrogen (see chapter 5). The temporary excess of carbohydrates gives rise to synthesis and accumulation of storage compounds, which under the conditions of an adequate oxygen supply are assumed to proceed more readily in *Arthrobacter*-like bacteria than in *Sphaerotilus* (see fig. 27). Then nitrogen compounds available from protein breakdown give rise to the synthesis of nitrogen-containing cell material at the expence of accumulated storage compounds. In this respect *Arthrobacter*-like bacteria are in a more favourable position because of a. the assumed higher polysaccharide content, and b. the capacity to assimilate inorganic nitrogen compounds, which are normally formed during protein decomposition. These inorganic nitrogen compounds are assimilated much less readily by *Sphaerotilus natans*

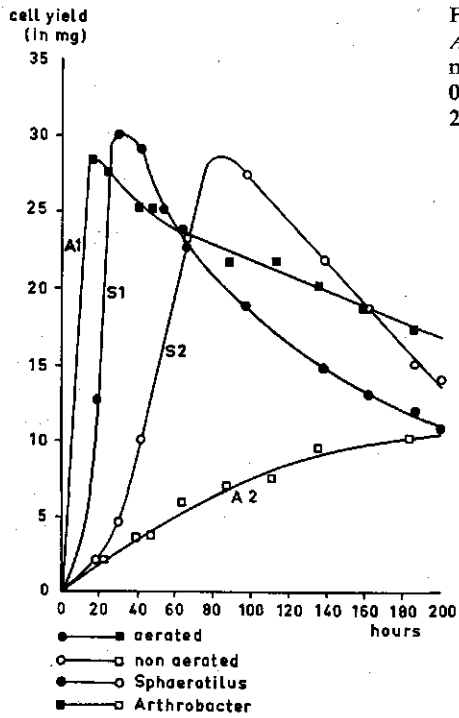

FIG. 27. Growth yield of a *Sphaerotilus* and an *Arthrobacter* species under well-aerated and non-aerated conditions. Substrate: lactose, 0.1% and casamino acids 0.05%. Temperature 25°C.

(MULDER, 1964). From these considerations it is concluded that under conditions of moderate supply of a normal dairy waste and of adequate aeration, *Arthrobacter*-like bacteria may maintain their predominant position in dairy waste activated sludge.

When the activated sludge suspension is overloaded with dairy waste of a normal composition, a prolonged period of oxygen deficiency results (see chapter 5). Under these conditions the growth of *Sphaerotilus* may proceed fairly steadily for a much longer period of time than is the case with *Arthrobacter*-like bacteria. The activity of the latter bacteria is suppressed by oxygen deficiency. Thus prolonged periods of oxygen deficiency may be expected to favour accumulation of storage compounds, and also growth, of *Sphaerotilus*, as part of the nitrogen may become available before the carbohydrate fraction is fully dissimilated. When all the carbohydrate has been utilized, the DO content of the liquid rises again. Although under these conditions *Arthrobacter*-like bacteria may show an increasing activity, with regard to growth *Sphaerotilus* is in a much better position competitively, owing to its relatively high quantity of storage compounds which, upon liberation of assimilable nitrogen compounds, are partly used for the synthesis of cell material. This leads to an increase of *Sphaerotilus* in the sludge flora, which finally may cause bulking of the activated sludge.

A high C/N ratio of the waste water has a similar effect, as especially the

decomposition of the carbohydrate fraction causes deficiency of oxygen. Under such conditions *Sphaerotilus* accumulates storage compounds, whereas the *Arthrobacter*-like bacteria show a decreased activity. When the carbohydrate fraction of the substrate has been dissimilated and DO has reappeared, *Sphaerotilus* benefits more from the small amount of available nitrogen than the *Arthrobacter*-like bacteria because of the reduced activity and the low dissimilation rate of the limited amount of storage compounds of the latter. When this type of waste water is repeatedly discharged, *Sphaerotilus* may finally predominate and bulking of the activated sludge may occur.

From the above considerations it may be concluded that bulking activated sludge could possibly be prevented or eliminated by controlling the amount and the actual C/N ratio of the waste water. The latter might be achieved by dosing an additional amount of an inorganic nitrogen compound immediately before the waste water is subjected to purification, or by adding an additional amount of an energy source when the carbohydrate fraction of the dairy waste has been consumed and protein decomposition still proceeds. It would be preferable to add the carbon compound when the nitrogen compounds have been mineralized, since *Arthrobacter* in contrast to *Sphaerotilus* readily assimilates inorganic nitrogen compounds when a carbon source is available.

Bulking of activated sludge is characterized by a poor settling of the floc suspension. In many cases it is attempted to overcome this by supplying $FeCl_3$, $FeSO_4$, $Al_2(SO_4)_3$, lime, $CaCO_3$ or even soil. Promotion of settling characters by increasing the specific gravity of the suspended matter may be possible to some extent, but the most logical and most effective remedy will be to restore the original sludge condition by promoting the growth of the desirable microorganisms, so that they may achieve a more predominant position. On the other hand a similar effect may be obtained by a selective destruction or inhibition of the undesirable microorganisms. For this purpose active chlorine is sometimes used (SMITH and PURDY, 1936; RENSINK, 1966). This destroys the bacteria on the surface of the activated sludge flocs and filamentous bacteria in the interstitial spaces, whereas bacteria inside the flocs survive and afterwards readily grow on the released organic matter of destroyed cells (RENSINK, 1966).

In the following section experiments are described which depend on the promotion of growth of desirable bacteria in bulking dairy waste activated sludge.

7.2. EXPERIMENTAL

Bulking activated sludge (sludge volume index approximately 600) was used half a day after supplying it with artificial dairy waste (1 gram of powder mixture per litre); the carbohydrate fraction had been dissimilated and part of the organic nitrogen fraction had presumably been mineralized. In this stage the sludge suspension was divided into 4 portions each of them contained in an aeration cylinder. To 3 of the 4 portions a carbon source was added for supplying energy and materials for the synthesis of cell material. As a readily utilizable

carbon source, one of the intermediates found in the course of the dissimilation of the carbohydrate fraction, viz. acetic or lactic acid, was supplied. Both acids were applied in the form of their calcium salts and in concentrations of 1 gram per litre of activated sludge suspension. The third suspension received 1 gram of powder mixture per litre, and a fourth was used as a blank. After one day of heavy aeration an equal amount of the respective doses was supplied. After a further 2 days the suspensions were allowed to settle. At the same time 25 ml samples of each treatment were transferred into colorimeter tubes for photographing the sludge volumes after a settling time of 30 minutes. The samples seen in Plate 1a, which were equal to the correspondingly larger volumes, indicate a striking difference between the four suspensions. In the blank (D) the bulking characters had somewhat decreased when compared with the original activated sludge (not demonstrated). In the suspension fed with powder mixture (A) the activated sludge was bulking as before. However, a markedly improved condition of the sludge was obtained with acetate (C). Addition of lactate (B) had also a favourable influence on the settling properties of the activated sludge, although somewhat less than with acetate.

A similar experiment with moderately bulking sludge was carried out in duplicate and on a larger scale. Two series of suspensions were present: with and without 300 mg of NH_4Cl per litre of activated sludge suspension used. Each series consisted of 5 suspensions: a blank; one supplied with powder mixture (1 g/l); one with Ca-lactate (1 g/l); one with Ca-acetate (1 g/l) and one with acetic acid (1 g/l). One of the duplicate blanks and one of the duplicate suspensions fed with powder mixture of the series without NH_4Cl were supplied with 300 mg of $CaCO_3$ per litre. After 24 hours' aeration, 25-ml samples were withdrawn from each suspension for photographing the results after 30 minute's settling of the sludge. Thereafter the suspensions again received a similar supply of the respective substrates. Aeration was applied for 48 hours. Then the series were again photographed after 30 minute's settling (Plate 1. b and c).

The sludge volumes of the blanks (A) were decreasing slowly in the course of the experiment. Addition of $CaCO_3$ (B) markedly reduced the settled sludge volume. When only NH_4Cl was supplied the settled sludge volume increased considerably (C). With a powder mixture supplied (D) no improvements of the settling characters were obtained. Additional supply of NH_4Cl (F) increased the settled volume, a result which is not in accordance with the consideration of section 7.1. Application of Ca-lactate (G) or Ca-acetate (I) resulted in a significant improvement of the settling characters of the activated sludge. When in addition to these substrates NH_4Cl was added, the excessive growth obtained resulted in the increase of the settled sludge volume (H and J), as compared with the suspensions without NH_4Cl. Addition of acetic acid (K) gave an excessive growth of moulds and yeasts especially when NH_4Cl was also added (L). In these cases bulking of the activated sludge was largely stimulated.

Improvement of the settling characters of the activated sludge is not always clearly demonstrated by the settled sludge volume, because certain treatments promote growth which may mask the settling properties of the sludge. This

can be easily seen by comparing the blank + $CaCO_3$ (B) with e.g. sludge treated with Ca-acetate + NH_4Cl (J). In the former case no growth occurred, so that the sludge condition – a relatively large proportion of *Sphaerotilus* – had not changed, although the settled sludge volume had decreased markedly, owing to the increase of the specific gravity of the sludge mass. In the suspensions treated with acetate (I and J) selective growth of desirable bacteria had taken place, whereas the development of *Sphaerotilus* was curtailed. As a result of this alteration in microflora, settling characters and structure of the activated sludge had essentially been improved. The latter can be seen in a series of microphotographs showing qualitative differences between sludges after different treatments (Plate 2).

In the sludge suspension supplied with the calcium salts of acetic and lactic acids, the added Ca ions by increasing its specific gravity, may have exerted an additional effect on improving te settling characters of the sludge.

In a subsequent experiment sludge dry weight and ash content of the treated activated sludge were also determined, so that the settling properties could be expressed in terms of sludge volume indices, calculated on sludge dry weight with and without its ash content, respectively.

A suspension of activated sludge was repeatedly supplied with large portions of whey powder until a moderate bulking of the sludge was obtained. This suspension was divided into 5 portions which were treated as follows: 1: blank (untreated); 2: blank + $CaCO_3$ (600 mg/l); 3: powder mixture (1 g/l); 4: powder mixture (1 g/l) + NH_4Cl (300 mg/l); 5: Ca-acetate (1 g/l). After 24 hours of aeration double amounts of the successive substrates were applied. After another 48 hours' aeration, settling was allowed for 30 minutes and the respective sludge volumes were measured and photographed (Plate 3). The corresponding sludge volume indices, calculated on the basis of the sludge dry weight, and those calculated on the basis of sludge dry weight minus ash, a more accurate measure of cellular growth, are listed in table 18.

TABLE 18. The effect of added substrates on settling properties of activated sludge.

Suspension	Substrate	Settled sludge volume (ml/l)	Sludge dry weight (g/l)	Dry weight minus ash (g/l)	Sludge volume index	Corrected sludge volume index	Remarks
1	Blank	672	4.80	3.46	140	194	No growth
2	$CaCO_3$	472	7.43	4.73	64	100	No growth[1])
3	Dairy waste	616	5.89	4.23	105	146	Growth
4	Dairy waste + NH_4Cl	800	8.24	6.70	98	119	Selective growth
5	Ca-acetate	372	7.34	4.85	51	77	Selective growth

[1]) The increase of the sludge dry weight minus ash compared with the blank can not be explained.

It will be seen from both table 18 and plate 3 that the addition of acetate had the most drastic effect on decreasing the sludge volume index. Supplement-

ing the dairy waste with NH_4Cl had also a distinct influence on this index, although the pronounced increase in volume resulting from its strong growth-promoting influence would suggest the contrary. The observed beneficial effect of added $CaCO_3$ on the settling properties must mainly have been due to its non-biological effect; no explanation can be given of the apparent sludge growth in this suspension.

7.3. Discussion

Bulking of activated sludge generally depends on the presence of relatively large amounts of *Sphaerotilus natans*, which adversely affects the settling properties of the sludge. It is considered to be a response to environmental conditions, which are known to affect the bacterial composition of the sludge. In the case of bulking these conditions are assumed to be more favourable to the development of *Sphaerotilus natans* than to that of desirable bacteria such as *Arthrobacter*.

Based upon pure culture experiments with both *Sphaerotilus* and *Arthrobacter* and on literature recordings, an effort was made to analyse the mechanism that causes bulking of dairy waste activated sludge. From the experiments described in chapter 5 it was clearly seen that food supply and the amount of dissolved oxygen in the activated sludge suspension are closely related. Dissimilation of the carbohydrate fraction of the applied artificial dairy waste caused a drop of pH and of DO content of the liquid. During this phase, oxygen demand exceeded oxygen supply, even with excessive aeration. An additional observation of this type of substrate related to the difference in carbohydrate and protein dissimilation. It resulted in a discrepancy between the potential and the actual C/N ratios of the substrate, leading to a difference in availability of readily assimilable carbon and nitrogen compounds to the activated sludge bacteria.

As to the growth characters of *Sphaerotilus* and desirable bacteria such as *Arthrobacter*, the two groups of microorganisms mainly involved in either bulking or normal activated sludge, the following can be noted.

1. *Sphaerotilus* grows well at low oxygen tensions, whereas the growth of *Arthrobacter* under such conditions is poor.
2. In well aerated cultures the growth rate of *Arthrobacter* exceeds that of *Sphaerotilus* (increase of cell dry weight; see fig. 27).
3. Both types of microorganisms are able to accumulate storage compounds when a proper substrate is supplied (high C/N ratio).
4. *Arthrobacter* readily utilizes inorganic as well as organic nitrogen compounds for growth, whereas *Sphaerotilus* grows much better with organic than with inorganic nitrogen sources.
5. Acetate is much more favourable as a carbon source for *Arthrobacter* than for *Sphaerotilus* (Mulder and van Veen, 1963).
6. Both types of microorganisms are assumed to utilize accumulated storage compounds for growth when the exogenous substrate has been consumed and nitrogen is still available. This is supported by the observed decrease of total car-

bohydrate content of *Arthrobacter* when subjected to pretreatment with excess of NH_4Cl (Chapter 6). MULDER (1964) showed that storage compounds of *Sphaerotilus* can rapidly be respired. It may be expected that the storage compounds of both microorganisms are used for cell growth if readily assimilable nitrogen compounds are available.

The processes during dissimilation of dairy waste water, in combination with the listed data concerning the response of both types of microorganisms to different environmental conditions enabled elucidation of the mechanism of bulking of activated sludge.

Supplying large amounts of dairy waste water or dosing waste water with a high C/N ratio, favours the growth of *Sphaerotilus* because of a. the low oxygen tension being obtained, b. the accumulation of storage compounds, and c. the discrepancy existing between the dissimilation rates of the carbohydrate and protein fractions. A low oxygen tension depresses the activity of *Arthrobacter*-like bacteria to a large extent, whereas the activity of *Sphaerotilus* is not affected. During dissimilation of the carbohydrate fraction of the substrate, *Sphaerotilus* accumulates large amounts of storage compounds which afterwards, when assimilable nitrogen compounds become available from protein decomposition, may be used for the assimilation of the latter and so for cell growth. Under the conditions of limited oxygen supply *Arthrobacter* shows a much lower activity and accumulates less storage compounds. Therefore it will be in a weaker competitive position concerning nitrogen assimilation and ultimate cell growth, when oxygen becomes available after the dissimilation of the lactose of the dairy waste.

The above explanation as to the interaction of food supply and availability of oxygen to the bacterial cells as the cause of bulking, is in agreement with the conception held by INGOLS and HEUKELEKIAN (1939, 1940), HEUKELEKIAN and INGOLS (1940, 1941), HEUKELEKIAN and LITTMAN (1939) as well as with those of others (RUCHHOFT and KACHMAR, 1941; LACKEY and WATTIE, 1958; see also DONDERO, 1961).

Based on the above considerations a number of efforts have been made to restore the settling properties of bulking dairy waste activated sludge by changing its microbial composition. This was done by a. the addition of a readily assimilable carbon source after the carbohydrate fraction of the dairy waste had already been taken up by the sludge microorganisms but part of the assimilable nitrogen had presumably still been left in the suspension, b. supplying an inorganic nitrogen compound along with the dairy waste. Both treatments were thought to improve the growth of the desirable bacteria, particularly *Arthrobacter*, as contrasted to that of *Sphaerotilus*. As carbon sources, acetate and lactate were supplied because these acids had been found as temporary intermediates (Chapter 6) in the lactose decomposition by dairy waste activated sludge bacteria.

As expected, both carbon compounds, and particularly acetate, were found to have a highly beneficial effect on the settling properties of the activated sludge, almost eliminating bulking. Although $CaCO_3$ formed after the con-

sumption of the Ca-salts of the organic acids may have contributed to the amelioration of the sludge quality, increased growth of desirable activated sludge bacteria upon the application of Ca-acetate or Ca-lactate, as contrasted with that of *Sphaerotilus natans*, has undoubtedly mainly been responsible for the observed effect.

The application of NH_4Cl along with dairy waste, although improving the settling properties of bulking activated sludge, gave less spectacular results than that of acetate and lactate. This was partly due to the growth-promoting effect of the nitrogen source. When calculated as sludge volume index the ameliorative effect was more clearly visible.

Improvement of the settling properties of bulking activated sludge by changing the composition of its bacterial flora, i.e. by stimulating the growth of the desirable bacteria, is supposed to be ultimately more effective than those resulting from an increase of the specific gravity of the activated sludge flocs by addition of Ca-, Fe- or Al-salts.

7.4. Summary

Based upon pure culture studies with the two main types of microorganisms that may be involved in bulking of dairy waste activated sludge, *Sphaerotilus* and *Arthrobacter*, described in section 7.2 and in the literature, an attempt was made to explain the mechanism of bulking.

Reduced oxygen supply, resulting from the high dissimilation rate of the carbohydrate fraction of the dairy waste, ability to accumulate storage compounds and discrepancy between the potential and actual C/N ratios of this waste were found to be the main factors in stimulating relative growth of *Sphaerotilus natans* and thus being responsible for bulking.

Alteration of the nutritional conditions caused an improved growth of the desirable bacteria (mainly *Arthrobacter*-like bacteria) and this led to a pronounced relative suppression of *Sphaerotilus natans*, as a result of which the settling properties of the activated sludge were restored.

The clearest effect was achieved by adding lactate, and particularly acetate, at the moment that the lactose of the dairy waste was exhausted, but assimilable nitrogen compounds were still available. Decreasing the actual C/N ratio by adding NH_4Cl along with the dairy waste favoured activated sludge growth and also tended to eliminate *Sphaerotilus*.

SUMMARY

A bacteriological study has been made of dairy waste activated sludge, including its formation, ultimate composition when fully adapted to the dairy waste applied, and response to different physiological conditions.

The bacteriological formation and the ultimate composition of the activated sludge were studied both in an oxidation ditch (Pasveer system) and in aeration flasks in the laboratory. The latter were supplied with an artificial dairy waste consisting of a mixed skim milk powder whey powder solution, the chemical composition of which was comparable with the mean composition of the dairy waste discharged into the oxidation ditch. Only in the case of artificial dairy waste supplied to the aeration flasks a mixed suspension of soil, manure and sewage bacteria was used as the inoculation material. Bacteria were isolated at 9, 28 and 56 days after the start of the experiment and identified, using an incubation temperature of 25°C.

A comparison of the bacterial flora of dairy waste activated sludge, developing from its initial to its optimal stage in the oxidation ditch and in the laboratory apparatus, respectively, showed a close similarity between both systems.

Adaptation of the bacterial flora to the supplied waste proceeded slowly. The composition of the flora altered with time until ultimately it consisted of three major groups of bacteria, viz. a predominant group of coryneform bacteria, a relatively large group of *Achromobacteraceae* and a group of *Pseudomonadaceae*. The latter bacteria, initially present in large numbers, ultimately represented the smallest of the three major groups. A minor rest group consisted of microorganisms only present in relative small numbers. About 85% of the coryneform bacteria, according to their morphological characters, were *Arthrobacter*-like bacteria. No significant differences in bacterial composition were observed between the activated sludge in its assimilative and in its endogenous phase.

In the stabilized activated sludge two-thirds of the isolated bacteria gave a neutral reaction on Hugh and Leifson media and more than half were non-proteolytic.

The brown colour of the dairy waste activated sludge was shown to be due to *Flavobacterium* species.

In a subsequent series of experiments representative species of the isolates of the three major groups of bacteria were examined for a number of properties thought to be important in explaining the changeing bacterial composition of developing activated sludge.

The *Arthrobacter* species was found to be the most active and effective of the three types, having the shortest generation time in pure culture, growing somewhat more slowly in mixed culture, but demonstrating a continued growth for a longer period of time, and ultimately outnumbering both other species. This was supposed to be due, partly to the conversion of rods into cocci at the end of the growth period, and partly to the ready accumulation of con-

siderable amounts of polysaccharides which were available as a source of energy when the original substrate had been consumed. The other two species hardly accumulated polysaccharides. The growth yield, calculated as mg of cell dry weight produced per unit of substrate consumed (in terms of chemical oxygen demand, COD), was slightly higher in the case of the *Arthrobacter* species. When after a period of aeration without added substrate, feeding was resumed, the *Arthrobacter* species showed a much higher oxygen uptake rate as compared with the *Achromobacter* and *Pseudomonas* species. These results agree with the ultimate predominance of the group of the *Arthrobacter*-like bacteria in the dairy waste activated sludge.

When activated sludge was fed a normal amount of artificial dairy waste, a sharp drop of pH and dissolved oxygen (DO) was observed immediately after feeding, due to the ready dissimilation of the carbohydrate fraction of the substrate. Decomposition of the protein fraction started later, proceeded at a much lower rate, and was accompanied by a slight fall of pH and DO for a more prolonged period of time. The drop of pH during the dissimilation of the carbohydrate fraction was caused by a transitory occurrence of acid intermediates in the suspension. This was assumed to be due to the fact that, in spite of an excessive aeration, the oxygen absorption rate was unable to meet the increased demand for oxygen and therefore became a limiting factor in the oxidation of the carbohydrate fraction of the substrate.

The difference found between the dissimilation rate of the carbohydrate fraction and that of the protein fraction caused a discrepancy between the potential and actual C/N ratios of the substrate.

Analyses of the acid intermediates showed that acetic acid was accumulated in the activated sludge suspension when DO was present, whereas lactic acid was found when DO was absent. Studying the dissimilation of these acids by dosing them to the activated sludge suspension with the aid of an automatic titrator, lactic acid was found to be dissimilated more readily than acetic acid. The dissimilation rate of the latter was almost equal to the apparent dissimilation rate of lactose, when calculated on a molar basis. The apparent dissimilation rate of artificial dairy waste was optimal when the activated sludge suspension had a pH of 6.5.

When lactose was added to activated sludge, approximately 10% was respired, up to 50% was accumulated as polysaccharides, while the remainder was partly used for synthesis of structural and vital cell constituents, and partly excreted as intermediates into the suspension.

The respiration percentage was independent of the amount of lactose supplied of the activated sludge concentration, of the total carbohydrate content of the sludge, and of the oxygen uptake rate. A high C/N ratio of the substrate resulted in a high total carbohydrate content of the sludge. Pre-aerating such a sludge with an excess of supplied NH_4Cl gave a large drop of the total carbohydrate content, followed by a considerably decreased oxygen uptake rate of the sludge.

The phenomenon of bulking of dairy waste activated sludge was studied in

relation to known and estimated data concerning two important types of microorganisms involved in the mechanism of bulking, viz. *Sphaerotilus* and *Arthrobacter*-like bacteria.

Reduced oxygen supply, as resulting from the high dissimilation rate of the carbohydrate fraction of the dairy waste, together with the ability to accumulate storage compounds and the discrepancy between the potential and actual C/N ratios of this waste, were considered to be the main factors in stimulating growth of *Sphaerotilus*, and thus to be responsible for bulking.

Alteration of the nutritional conditions caused an improved growth of *Arthrobacter*-like bacteria and this led to a pronounced suppression of *Sphaerotilus*, as a result of which the settling properties of the sludge were restored. The clearest effect was achieved by adding Ca-acetate, and, to a somewhat smaller extent, by adding Ca-lactate at the moment that the lactose of the dairy waste was exhausted, and when assimilable nitrogen compounds were still available. Decreasing the actual C/N ratio by adding NH_4Cl along with the dairy waste favoured activated sludge growth and also tended to eliminate *Sphaerotilus*.

SAMENVATTING

Zuivelafvalwater kan met goed gevolg worden gezuiverd volgens de actief-slib methode. Door de werkzaamheid van overwegend aërobe microörganismen worden de verontreinigende stoffen uit het afvalwater verwijderd.

Een onderzoek werd verricht naar het ontstaan van en de uiteindelijke samenstelling van de bacterieflora van een dergelijk actief slib. Daarnaast werd de invloed van verschillende fysiologische factoren op de samenstelling en de werking van het actief slib nagegaan.

De bacteriologische ontwikkeling van actief slib en zijn uiteindelijke samenstelling werden bestudeerd zowel in een oxydatiesloot (Pasveer-sloot), als in beluchte kolven in het laboratorium. Deze kolven werden voorzien van een kunstmatig zuivelafvalwater bestaande uit een mensel van ondermelkpoeder en weipoeder, opgelost in water, en in scheikundige samenstelling overeenkomend met het zuivelafvalwater dat op de oxydatiesloot werd geloosd. Waar kunstmatig afvalwater werd gebruikt, werd een weinig entmateriaal toegevoegd, bestaande uit een suspensie van bacteriën uit grond, mest en rioolwater. Op de tijdstippen 9, 28 en 56 dagen na aanvang van de proefnemingen werden actief-slib monsters genomen waaruit bij 25 °C bacteriën werden reingekweekt en in groepen gerangschikt op grond van een reeks morfologische en fysiologische kenmerken.

Een vergelijking van de bacterieflora van actief slib, zoals die zich ontwikkelde in de beluchte kolven, met die in de oxydatiesloot, liet een opmerkelijke overeenkomst in ontwikkeling zien.

De aanpassing van de bacterieflora aan het betreffende afvalwater verliep langzaam. Het goed aangepaste slib dat uiteindelijk werd verkregen, bleek hoofdzakelijk te bestaan uit drie groepen bacteriën, nl. een overheersende groep van corynebacterie-achtigen, voorts een belangrijk aantal bacteriën behorende tot de *Achromobacteraceae* en verder een groep te rekenen tot de familie der *Pseudomonadaceae*. De laatste waren aanvankelijk in veel grotere aantallen aanwezig, doch namen na verloop van tijd aanzienlijk af. De overblijvende microörganismen, normaal nimmer in belangrijke aantallen aanwezig, werden in een restgroep ondergebracht. Ongeveer 85 % van de corynebacterie-achtigen vertoonde de morfologische kenmerken van het genus *Arthrobacter*.

Ongeveer tweederde deel van de bacteriën van het uiteindelijk verkregen gestabiliseerde actief slib reageerde neutraal op de voedingsbodems volgens Hugh en Leifson en meer dan de helft was niet-proteolytisch.

De bruine kleur van het actief slib van zuivelafvalwater moet worden toegeschreven aan flavobacteriën.

In een volgende reeks proefnemingen werd van elk der drie belangrijke groepen bacteriën een karakteristieke vertegenwoordiger onderzocht op een aantal eigenschappen, die van belang werden geacht voor het verklaren van de waargenomen bacteriologische veranderingen tijdens de ontwikkeling van het actief slib.

Gevonden werd dat van de drie onderzochte typen, de arthrobacter de actiefste en de meest efficiënte was en de kortste generatietijd had. Gekweekt in mengculture, bleef de arthrobacter aanvankelijk bij de beide andere bacteriën ten achter, maar tenslotte vormde hij het grootste aantal cellen, doordat de groei gedurende een langere periode doorging. Verondersteld werd dat dit ten dele het gevolg was van het in coccen uiteenvallen van de staven, en ten dele van het vermogen van deze arthrobacter om grote hoeveelheden reservestoffen op te slaan, die gebruikt werden toen het oorspronkelijk versterkte voedsel was verbruikt. De beide andere bacterie-typen vertoonden nauwelijks enige opslag van reservestoffen bij voeding met zuivelafvalwater. De groeiopbrengst, berekend als mg droge-celopbrengst per eenheid verbruikt substraat (de laatste uitgedrukt als mg COD (chemische zuurstofbehoefte)), was een weinig hoger in het geval van *Arthrobacter*. Als na een periode van beluchting, gedurende welke geen voedsel beschikbaar was, de voedseltoediening werd hervat, vertoonde *Arthrobacter* een aanmerkelijk snellere zuurstofopname dan de *Achromobacter-* en *Pseudomonas*-typen. Deze resultaten verklaren de belangrijke plaats die de arthrobacters uiteindelijk in het slib innemen.

Wanneer actief slib werd voorzien van een normale hoeveelheid kunstmatig zuivelafvalwater, werd onmiddellijk na het toedienen van het voedsel een snelle daling van de pH en van het gehalte aan opgeloste zuurstof van de suspensie waargenomen. Dit werd veroorzaakt door een snelle omzetting van het koolhydraatgedeelte van het afvalwater. De afbraak van het eiwitgedeelte begon later en verliep veel minder snel. Zowel de pH als het gehalte aan opgeloste zuurstof werd in dit geval slechts weinig verlaagd, terwijl deze geringe verlaging gedurende een veel langer tijdsverloop aanhield.

De pH-daling tijdens de koolhydraatafbraak werd veroorzaakt door het vrijkomen van zure tussenprodukten. Op grond hiervan werd verondersteld dat, niettegenstaande een heftige beluchting, de zuurstof-absorptiesnelheid van de vlok niet kon voldoen aan de toegenomen zuurstofbehoefte van de bacteriën en daardoor een beperkende factor was geworden bij de oxydatie van het koolhydraatgedeelte van het substraat.

De verschillen tussen koolhydraat- en eiwitafbraak in zuivelafvalwater veroorzaakten een belangrijke afwijking tussen de potentiële en de actuele C/N-verhouding van het afvalwater.

Een nader onderzoek van de zure tussenprodukten toonde aan dat er, zolang er nog opgeloste zuurstof in de suspensie aanwezig was, azijnzuur werd opgehoopt, terwijl melkzuur werd gevormd bij afwezigheid van opgeloste zuurstof. Nadat de koolhydraatvoorziening was uitgeput, werden de opgehoopte zuren op hun beurt afgebroken, met het gevolg dat de pH en het gehalte aan opgeloste zuurstof weer gingen stijgen.

Uit een onderzoek over de afbraak van deze zuren, waarbij ze met een automatische titrator aan actief slib werden gedoseerd, bleek, dat melkzuur sneller werd omgezet dan azijnzuur. De snelheid (in m.mol/l/u) waarmee de laatstgenoemde verbinding werd afgebroken was vrijwel gelijk aan die waarmee lactose schijnbaar werd omgezet. De schijnbare afbraaksnelheid van kunstmatig afval-

water, door de titrator gedoseerd, was het hoogst bij pH 6,5 van de actief-slib suspensie.

Wanneer lactose aan het actief slib werd toegediend, werd ongeveer 10% verademd, ongeveer 50% opgehoopt als polysacchariden, terwijl de rest ten dele voor cel-synthese werd gebruikt en ten dele als tussenprodukten in de suspensie werd afgescheiden.

Het verademingspercentage was onafhankelijk van de verstrekte hoeveelheid lactose, de actief-slib concentratie, het polysaccharidegehalte van het slib en de snelheid van zuurstofopname. Een hoge C/N-verhouding van het afvalwater bevorderde de ophoping van polysacchariden in het slib. Werd een dergelijk slib voorbelucht in aanwezigheid van een overmaat aan NH_4Cl, dan verdwenen de opgehoopte polysacchariden, terwijl vervolgens de snelheid van zuurstofopname van het slib aanzienlijk daalde.

Het verschijnsel van opgeblazen actief slib werd bestudeerd aan de hand van reeds bekende en met behulp van in dit onderzoek verkregen feiten, betrekking hebbende op twee typen microörganismen die veelal betrokken zijn bij dit verschijnsel, nl. bacteriën van het *Sphaerotilus*- en *Arthrobacter*-type.

Een beperkte zuurstofvoorziening, zoals die optreedt als gevolg van de snelle omzetting van het koolhydraatgedeelte van zuivelafvalwater, het vermogen om reservestoffen op te hopen en het verschil tussen de potentiële en actuele C/N-verhouding van dit afvalwater werden beschouwd als de belangrijkste factoren die de groei van *Sphaerotilus* bevorderen en daarom verantwoordelijk zijn voor het optreden van opgeblazen actief slib.

Veranderingen aangebracht in de voedingsomstandigheden bleken de groei van gewenste *Arthrobacter*-achtige bacteriën te kunnen bevorderen, waardoor *Sphaerotilus* werd overgroeid en een verbeterde bezinking van het slib kon worden verkregen. Het beste geschiedde dit door Ca-acetaat, en in iets mindere mate door Ca-lactaat, aan het slib toe te voegen op het tijdstip waarop het koolhydraatgedeelte van het afvalwater was verbruikt, doch opneembare stikstofverbindingen nog beschikbaar waren.

Verlaging van de actuele C/N-verhouding door met het zuivelafvalwater tegelijkertijd NH_4Cl te verstrekken, bevorderde eveneens selectief de groei van het actief slib, en wel zodanig dat *Sphaerotilus* geleidelijk in een minder gunstige positie werd gedrongen.

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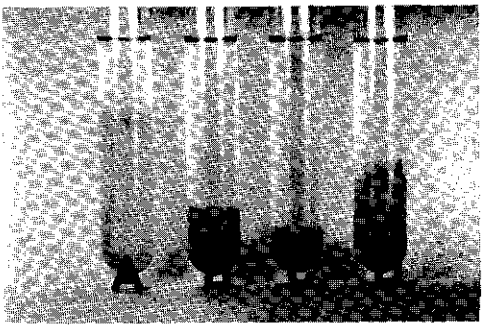

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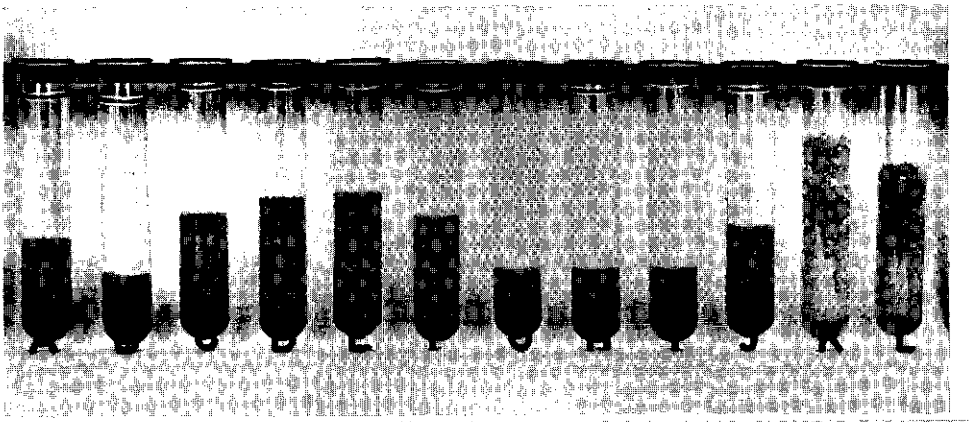

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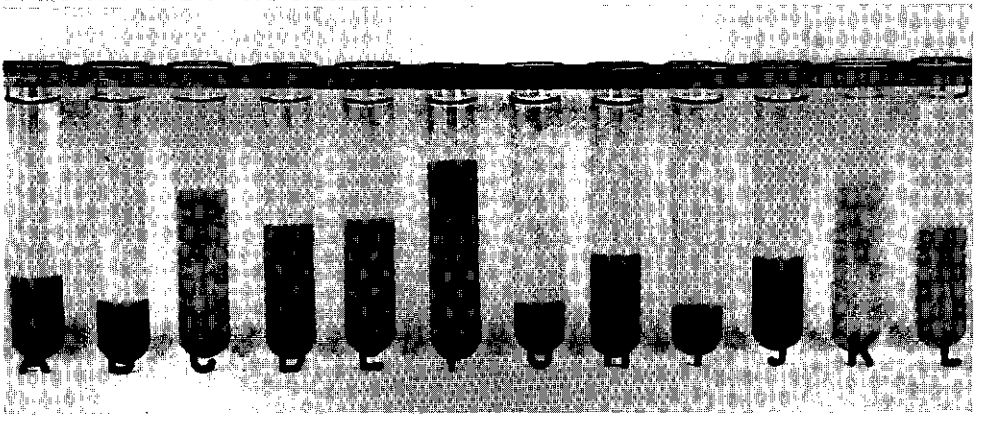


PLATE 1a. Settled sludge volumes after addition of varying substrates and subsequent aeration. Substrate: A: powder mixture; B: Ca-lactate; C: Ca-acetate; D: blank, only aerated; b.,c. Settled sludge volumes after 1 day (b) and after 3 days (c) upon addition of varying compounds and subsequent aeration.

A = blank (only aerated)
B = blank + $CaCO_3$
C = blank + NH_4Cl
D = powder mixture
E = powder mixture + $CaCO_3$
F = powder mixture + NH_4Cl
G = Ca-lactate
H = Ca-lactate + NH_4Cl
I = Ca-acetate
J = Ca-acetate + NH_4Cl
K = acetic acid
L = acetic acid + NH_4Cl

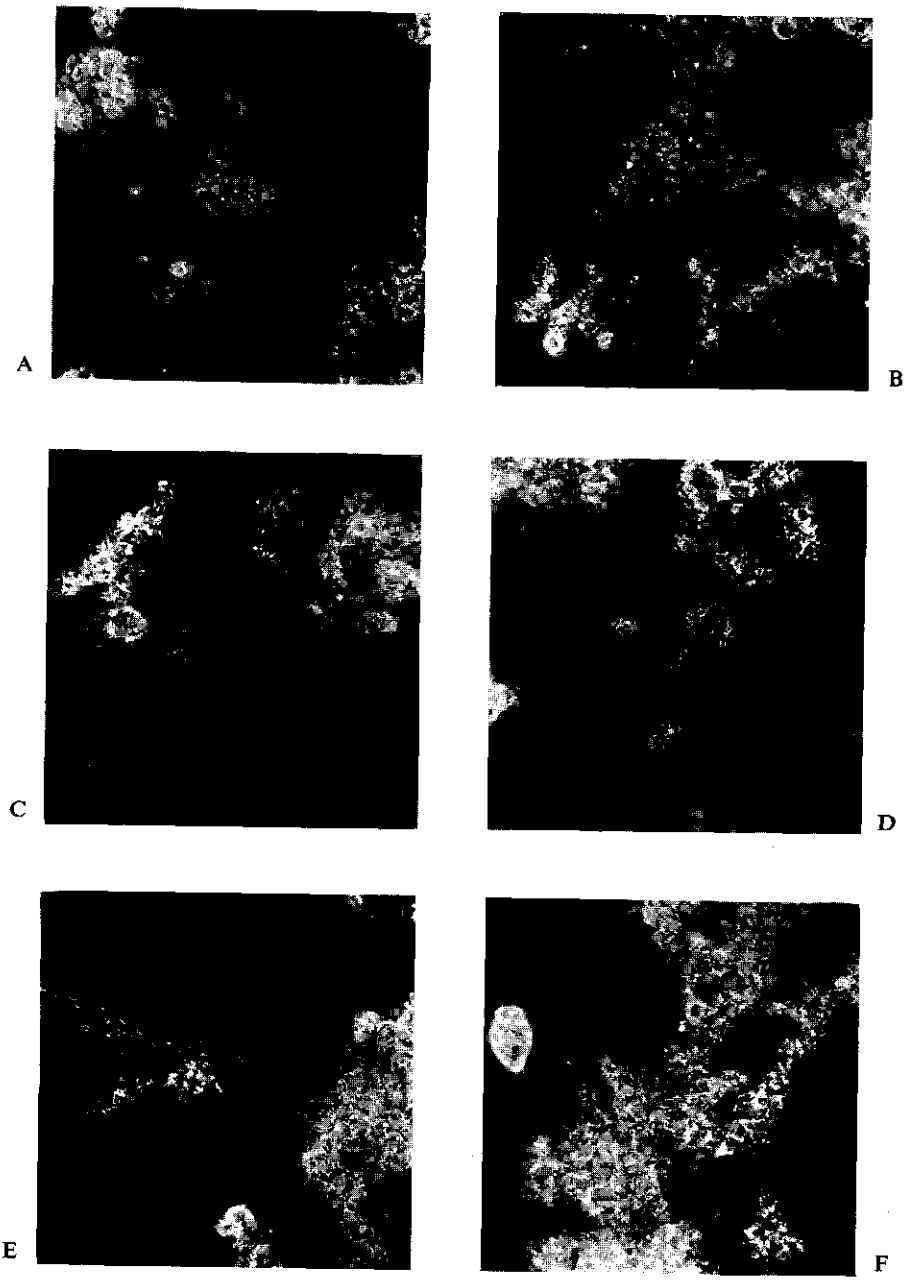
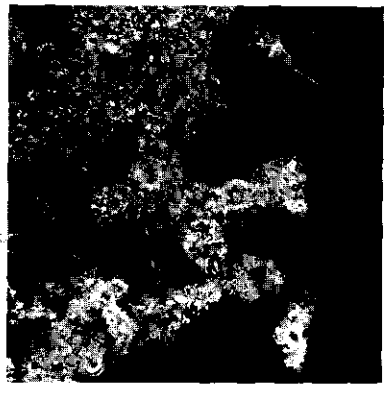


PLATE 2. Photomicrographs of activated sludge after treatment with varying compounds (phase contrast, 100 ×).

A = blank (only aerated)
B = blank + $CaCO_3$
C = blank + NH_4Cl
D = powder mixture
E = powder mixture + $CaCO_3$
F = powder mixture + NH_4Cl



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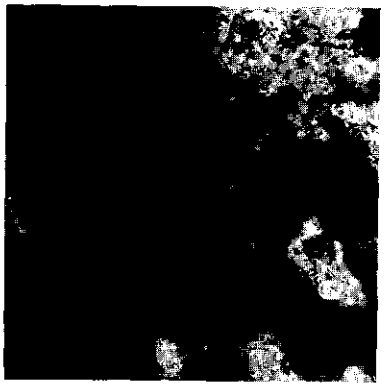

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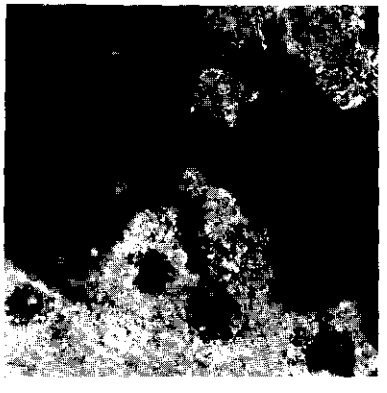

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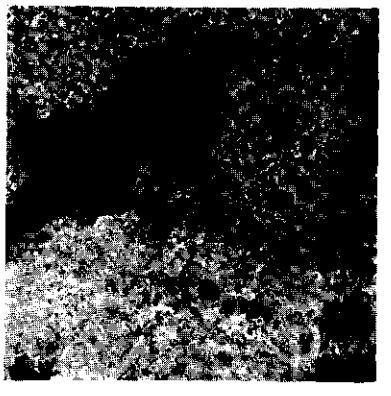

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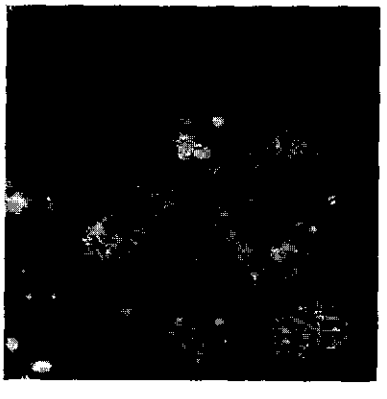

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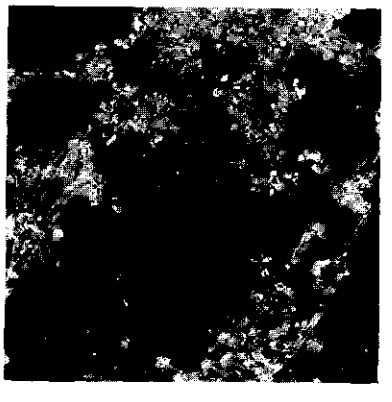

L

G = Ca-lactate
H = Ca-lactate + NH_4Cl
I = Ca-acetate
J = Ca-acetate + NH_4Cl
K = acetic acid
L = acetic acid + NH_4Cl

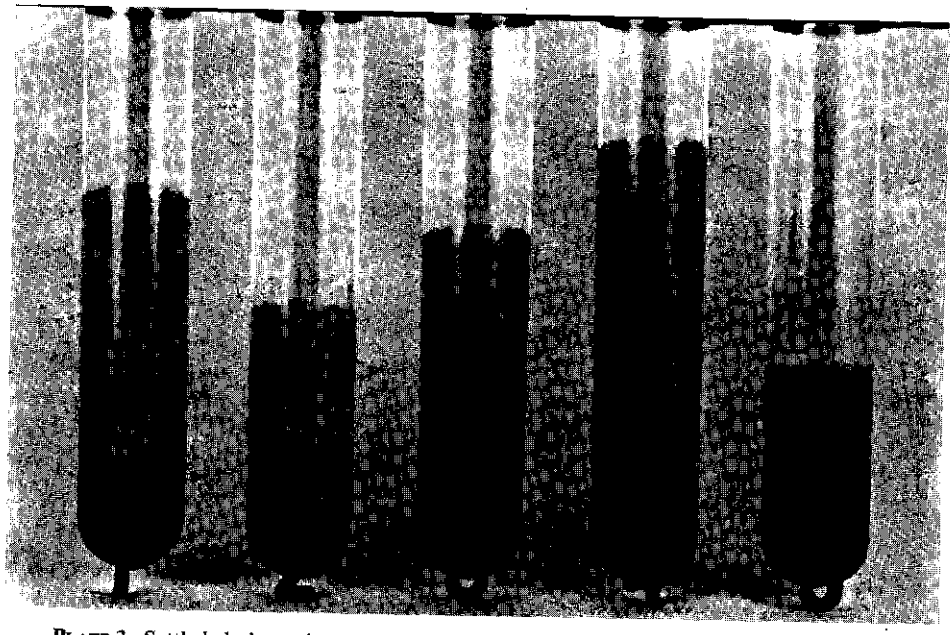

PLATE 3. Settled sludge volumes of activated sludge upon addition of varying compounds and subsequent aeration. 1 = blank (*only aerated*); 2 = blank + $CaCO_3$; 3 = powder mixture; 4 = powder mixture + NH_4Cl; 5 = Ca-acetate.