CASCADES OF BIOREACTORS

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CASCADES OF BIOREACTORS

Proefschrift
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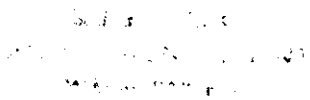


Cover :

A schematical representation of a series of bioreactors.

Ontwerp kaft :

met dank aan Boukje de Gooijer.

STELLINGEN

1 De door Yoshikawa et al. gepresenteerde redenering dat externe diffusielimitatie is uit te sluiten als de specifieke produktiesnelheid constant blijft bij een oplopende celconcentratie in suspensiecultures is fundamenteel onjuist.

Yoshikawa N., Ohta K., Mizuno S. and Ohkishi H. Production of cis-cis-Muconic Acid from Benzoic Acid. In : Tanaka A., Tosa T., Kobayashi T. (Eds), Industrial Application of immobilized biocatalysts, Marcel Dekker, New York, 1993, 137.

2 De door Tyagi en Ghose gesuggereerde procedure voor ontwerp van series heeft met optimalisatie niets van doen.

Tyagi, R.D. and Ghose, T.K. Batch and multistage continuous ethanol fermentation of cellulose hydrolysate and optimum design of fermentor by graphical analysis. Biotechnol. Bioeng. 1980, **22**, 1907-1928.

3 Dat met de slogan "Doodt 99% van de huishoudbacteriën" een schoonmaakmiddel succesvol kan worden verkocht geeft aan dat er voor de biotechnologie op het gebied van de publieksvoorlichting nog een lange weg te gaan is.

4 Daar het ecologisch gevaar bij de toepassing van recombinant-DNA-technologie omgekeerd evenredig is met de omvang van het organisme, terwijl de weerstand tegen recombinant-DNA-technologie in de samenleving evenredig is met de omvang van het organisme, is enige publieksvoorlichting gewenst.

5 De definitie van biotechnologie zoals voorgesteld door Houwink is onjuist, omdat Bioprocestechnologie daarin een basisdiscipline wordt genoemd.

Houwink E. Biotechnology - controlled use of biological information. Kluwer Academic Publishers, Dordrecht, the Netherlands, 1990.

6 De titel "Beter dan God" voor een programma over recombinant-DNA-technologie zegt meer over de makers van het programma dan over de technologie of over God.

7 De huidige hoogte van de arbeidsbeloning leidt tot een merkwaardige driedeling in de westerse samenleving : werkenden, niet-werkenden, en overwerkten.

8 Het koppelen van de zuiveringsheffing aan het waterverbruik bestraft milieuvriendelijke teeltwijzen in volkstuinen.

Sikkema, J. Microbial transformation of tetralin, PhD thesis, Wageningen Agricultural University, 1993.

9 In het algemeen is een fotomodel een te mooie weergave van de werkelijkheid.

10 Nu binnen de Acol-conventie elementen uit de Klaver-conventie worden opgenomen wordt het tijd beide biedsystemen kritisch te evalueren.

11 De gebruikelijke eenheid voor regenval, mm, zou beter kunnen worden veranderd in $m^3.m^{-2}.h^{-1}$.

12 De ambtelijke salaristabel dient verticaal omgekeerd te worden uitgevoerd om het taalgebruik "een periodiek omhoog" beter bij de werkelijkheid aan te laten sluiten.

13 De aanvangstijden van feesten zijn omgekeerd evenredig met de leeftijd van de gastheer of gastvrouw.

14 Om als consument een ecologisch verantwoorde keuze te kunnen maken tussen vliegen en reizen per trein, bus of auto is het noodzakelijk dat op kerosine dezelfde accijns wordt geheven als op dieselolie.

15 Een modern, gemiddeld bezet, vliegtuig op kruissnelheid verbruikt per persoon per kilometer minder brandstof dan een auto met twee inzittenden.

16 De positionering van de regelkranen bij gasfornuizen is een ergonomische blunder.

17 Wegens de onnauwkeurigheid in de beginsituatie bij zwangerschap verdient het aanbeveling het begrip 'uitgerekende datum' te vervangen door 'uitgerekende week'.

18 Bij een prijsvergelijking tussen papieren en katoenen luiers dient de aanschaf van een wasmachine als 'sunk cost' te worden beschouwd.

19 Het vermelden van de mogelijkheid van kinderopvang dient bij alle personeelsadvertenties in gelijke mate te gebeuren.

20 Dat gepureerde tauge geschikte babyvoeding zou zijn, is onjuist.

Ten Hoopen, E. Groeiboek. GVO, Den Haag, 1993, 36.

21 Bij een stijgende participatie van mannen in het huishoudelijk bedrijf is een stijging van de werkbladhoogte van aanrechten en commodes dringend gewenst.

22 Binnen het emancipatiebeleid dient meer aandacht te worden gegeven aan de invoering van zwangerschapsverlof voor mannen.

23 De regels om in het Engels woorden door een koppelteken te verbinden zijn zo verwarrend, dat het de hoogste tijd wordt voor een streepjes-code.

Weiner, E.S.C. and Delahunty, A. The oxford guide to english usage. 2nd edition, BCA, London, United Kingdom, 1994, 27.

24 De suggestie die wordt gewekt dat door een groot aantal auteurs bij een publikatie de bijdrage per auteur steeds minder wordt, berust, zeker voor de eerste auteur, op gezichtsbedrog.

25 De invoering van het dubbelblind-principe bij de beoordeling van ter publicatie aangeboden wetenschappelijke artikelen zal de objectiviteit in het wetenschappelijk bedrijf doen toenemen.

26 In tegenstelling tot wat de positie doet vermoeden, wordt na de eerste auteur van een publicatie de meeste aandacht geschonken aan de laatste auteur.

27 De benamingen Northern, Western and Southern blotting doen ernstige twijfels rijzen omtrent het geografisch inzicht van moleculair-biologen.

28 Het aantal in omloop zijnde verschillende BIOSIM pakketten rechtvaardigt ernstige twijfels aan de creatieve vermogens van software producerende biotechnologen.

29 Hoe geavanceerder de software, hoe groter de kans op fouten. Gelukkig dat tevens de mogelijkheden zo groot worden dat de kans om die fouten te vinden steeds kleiner wordt.

30 Wáren het maar tekst***ver***werkers !

31 De tarieven van de inkomstenbelasting zijn er om de politieke wens tot nivellering uit te voeren. Elke andere van overheidswege voorgeschreven inkomensafhankelijke bijdragentabel dient daarom te worden afgeschaft.

32 En toch lijkt het verstandig geweest de minister-president van een land dat officieel per 15-7-94 461000 werklozen en tegelijkertijd 739000 werkloosheidsuitkeringen telt, geen voorzitter van de Europese Unie te maken.

De Volkskrant, 15-7-1994, 1.

33 Om techniek in bredere lagen van toekomstige studenten als gemeengoed geaccepteerd te krijgen verdient het aanbeveling om in navolging van Duitsland basisfiguren en vergelijkingen op de bankbiljetten af te drukken.

Zehn Deutsche Mark, Deutsche Bundesbank, Frankfurt am Main, 1989.

34 Aangezien bij onderwijsevaluaties studenten wellicht niet in staat zijn tot reflectie, en docenten niet meer onbevangen staan ten opzichte van de stof, zit er niets anders op dan beide meningen serieus te nemen.

35 Vanuit proceskundig oogpunt verdient het aanbeveling op drukke baanvakken de geldende maximumsnelheid te veranderen in een minimumsnelheid met dezelfde waarde.

36 Het advies van de Veluwse Nutsbedrijven om bij afwezigheid de verwarming wat lager te zetten staat haaks op het geafficheerde energiebesparingsimago: uitzetten is altijd voordeliger.

37 Het was correcter geweest als in het voorbeeld waarmee Bird, Stewart en Lightfoot partiële en volledige afgeleiden toelichten gebruik was gemaakt van een duikboot in plaats van een motorboot.

Bird R.B., Stewart W.E. and Lightfoot E.N. Transport phenomena. Wiley, London, United Kingdom, 1960, 73.

Stellingen behorende bij het proefschrift 'Cascades of bioreactors'.

C.D. de Gooijer
Wageningen, 26 juni 1995.

ter nagedachtenis aan mijn vader.

VOORWOORD

Het is verbazend hoe lang het antwoord "volgend jaar" gebruikt kan worden op de veelvuldig gestelde vraag wanneer ik nou eens ging promoveren. Nu is het dan zover.

Tijdens de afgelopen jaren ben ik bij een aantal projecten betrokken geweest waar ik vanaf de zijlijn mocht toekijken en meedenken. Dit heeft in een aantal gevallen geleid tot het maken van een model. Deze modellen hebben steeds als doel gehad het verkrijgen van inzicht in de fenomenen die zich voordeden bij de verschillende processen. Buitengewoon bevredigend was het als een model iets voorspelde wat nog niet was waargenomen of opgevallen, en dat in werkelijkheid ook zo bleek te zijn.

Modellen hebben geen enkele waarde zonder experimentele gegevens. Anderen hebben mij ruimhartig gegevens verstrekt, zodat die gebruikt konden worden bij validatie en verdere verfijning van de modellen. Met name Rene Wijffels, Wilfried Bakker, en Frank van Lier van de sectie proceskunde, en Marcel Kool en Magda Usmany van de vakgroep virologie hebben mij die, vaak zeer moeizaam verkregen, gegevens verstrekt. Bij deze wil ik hen daarvoor bedanken, wees overtuigd van mijn diepe respect voor jullie werk.

In die modellen bleek een duidelijke rode draad aanwezig : alle processen speelden zich af, of konden zich afspelen, in series van bioreactoren. Het is aan Hans Tramper te danken geweest dat ik de ruimte kreeg om dit verder uit te werken in dit proefschrift. Hans, kort en goed : bedankt.

Ruimte alleen bleek echter niet toereikend. Mijn schromelijk tekortschietende kennis van de virologie in zijn algemeenheid en van baculovirussen in het bijzonder werd op altijd boeiende wijze aangevuld door Just Vlak van de vakgroep virologie. Tevens bleek, op het moment dat het laadste loodje spreekwoordelijk zwaar begon te wegen, de steeds mild nuancerende invloed van Rik Beeftink van doorslaggevend gewicht, en zonder zijn behendigheid met Maple had het nog een jaar langer geduurd. Just en Rik : dank !

De basis voor hoofdstuk 7 is al in een zeer vroeg stadium gelegd door Marcel Zwietering. Tevens is zijn kennis van notaties en schrijfwijzen beroemd, waarvan ook ik dankbaar gebruik heb gemaakt.

Wetenschap bedrijven is het stellen van vragen. Van de talloze discussies met Hans, Klaas, Just, Rene, Rik, Marcel, Frank, Fred, Jan, Marcel, Arie, Vitor en Imke heb ik niet alleen veel geleerd, maar ook erg genoten.

Zonder de inbreng van studenten was dit allemaal niks geworden. Harry Hens, Sjon Kortekaas, Gertjan Smolders, Leonard Mallee, Meinard Eekhof, Evelien Beuling, Serge Lochtmans, Hans van 't Noordende, Aldo Schepers, Mirjam den Boer, Hans van den Homberg, Wiebe Kroon, Albert Hamming, Joost Knitel, en uiteraard Rick Koken, bedankt voor jullie bijdragen.

Wilfried, de af en toe 'harde knallers' die we over en weer sloegen hebben mijn conditie op peil gehouden, tussen het zitten achter een buro en zitten in de auto door. De draad is met Marco weer opgepakt.

Hendrik, je kennis van computers en het beschikbaar stellen van botte rekenkracht spaarden mij weken getob. Bedankt !

Henk, grafisch ontwerpen is toch ietsje meer dan wat met tekstverwerkers stoeien. Zonder jou had dit proefschrift er anders uitgezien !

Fred, je hebt de afgelopen jaren heel wat te verduren gehad als medebewoner van kamer 610. Maar zonder ons Algemeen Beschaafd Labs en Inheems Aziatisch als uitlaatklep zou het niet alleen een stuk minder gezellig zijn geweest ! Dat we dat nog lang mogen volhouden.

Hedy, Joyce en Maria : jullie administratieve ondersteuning was meer dan welkom.

Medewerkers van de sectie Proceskunde : wat een club !

Frank, Nettie, Marjet en Ed, afgezien van de verkregen broodnodige gezonde geest na onze avonden pas-pas-pas-doublet, de roddels over het Wageningse biotechnologie-circuit waren me een genoegen.

Elsje, het leven dat wij leiden bestaat niet uit tijd verdelen maar uit tijd vermenigvuldigen. Elkaars werk en kinderen serieus nemen is een moeilijk en heerlijk gebeuren tegelijkertijd. Ik zou het niet anders willen.

CONTENTS

This chapter has been accepted for publication in Enzyme Microbial Technology, by the authors De Gooijer, C.D., Bakker, W.A.M., Beeftink, H.H. and Tramper, J.

CHAPTER 1

BIOREACTORS IN SERIES: AN OVERVIEW OF DESIGN PROCEDURES AND PRACTICAL APPLICATIONS

"*If one fermenter gives good results,*
two fermenters will give better results and three fermenters better still.
This is sometimes true, but often false."

Herbert, D., 1964[A]

INTRODUCTION

Over the last decades, many papers described the design or application of series of bioreactors. Usually, these bioreactors in series are of the continuous stirred tank reactor (CSTR) type. This most widely used bioreactor is easy to

operate, of simple construction, and replacement of biocatalysts and maintenance is not troublesome (Hill, 1977).

The pertinent processes described in literature can be divided into two main groups: processes with a constant overall reaction stoichiometry that can be described by a single kinetic equation, and processes where the stoichiometry is variable and the descriptive kinetic equation changes. The first group consists of those bioprocesses that may well be performed in one bioreactor, but where segregation into two or more bioreactors may lead to a higher product concentration, a larger degree of conversion, or a higher volumetric productivity (also known as space-time yield), or a combination of these factors. The second group is by nature heterogeneous in time or space, and is characterized by two or more independent reactions each governed by its own kinetics, as in biogas production or nitrification/denitrification.

For processes with a fixed overall reaction stoichiometry, this paper first will present a general theoretical outline that enables one to decide *if* a series of bioreactors is favourable. After that, the question of *how* such a series should be designed will be addressed. Subsequently, the general theory will be applied to catalytic reactions (enzymatic conversions), and also to autocatalytic reactions (cells in suspension). This will be done for different types of kinetics. After each theoretical treatise, a number of applications of series of bioreactors will be presented.

For processes with a variable stoichiometry some examples will be presented, since up to now no design rules exist for series with these types of processes. The paper concludes with a short description of bioreactors suitable for bioprocesses in series, both with constant and variable stoichiometry.

The descriptions of a single plug-flow type bioreactor with the tanks-in-series model (Powell & Lowe, 1964, Kleinstreuer 1987) are omitted, as

well as the numerous papers devoted to this subject in the field of chemical engineering. This paper will also solely focus on single-feed series of bioreactors.

CONSTANT STOICHIOMETRY PROCESSES

Processes with a constant overall reaction stoichiometry may show a higher product concentration, a higher degree of conversion, a higher volumetric productivity, or a combination of those if executed in a series of CSTR's when compared to a single bioreactor.

Theory

As a process with a constant stoichiometry, Bischoff (1966), citing Herbert (1964A), describes an optimal series for biomass production, consisting of a CSTR followed by a plug-flow reactor. Such a system may be conceived of as one large CSTR, followed by an infinite number of infinitesimally small CSTR's (Figure 1A; for the calculations underlying figure 1 see appendix). This combination of a CSTR followed by a PFR has the lowest total residence time to achieve a certain degree of conversion (Figure 1A-G). Since for biomass production in most cases oxygen is required, and an aerated PFR does not exist, an alternative to this combination is a series of CSTR's with equal volume (Figures 1B and 1C), or a series of unequal-volume CSTR's (Figures 1D and 1E). Implicitly, figure 1, which holds with no biomass in the influent, imposes that the desired degree of conversion of the process determines whether a series of bioreactors is favourable or not : if the substrate concentration at the exit of the

series is to the left-hand side of point A in figure 1A (a high degree of conversion) then a series is favourable, if it would be to the right-hand side of point A (a low degree of conversion), a single CSTR would be the best solution. It should be noted, as also stated by Moser (1985), citing Topiwala (1974), that the PFR/CSTR volume ratio for such an optimal configuration is strongly dependent on the ratio $\kappa = K_s / S_0$, with K_s the Monod constant (mol.m^{-3}) and S_0 the substrate concentration at the inlet of the series (mol.m^{-3}) (Figure 2).

For Monod kinetics, and a quite common influent concentration $\kappa = 0.01$ (Van 't Riet & Tramper 1991), the minimum in figure 1C is attained at $\alpha = S / S_0 = 0.1$, indicating that up to conversions of 90% a single CSTR (without any PFR) would perform optimally.

As far as the design of a reactor series is concerned, a distinction has to be made between the design of a series of equal volume CSTR's, and a series of non-equal volume CSTR's, the latter also referred to as optimal design.

Figure 1. For a continuous-flow system with a autocatalytic processes (microbial reaction, Monod kinetics, single feed and no biomass in the influent), at a fixed stoichiometry, optimal design is inferred from a plot of the reciprocal dimensionless rate ($1 / \rho = \mu_{max} S_0 / r_s$) versus the dimensionless concentration ($\alpha = S / S_0$). Due to the existence of a minimum in the curve, minimalization of overall holding time may require a CSTR and a PFR in series. Shaded areas: holding times. A 95% degree of conversion is aimed for, and $\kappa = K_s / S_0 = 1$. The number listed at the top of the graph is the total dimensionless holding time of each configuration.

A: one CSTR followed by a Plug-flow reactor. Point A is the minimum of the curve, corresponding to α_{crit}.

B: series of 3 equal-sized CSTR's designed by the procedure described by Fiechter (1981); the first vessel operates at the maximum rate. Note that more than 95% is converted.

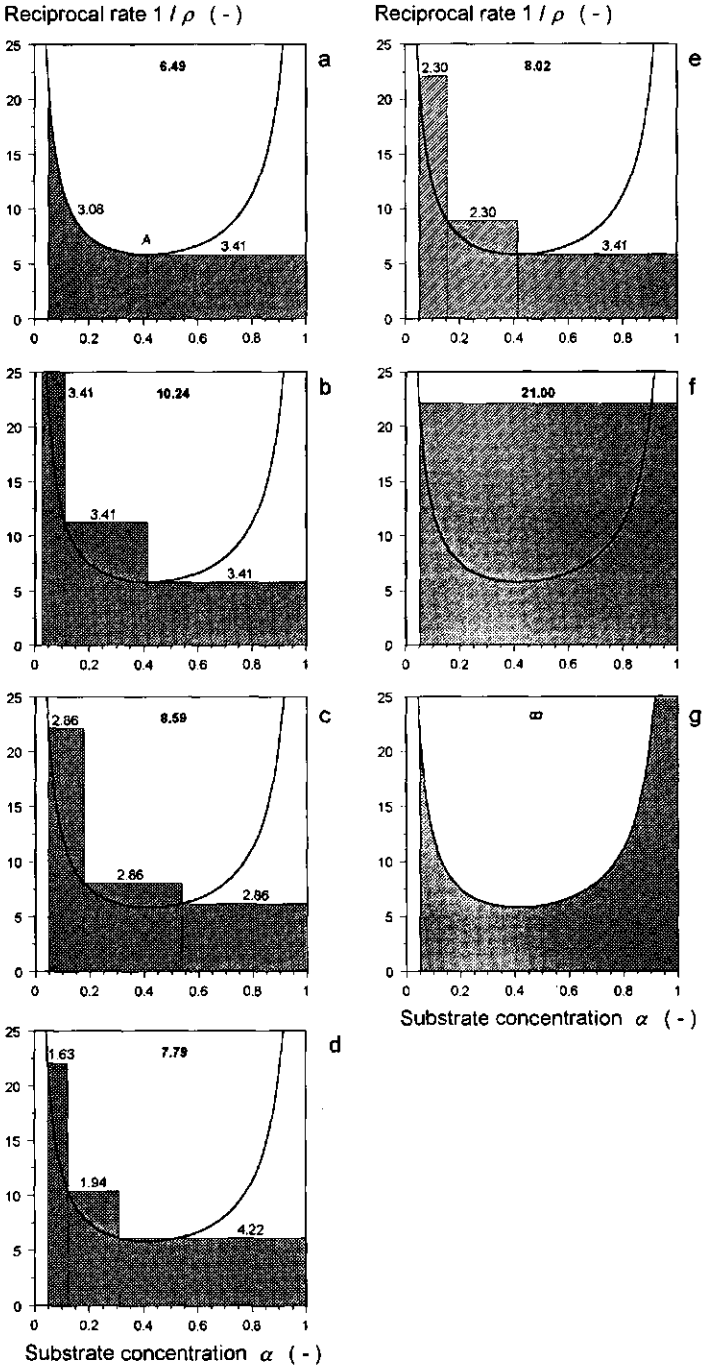
C: series of three equal-sized CSTR's where the first vessel operates at less than the maximum rate.

D: series of three unequal-sized CSTR's, optimal design according to Hill & Robinson (1989).

E: series of three unequal-sized CSTR's, where the first vessel operates at the maximum rate and the subsequent vessels have an equal volume.

F: one single CSTR.

G: one single PFR.


Reciprocal rate 1 / ρ (-)
a
6.49
3.08
A
3.41
b
3.41
10.24
3.41
3.41
c
2.86
8.59
2.86
2.86
d
1.63
7.79
1.94
4.22
Substrate concentration α (-)
Reciprocal rate 1 / ρ (-)
e
2.30
8.02
2.30
3.41
f
21.00
g
∞
Substrate concentration α (-)

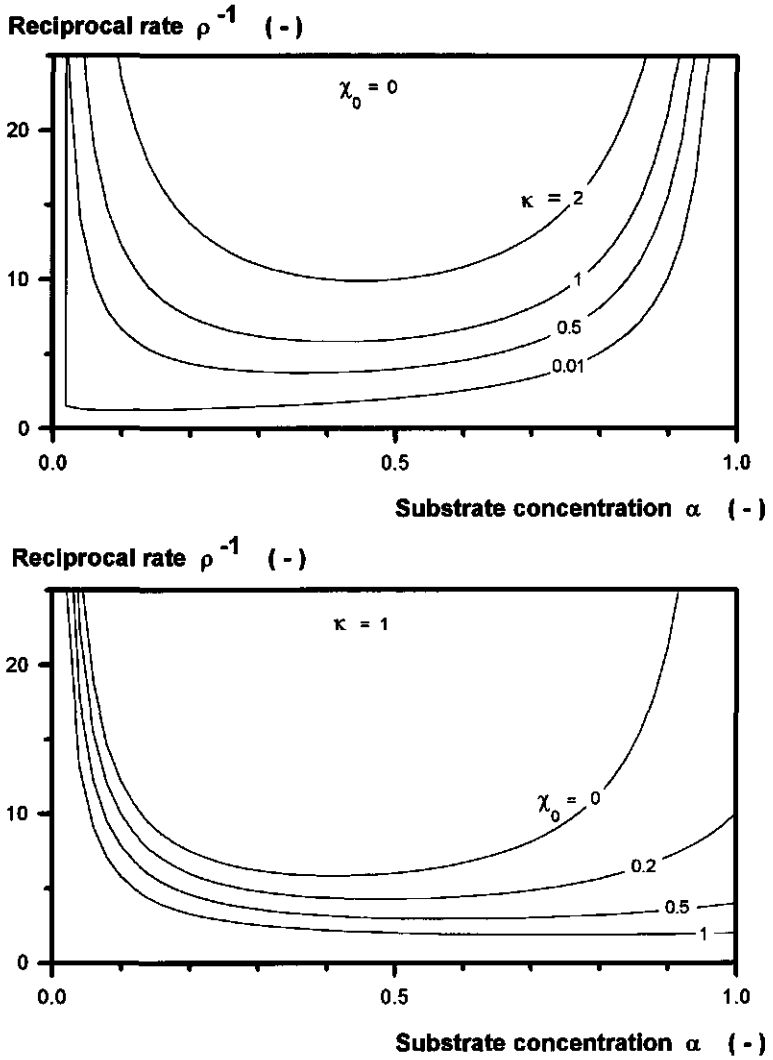


Figure 2. Dimensionless rate ($1 / \rho = \mu_{max} S_0 / r_s$) versus the dimensionless concentration ($\alpha = S / S_0$). Upper graph: parameter $\kappa = K_s / S_0$, with no biomass in the influent ($\chi_0 = 0$). At low κ, the minimum in the curves (α_{crit}) decreases to a low value, indicating that a single CSTR performs best for most exit concentrations. Lower graph: parameter $\chi_0 = X_0 / Y_{xs} S_0$, with $\kappa = 1$. At high biomass concentrations at the inlet of the series, the minimum of the curves (α_{crit}) shifts to 1, indicating that series of CSTR's perform best for all exit concentrations.

Design of a finite equal-volume series

The theory of a series of equal-sized CSTR's was first treated by Herbert (1964B), and later cited by Moser (1988). Two performance criteria were defined: biomass productivity ($kg.m^{-3}.s^{-1}$), and effective yield, defined as the ratio of biomass concentration at the outlet and the substrate concentration at the inlet of a bioreactor system. For the first criterion he showed that for a single-stage system this productivity equals $D.X$ with D the dilution rate (s^{-1}) and X the biomass concentration ($kg.m^{-3}$), whereas for a two-stage system this is $X_2.D_{ave}$, with $D_{ave} = D_2/2$ (or, in general terms, $D_{ave} = F / (NV)$, with V the volume (m^3) of each vessel in the series of N reactors, and F the flow rate ($m^3.s^{-1}$)). For single-feed series, three conclusions could be drawn, as illustrated in figure 3:

i) the maximum possible overall productivity is higher in a single-stage system,

ii) at lower dilution rates the overall productivity of a two-stage system is slightly larger than that of a single-stage system, and, derived from this,

iii) at lower dilution rates, the effective yield (or the utilization of substrate) is slightly higher in a two-stage system.

Herbert (1964B) also stated that more than two bioreactors in series have no practical advantage as far as the volumetric productivity is concerned. However, later stages might improve product quality, since the endogenous metabolism will continue in a third stage, leading to changes in the chemical and physiological state of the cells. Note that this in fact implies a change in stoichiometry.

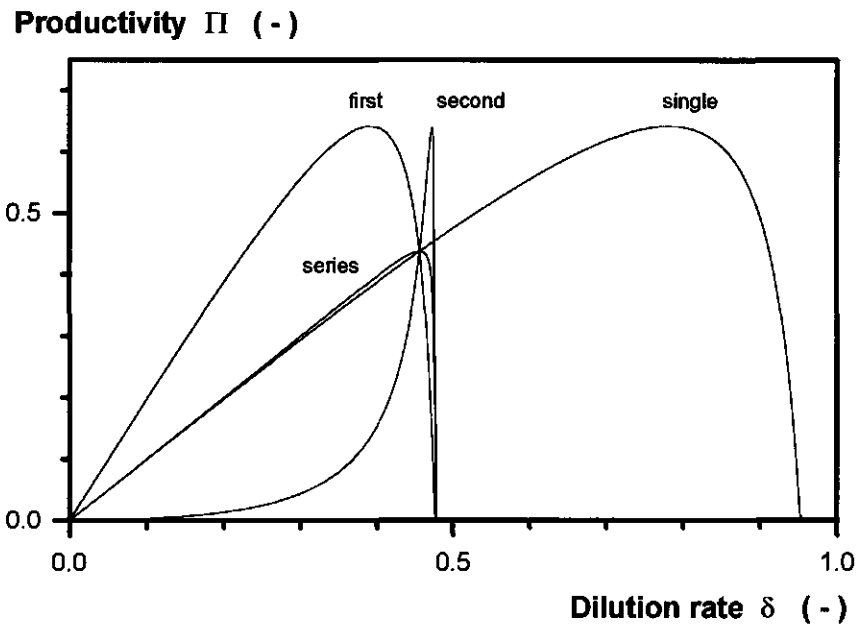


Figure 3. Dimensionless production rate per unit of reactor volume ($\Pi = D.X / \mu_{max} Y_{xs} S_0$) in an equal-volume two-stage series versus dimensionless dilution rate ($\delta = D / \mu_{max}$) as compared to a single CSTR. Each reactor on itself (the first and second reactor in the series, and the single CSTR) shows the same maximum in productivity. At low overall dilution rates, the series shows a slightly higher overall productivity; maximum productivity is attained in a single reactor at high dilution rates. Monod kinetics, with $\kappa = K_s / s_0 = 0.05$.

For multiple-feed series of bioreactors, Fencl et al. (1969, 1972) showed that under certain conditions the productivity can be higher than for a single CSTR. Herbert (1964B) gives mathematical descriptions of a multiple-feed reactor system. For an extensive theoretical treatise of series with multiple-feed operation we refer to Fencl (1966).

Fiechter (1981), citing Fencl (1966) and Deindoerfer & Humphrey (1959), describes a four-step graphical procedure for the design of a series of chemostats of equal volume for biomass production. The first vessel should, in

their view, operate at the maximal volumetric productivity, and subsequently the number of (equal-sized) vessels is determined in order to achieve a certain degree of conversion or biomass concentration (Figure 4):

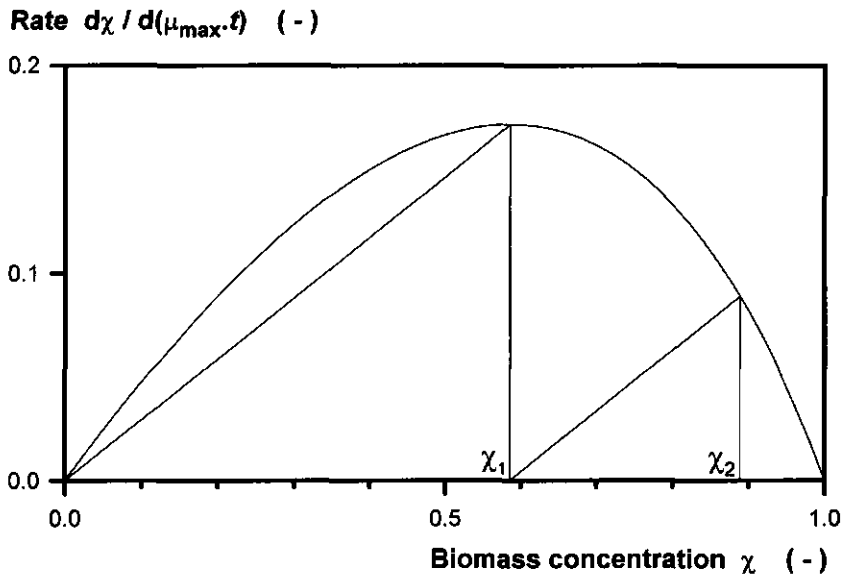


Figure 4. For a cascade of equal-volume CSTRs and a microbial reaction with Monod kinetics, the number of vessels in the series may be determined from a dimensionless rate vs. dimensionless concentration plot according to Fiechter (1981). Slopes represent dilution rates; the first reactor operates at the maximum rate. See text for details.

i) Obtain $d\chi/d\mu_{max}t$ as a function of χ, the dimensionless biomass concentration $X / Y_{xs}S_0$ with Y_{xs} the yield of biomass on substrate ($kg.mol^{-1}$), from a batch experiment or from mass balances over substrate and biomass if the parameters in the kinetic equation are known.

ii) Plot $d\chi/d\mu_{max}t$, with μ_{max} the maximum specific growth rate (s^{-1}) and t the time (s) versus χ.

iii) Draw a line from $\chi=0$ to $\chi=\chi_1$, the dimensionless concentration of biomass in the first stage, which is found at the maximum of the curve. The slope of this line is equal to F/V_i.

iv) Keep the slope of this line constant, start at $\chi=\chi_1$ and determine χ_2, and so forth.

Similarly, if product formation is the aim of the process, dP/dt versus the product concentration P ($mol.m^{-3}$) can be plotted, as applied by Tyagi & Ghose (1980) for the design of an ethanol fermentation with cellulose hydrolysate as substrate. For enzymatic conversions the latter procedure is also adequate.

As shown in figures 1B and 1C, this procedure may lead to a higher degree of conversion than needed. The alternative design of series of equal-sized CSTR's can be done iteratively, starting with a desired rate of conversion calculating back along the series, or by means of a zero-finding routine, solving the set of equations for each CSTR in the series, in a similar way as will be discussed below.

Optimal Design of a finite series of non-equal-volume-CSTR's

The theory on cascade design may be quite perplexing, due to the plethora of performance criteria and kinetic equations (and combinations thereof). Although quite relevant, economic criteria will be ignored here in favour of an engineering measure : Luyben & Tramper (1982) defined optimal design of a finite series of CSTR's as that configuration that has the minimal total holding time at a given degree of conversion in a series consisting of N reactors. This means that the volumes for all reactors along the series are varied, with a subsequent change in the intermediate substrate concentrations, until a minimal total volume is

reached. Mathematically this leads to:

$$\frac{\partial\left[\sum_{j=1}^{N}\tau_j\right]}{\partial\alpha_i}=0 \qquad i=1, 2, \ldots (N-1) \qquad [1]$$

where α_i is the dimensionless substrate concentration in the i-th vessel, S / S_0, so $\alpha_0=1$, N is the pre-defined number of vessels, and τ_j is the dimensionless residence time in the j-th vessel. τ is defined as $V_i v_{max} e / F S_0$ for enzymes with v_{max} the maximum specific velocity per unit amount of enzyme ($mol.kg^{-1}.s^{-1}$) and e the enzyme concentration ($kg.m^{-3}$), and as $V_i \mu_{max} / F$ for autocatalytic reactions. Although Luyben & Tramper (1982) defined this optimal design for a dissolved enzyme following Michaelis-Menten kinetics, equation [1] is independent of the kinetics involved, and has been used by many authors (Malcata 1988, Hill & Robinson 1989, De Gooijer et al. 1989, Malcata & Cameron 1992, Lopes & Malcata 1993, Paiva & Malcata 1993).

For autocatalytic systems (suspended micro-organisms), Schügerl (1987) and Hill & Robinson (1989) introduced the concept of a dimensionless critical effluent substrate concentration α_{crit}. At dimensionless effluent concentrations up to or equal to α_{crit}, the optimal "series" is the single tank. This critical concentration can be found by assuming that the dimensionless effluent concentrations of the first and the second vessel in the optimal series are the same, or $\alpha_1 = \alpha_2$ (= α_{crit}), meaning that the volume of the second vessel is zero. If it is found that the desired dimensionless effluent concentration α_N is smaller than α_{crit}, an optimal design is feasible. In figure 1 and 2, α_{crit} is the minimum of the curve.

Below, the α_{crit} concept and the optimal design of a series will be described for both autocatalytic and catalytic processes. To further classify the latter group,

a division is made between non-growing and growing biocatalysts. Within each division, a subclassification is made between freely suspended and immobilized biocatalysts.

AUTOCATALYTIC SYSTEMS

In this section papers dealing with growing cells (Figures 1-4), freely suspended or immobilized, will be reviewed.

Theory

The design of a reactor cascade for autocatalytic reactions depends on the optimization criterion. The minimum overall residence time at an arbitrary but fixed exit substrate concentration will be used as a criterion. To investigate *if* a series is worth considering, the critical effluent concentration at which the reaction order changes from positive to negative (point A in figure 1A) and at which the optimal cascade in fact is a single tank (α_{crit}), may be obtained from a procedure given by Hill & Robinson (1989) and by Schügerl (1987), as discussed below.

A generalized form of the growth equation is used:

$$\frac{\mu}{\mu_{max}} = \frac{p\alpha + q\alpha^2}{f + g\alpha + h\alpha^2} \qquad [2]$$

Here p, q, f, g, and h are dimensionless parameter groups depending on the particular kinetic equation (Table1). Combining the specific growth rate μ (s^{-1})

Table 1. Values of the parameters *f*, *g*, *h*, *p*, and *q* in the general rate equation for autocatalytic reactions (Equation 2) and the critical substrate concentration α_{crit} at which an optimally designed series of bioreactors consists of a single vessel. For lethal product inhibition kinetics the implicit equation for α_{crit} has to be used, for all other kinetics a more convenient expression can be found, as shown.

	MONOD	SUBSTRATE INHIBITION	NON-LETHAL PRODUCT INHIBITION	LETHAL PRODUCT INHIBITION
$\frac{\mu}{\mu max} =$	$\frac{s}{K_S+s}$	$\frac{s}{K_S+s+\frac{s^2}{K_i}}$	$\frac{s}{K_S+s}\frac{1}{1+\frac{p}{K_p}}$	$\frac{s}{K_S+s}\left(1-\frac{p}{K_p}\right)$
	$\frac{p\alpha+q\alpha^2}{f+g\alpha+h^2}$			
p	1	1	1	$1-\frac{p_0}{K_p}-\frac{Y_p s_0}{K_p}$
q	0	0	0	$\frac{Y_p s_0}{K_p}$
f	$\frac{K_S}{s_0}$	$\frac{K_S}{s_0}$	$\left(1+\frac{p_0}{K_p}+\frac{Y_p s_0}{K_p}\right)\frac{K_S}{s_0}$	$\frac{K_S}{S_0}$
g	1	1	$1+\frac{p_0}{K_p}+\frac{Y_p s_0}{K_p}\left(1-\frac{K_S}{s_0}\right)$	1
h	0	$\frac{s_0}{K_i}$	$-\frac{Y_p s_0}{K_p}$	0
α_{crit} (implicit)	$(1-\alpha_{crit})\left(1+\frac{(1+\chi_0-\alpha_{crit})(g+2h\alpha_{crit})}{(f+g\alpha_{crit}+h\alpha_{crit}^2)}-\frac{(1+\chi_0-\alpha_{crit})(p+2q\alpha_{crit})}{p\alpha_{crit}+q\alpha_{crit}^2}\right)=0$			
α_{crit}	$\sqrt{f^2+f(1+\chi_0)}-f$	$\frac{\sqrt{f^2+f(1+\chi_0)(1+h(1+\chi_0))}-f}{1+h(1+\chi_0)}$	$\frac{\sqrt{f^2+f(1+\chi_0)(g+h(1+\chi_0))}-f}{g+h(1+\chi_0)}$	

with the yield factor Y_{xs} (kg.mol^{-1}) and the biomass concentration X (kg.m^{-3}), the substrate consumption rate r_s (mol.m^{-3}.s^{-1}) is obtained :

$$r_S = \frac{\mu}{Y_{xs}} X \qquad [3]$$

A general mass balance over a single CSTR in a series yields :

$$\theta_i = \frac{V_i}{F} = \frac{(\alpha_{i-1} - \alpha_i)}{r_{s,i}/s_0} \qquad [4]$$

where θ_i is the residence time in the *i*-th reactor (s). Substituting equation [2] and [3] in equation [4] results in an expression for the dimensionless residence time for each vessel in the series in terms of substrate concentrations and kinetic parameters:

$$\tau_i = \frac{V_i \mu_{max} X_i}{F S_0 Y_{xs}} = \frac{(\alpha_{i-1} - \alpha_i)(f + g\alpha_i + h\alpha_i^2)}{p\alpha_i + q\alpha_i^2} \qquad [5]$$

which, with the result of a mass balance over all vessels in the series:

$$X_i = Y_{xs}(S_0 - S_i) + X_0 \qquad [6]$$

can be written as :

$$\tau_i = \frac{V_i \mu_{max}}{F} = \frac{(\alpha_{i-1} - \alpha_i)(f + g\alpha_i + h\alpha_i^2)}{(1 + \chi_0 - \alpha_i)(p\alpha_i + q\alpha_i^2)} \qquad [7]$$

For a two-reactor cascade (*N*=2 in equation [1]), operated at a fixed, arbitrary effluent concentration α_2, optimization requires:

$$\frac{d(\tau_1 + \tau_2)}{d\alpha_1} = 0 \qquad [8]$$

and from this equation, an optimal α_1 value is obtained as a function of the effluent concentration chosen (α_2). Implicitly, the volumes of both reactors are defined by this result.

In order to answer the question as to whether or not an optimally designed series is worthwhile, in other words if the desired effluent concentration is to the left of point A in figure 1A, the minimum of this curve has to be found.

Substituting equation [7] in equation [8], and applying $\alpha_1 = \alpha_2 = \alpha_{crit}$ for this general case of 2 reactors in series, results in the following implicit equation:

$$(1-\alpha_{crit})\left(1+\frac{(1+\chi_0-\alpha_{crit})(g+2h\alpha_{crit})}{f+g\alpha_{crit}+h\alpha_{crit}^2}-\frac{(1+\chi_0-\alpha_{crit})(p+2q\alpha_{crit})}{p\alpha_{crit}+q\alpha_{crit}^2}\right)=0 \quad [9]$$

Table 1 shows the expression for α_{crit} for different types of kinetics. If indeed two reactors in series are superior to a single vessel, that is $\alpha_2 < \alpha_{crit}$, then this procedure may be used to show the favourablity of multi-reactor cascades (Hill & Robinson 1989). This means that :

i) if two reactors in series are superior to a single vessel then any series of reactors will be superior, and

ii) the optimization of the design requires an infinite number of vessels that areincreasingly smaller along the series, or, the optimal reactor configuration is a single CSTR followed by a PFR.

It can be derived from table 1 that a cascade is particularly suited for product-inhibited autocatalytic reactions: increasing the severity of the inhibition (i.e. decreasing K_p, the inhibition constant (mol.m^{-3})), results in an increase in α_{crit}, thus widening the feasibility range for a cascade.This may also be clarified by taking the limit for K_p to zero of α_{crit}. To find this expression, the realtion for α_{crit} from the bottom row in table 1 is used, and appropriate expressions for f,g,h,p, and q are substituted. Thereby, for the sake of simplicity, it is assumed that both the biomass and product concentration are zero at the inlet of the first reactor (χ_0=0 and P_0=0). For non-lethal product inhibition it can then be found that:

$$\lim_{K_p\to 0}\alpha_{crit}=\lim_{K_p\to 0}\frac{(K_p+Y_pS_0)K_s+\sqrt{K_sK_p(K_p+Y_pS_0)(K_s+S_0)}}{S_0(Y_pK_s-K_p)}=1 \quad [10]$$

Hence at very strong inhibition (K_p approaching zero) a cascade becomes superior at all effluent concentrations (α_{crit} approaching one).

In case of substrate inhibition, the reverse is true: low K_i values entail low α_{crit} values, or, the more severe the substrate inhibition is, the less favourable series of reactors will become.

Now having the answer to the question *if* a series is worthwhile, that is after the determination of α_{crit}, the optimal design of the series according to equation 1 (*how*) can generally be done as suggested by Hill & Robinson (1989). For N reactors in series, N-1 equations with N-1 unknown substrate concentrations can be derived by substituting equation [7] in equation [1] and taking the differentiation:

$$\frac{B(g+2h\alpha_i)-A}{CD}+\frac{BA}{C^2D}-\frac{BA(p+2q\alpha_i)}{CD^2}+\frac{f+g\alpha_{i+1}+h\alpha_{i+1}^2}{(1+\chi_0-\alpha_{i+1})(p\alpha_{i+1}+q\alpha_{i+1}^2)}=0 \quad [11]$$

with

$$A=f+g\alpha_i+h\alpha_i^2;\ B=\alpha_{i-1}-\alpha_i;\ C=1+\chi_0-\alpha_i;\ D=p\alpha_i+q\alpha_i^2 \quad [12]$$

and i = 1..N-1.

This set of equations can be solved by a suitable algorithm with a zero-finding routine on a PC. Alternatively, for the first two reactors in series, a more convenient design equation can be found for all kinetics with q=0 (all kinetics except lethal product inhibition), and no biomass in the influent of the series (χ_0=0). Substition of equation [7] in equation [8] will then lead to an expression for α_1 in terms of α_2 :

$$\alpha_1^2=\frac{f\alpha_2(1-\alpha_2)}{\alpha_2(h+g)+f} \quad [13]$$

which is the same as found by Hill & Robinson (1989). Hence, for a two-reactor cascade, equation [13] is sufficient, and if the series consist of more than two reactors, equation [13] combined with equations [11] and [12] has to be used.

After determination of the intermediate substrate concentrations, the required residence times are easily obtained through equation [7].

Hill & Robinson (1989) showed for Monod kinetics, Aiba kinetics (product inhibition) and Haldane kinetics (substrate inhibition) that if indeed a series design is favourable (i.e. if $\alpha_N<\alpha_{crit}$), three optimally designed non-equal volume CSTR's will provide an overall residence time that is close to the possible minimum, i.e. the CSTR-PFR sequence suggested by Bischoff (1966). Also, it was shown that if three equal-sized CSTR's are used, the decrease in overall residence time is less than with an optimally designed series of non-equal-volume-CSTR's. If a series of equal-sized CSTR's is used, one should consider washout problems in the first vessel. It is also pointed out that although for substrate inhibition kinetics one would intuitively choose a single CSTR, depending on α, series of CSTR's might still be advantageous (Table 1).

A complication is the fact that with for example Haldane kinetics, the first reactor in the series operating at a substrate concentration beyond that concentration where the rate is maximal becomes inherently unstable (operating at a point to the right from point A in figure 5 : a small increase in the substrate concentration will result in washout, whereas a small decrease will result in an operating point to the left of point A at the same rate).

In the theory above, the assumption was made that all reactors in the series can be described by the same kinetic parameters. Lo et al. (1983) discuss the situation where the parameters of the Monod equation are non-identical in the different vessels of the series. They showed, for that case, with the use of two equal-sized bioreactors in series, that design according to the rules

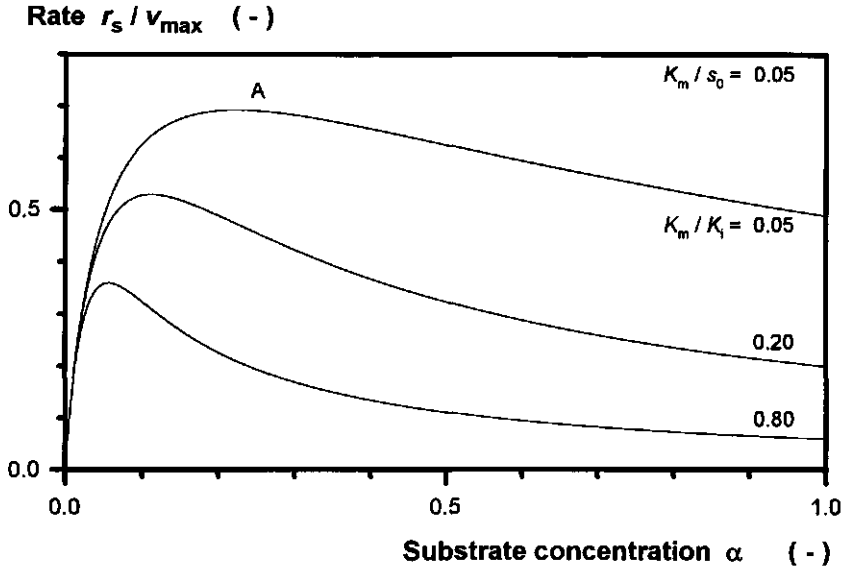


Figure 5. Dimensionless rate (r_s / v_{max}) versus dimensionless substrate concentration (α = S / S_0) for substrate inhibition kinetics. The dimensionless substrate concentration at which the maximum rate is attained (point A) equals α_{crit}; it decreases with increasing severity of the inhibition (i.e. lower Ki values).

discussed above is no longer possible.

Cells in suspension

In this section some examples of freely suspended cells will be discussed, whereas quantitative data on ethanol production are given in table 2, and a review of other processes described in literature is given in table 3.

For the production of lactic acid from whey permeate Aeschlimann et al. (1990) conclude that the dilution rate of the series affects all important fermentation parameters. In a single vessel, a maximum volumetric productivity

of 2.3 10^{-3} kg.m^{-3}.s^{-1} could be reached at a dilution rate of 1.1 10^{-4} s^{-1} with a degree of conversion of 50%. The addition of a second (non optimally designed) bioreactor resulted in an increase of the degree of conversion, but also in a decrease of the volumetric productivity, due to the additional reactor volume, which is consistent with theory (Figure 3). From their data of the first reactor only, plotted as r_s^{-1} versus S (compare figure 1D), they simply searched for the minimum area under the curve for two reactors in series, which is a correct approach. Further assuming that the data of the first reactor should also be valid for the second reactor, they conclude that for their case the total reactor volume may be reduced by almost 50% as compared to one fermentor (overall residence times of 3.2 10^4 s for the series as compared to 5.9 10^4 s for the single vessel, for a degree of conversion of 98%). They further report values for μ_{max}, S_0, and S_N of 1.9 10^{-4} (s^{-1}), 49.2, and 0.9 (kg.m^{-3}), respectively. In order to be able to illustrate the design procedure discussed above, Monod kinetics were assumed, and a value for K_s was obtained from a fit to their r_s^{-1} versus S plot for the first vessel in the series : 11.6 (kg.m^{-3}). Using table 1 (Monod kinetics) a value of a_{crit} of 0.3 can be calculated, indicating that indeed a series is favourable. Subsequently, α_1 is found through equation [13] to be 0.13 (α_2 = 0.018, fixed by the desired degree of conversion). Equation [7], for the case of Monod kinetics, reduces to $V_2\mu_{max}/F=(\alpha_1-\alpha_2)(K_s/S_0+\alpha_2)/((1-\alpha_2)\alpha_2)$ and $V_1\mu_{max}/F=(K_s/S_0+\alpha_1)/\alpha_1$ for the second and first vessel of the series, respectively. This then enables the calculation of the residence times : 1.5 10^4 and 0.8 10^4 s for the first and second vessel in the series, respectively. This results in an overall residence time of 2.3 10^4 s. If α_1 = 0.018 is substituted in the latter derivation of equation [7], the residence time in a single vessel can be calculated to be 7.1 10^4 s. The differences in residence times found here compared to those found by Aeschlimann et al. may be explained by the organism not

Table 2: Quantitative data on ethanol fermentation by several species of *Saccharomyces* and *Zymomonas*. *N* is the number of vessels in the series, x_a is the attained degree of conversion (%), *P* is the product concentration (kg.m^{-3}), and Q_p is the reported volumetric productivity (10^{-3} kg.m^{-3}.s^{-1}). a: S is Single feed at the entrance of the series, M is multiple feed in all reactors, b: 5 10^{-3} is with cell recycle, c: if a batch reactor requires 100 capital investment and 100 running costs, they calculate 62 and 89 for their series, respectively, d: two fluidized beds with recirculation followed by a plug-flow reactor, e: multistage fluidized bed with sieve plates, f: both vessels with settler and recycle.

N	X_A	*P*	Q_p	IMMOB. TECHN.	S[A] M	SUBSTRATE	MICRO-ORGANISM	REFERENCE
2	-	106	1.4/5[b]	-	S	glucose	*Z.mobilis*	Charley *et al.* (1983)
2	97	60	0.8	-	M	sugar beet molasses	*Z.mobilis*	Park & Baratti (1992)
10	99	90	-	-	S	glucose	*S.sake, S.c* UG5, *S. bayanus*	Bovee & Sevely (1982)
2	80	90	1.1	-	S	glucose	*S.cerevisiae*	Lee *et al.* (1983)
6	99	80	-	-	M	glucose	*S.bayanus*	Dourado *et al.* (1987)
4	86	98	-	-	S	glucose	*S.cerevisiae* UG5	Chattaway *et al.* (1988)
8	-	-	0.4	-	S	glucose	*S.cerevisiae* UG5	Moreno & Goma (1979)
-[e]	96	78	-	alginate	S	cane molasses	*S.formosensis* M111	Fukushima & Hanai (1982)
5[c]	92	85.4	1.4	-	M	cane molasses	*S.uvarum*	Chen & Mou (1992)
3/5	81	-	-	-	M	cane molasses	*S.uvarum*	Chen (1990)
2	80	-	13.4	sintered glass	S	hydrolized waste starch	*Z.mobilis*	Weuster *et al.* (1990)
2	95	-	8.3	sintered glass	S	hydrolized waste starch	*Z.mobilis*	Weuster *et al.* (1990)
3	98	74	15.8	alginate	S	glucose	*Z.mobilis* DSM424	Klein & Kressdorf (1983)
3[d]	100	72	30	alginate	S	glucose	*Z.mobilis*	Klein & Kressdorf (1986)
2	97	81	5.6	flocc.	S	cane molasses	*S.cerevisiae* HA2	Kida *et al.* (1990)
2[f]	99	93	2.6	flocc.	M	cane molasses	*S.cerevisiae* IR-2	Kuriyama *et al.* (1993)
8[e]	98 82	93 77	3.9 4.7	alginate	S	glucose	*S.carlsbergensis*	Tzeng *et al.* (1991)
6	-	130	1.1	wood chips	S	glucose	*S.carlsbergensis* STV89	Ryu *et al.* (1982)

Table 3: Examples of autocatalytic processes described in literature. N is the number of reactors in series. a: a theoretical study. b: 2 CSTR's followed by one PFR, galactose is added to the PFR.

PRODUCT	SUBSTR.	BIOCATALYST	N	REFERENCE
Lactic acid	Lactose	*Streptococcus cremoris* 2487	3	Mulligan *et al.* (1991)
Lactic acid	Lactose		7	Kulozik *et al.* (1992)
Lactic acid	Whey permeate	*Lactobacillus helveticus*	2	Aeschlimann *et al.* (1990)
Monoclonal Antibodies	-	Hybridoma cells	2	Reuveny *et al.* (1986)
Monoclonal Antibodies	-	Hybrydoma cells	2	Venables *et al.* (1993)
Streptomycin	Glucose	*Streptomyces griseus*	3	Sikyta *et al.* (1959)
Protease	-	*Bacillus pumilus*	2	Fabian (1969)
3-ketosteroid-Δ^1-dehydrogenase	Glucose	*Arthrobacter simplex*	2	Ryu & Lee (1975)
Biomass	Ethanol	*Candida utilis* IAM 4215	3	Goto *et al.* (1973)
Biomass	Ethanol	*Candida utilis* IAM 4215	3	Paca & Gregr (1979 A,B), Paca (1980,1982)
Biomass	Molasses	*Torula utilis*	2	Fencl *et al.* (1961), Fencl (1964)
Biomass	D-Sorbitol	*Acetobacter suboxydans*	2	Ricica (1969)
Biomass	-	*Streptococci*	2	Holström & Rose (1964)
Biomass	Sugar	*Saccharomyces cerevisiae*	8	Prokop *et al.* (1969)
Biomass	Glucose	*Aspergillus niger*	2	Fencl *et al.* (1980)
Biomass	Glucose or ethanol	*Hansenula polymorpha* CBS4732	3, 10	Schügerl (1982)
Biomass	Glucose	*Escherichia coli* ATCC 11105	3, 10	Schügerl (1982)
Gramicidin S[a)]	-	-	3	Blanch & Rogers (1972)
α-galactosidase[b)]	Glucose, galactose	*Monascus*	3	Imanaka *et al.* (1973)
Acetone, Butanol	Glucose	*Clostridium acetobutylicum*	2	Bahl *et al.* (1982)
Wine	Koshu-grape must	*Saccharomyces cerevisiae* 2HY-1	5	Ogbonna *et al.* (1989)
Mead	Honey mash	*Saccharomyces cerevisiae*, *Hansenula anomala*	2	Qureshi & Tamhane (1986)

completely obeying Monod kinetics. This shows that one should take care on how kinetic data are obtained and used.

An interesting theoretical study of the application of series of CSTR's (up to three reactors) for ethanol production is presented by Shimizu & Matsubara (1987). They use production kinetics with absolute inhibition ($1-P/P_m$), in combination with either growth-associated or non-growth-associated production, and the condition of a zero-maintenance level. Based on the kinetics and parameter values of Shimizu & Matsubara (1987), the productivity as a function of the product concentration of one, two and three reactors in series was calculated, as shown in figure 6. Obviously it is not possible to enhance the

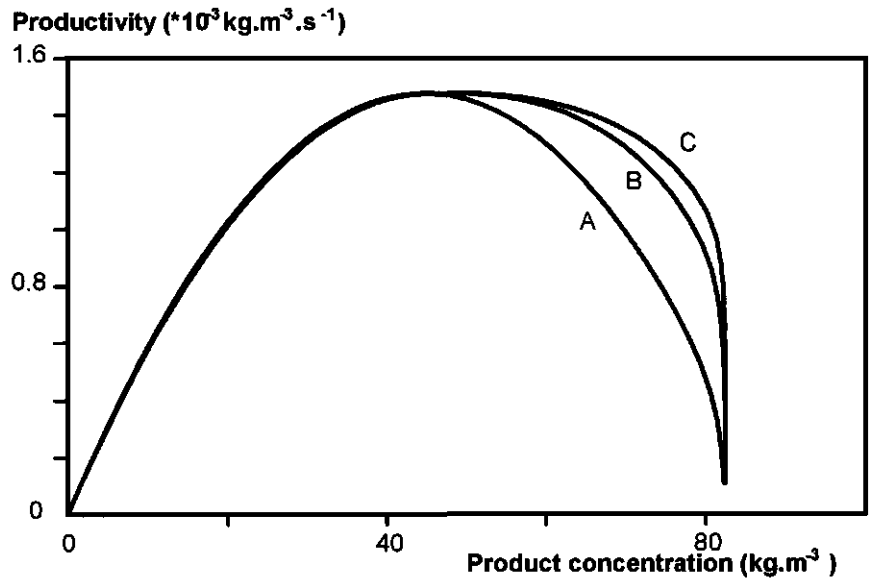


Figure 6. Volumetric productivity of an ethanol fermentation versus product concentration. A = 1, B = 2, C = 3 reactors in series, respectively. Parameter values were as used by Shimizu and Matsubara (1987), with K_s = 1.6 kg.m^{-3}, μ_m = 6.7 10^{-5} s^{-1}, Y_{xs} = 0.06, Y_{xp} = 0.16, P_m = 90 kg.m^{-3}, and S_0 = 220 kg.m^{-3}. Note that at a product concentration of 82.5 kg.m^{-3} all substrate is converted.

product concentration beyond P_m if this type of kinetics is involved. However, an improved volumetric productivity is possible at higher degrees of conversion by using more than one reactor. Note that, although the differences are marginal, for product concentrations below the point where the productivity is maximal, one single CSTR has the highest volumetric productivity. In other words, for substrate concentrations above α_{crit}, a single vessel is superior, as expected.

Shama (1988) reviewed the reactor development for fuel ethanol production. For a batch process with glucose as substrate and *Saccharomyces cerevisiae* as microbial strain, volumetric productivities of 2.8-6.7 10^{-4} $kg.m^{-3}.s^{-1}$ are typical, whereas 19.4 10^{-4} $kg.m^{-3}.s^{-1}$ can be achieved in continuous processes. As can be seen from table 2, continuously operated series of bioreactors can lead to much higher productivities combined with high degrees of conversion, as for example the studies of Klein & Kressdorf (1983, 1986) showed : at almost complete conversion productivities of 158.3 - 300 10^{-4} $kg.m^{-3}.s^{-1}$ were reported. Also, compared to a batch process, the ease of control, and the absence of peak loads upon up- and downstream processes favour continuous processes.

Few papers could be found dealing with animal cells, whereas theoretically (an autocatalytic system with growth-associated production, and very often by-product inhibition) series of bioreactors can be used advantageously for monoclonal-antibody production by hybridomas (Venables et al. 1993, Reuveny et al. 1986, Shimizu & Matsubara 1987, Pirt 1975). Reuveny et al. (1986) found that for a semi-continuous two-stage system with an extra feed of glucose and glutamine in the second stage, both the monoclonal-antibody concentration and the productivity doubled in the two-stage system as compared to the single semi-continuous bioreactor.

Immobilized growing cells

By immobilization, high cell densities and high volumetric productivities can be achieved. Immobilization also introduces simultaneous diffusion, consumption of substrates, and growth, which is troublesome to relate mathematically to design of a series. Therefore, it is not surprising that no papers could be found that deal with the theory of optimal design of series of CSTR's with immobilized growing cells. However, one paper presents a graphical procedure to provide design rules and estimation of kinetic constants for activated sludge processes for waste-water treatment (Braha & Hafner, 1985).

Godia et al. (1987) present a thorough review of the use of immobilized cells for continuous ethanol production. Table 2 shows experimental data on production of ethanol with series of immobilized-cell bioreactors. With ethanol production, three goals have to be met (preferably simultaneously) :

i) high volumetric productivities to reduce reactor costs,

ii) high product concentrations to reduce downstream processing costs, and

iii) high conversion degrees to reduce feed costs.

In a single vessel, these three goals can hardly be met simultaneously. Godia et al. (1987) show for example, that in a single vessel a productivity of 150 10^{-4} $kg.m^{-3}.s^{-1}$ was obtained at a degree of conversion of 63%, wheras 25 10^{-4} $kg.m^{-3}.s^{-1}$ was observed at a degree of conversion of almost 100%. It is still, however, a considerable improvement when compared with the review data of Shama (1988) where typical productivities of 2.8-6.7 10^{-4} and 19.4 10^{-4} $kg.m^{-3}.s^{-1}$ were reported for batch and continuous processes with freely suspended cells. From table 2 it is clear that the use of series of reactors with immobilized cells are the most promising prospect to meet the three goals for ethanol production (Klein & Kressdorf 1983, 1986).

CATALYTIC SYSTEMS

Non-growing biocatalysts may consist of enzymes, cell organelles, whole non-viable cells, or viable non-growing cells (Van 't Riet & Tramper 1991). In literature however, only reports based on free or immobilized enzymes, applied in bioreactor series, can be found.

Theory

The same optimization criterion as with autocatalytic processes is used : the minimization of the overall cascade residence time at a given final exit concentration of substrate (equation [1]). A fixed reaction stoichiometry is assumed, and the enzyme concentration in each reactor is assumed to be equal and constant. A generalized rate expression is used to represent various types of kinetics (Table 4). As a result, the reaction rate per unit volume r_s equals:

$$r_s = v_{max} e \frac{k + l\alpha + m\alpha^2}{f + g\alpha + h\alpha^2} \qquad [14]$$

where parameters f, g, h, k, l, and m are kinetic characteristics depending on the kinetic equation (see table 4).

Substitution of equation [14] in the general mass balance over a single CSTR in a series (equation [4]) yields:

$$\tau_i = \frac{V_i v_{max} e}{F S_0} = \frac{(\alpha_{i-1} - \alpha_i)(f + g\alpha_i + h\alpha_i^2)}{k + l\alpha_i + m\alpha_i^2} \qquad [15]$$

Table 4: Values of the parameters *f, g, h, k, l, m* in the general rate equation for enzyme catalysed reactions (Equation 14) and the critical substrate concentration α_{crit} at which an optimally designed series of bioreactors consists of a single vessel.

	MICHAELIS MENTEN	UNIMOLECULAR EQUILIBRIUM	SUBSTR. INHIBIT.	PRODUCT INHIBITION	ZERO ORDER	FIRST ORDER
$\frac{v}{v_{max}e} =$	$\frac{s}{K_m+s}$	$\frac{\frac{s}{K_m}-\frac{v_{max\,b}}{v_{max}}\frac{p}{K_p}}{1+\frac{s}{K_m}+\frac{p}{K_p}}$	$\frac{s}{K_m+s+\frac{s^2}{K_i}}$	$\frac{s}{K_m+s}\frac{K_p}{K_p+p}$	1	$\frac{s}{K_m}$
	$\frac{k+l\alpha+m\alpha^2}{f+g\alpha+h\alpha^2}$					
k	0	$\frac{v_{max,b}K_m}{v_{max}K_b}\left(\frac{p_0}{s_0}+Y_p\right)$	0	0	1	0
l	1	$1+\frac{v_{max,b}K_m}{v_{max}K_b}Y_p$	1	1	0	1
m	0	0	0	0	0	0
f	$\frac{K_m}{s_0}$	$\frac{K_m}{K_b}\left(\frac{K_b}{s_0}+\frac{p_0}{s_0}+Y_p\right)$	$\frac{K_m}{s_0}$	$\frac{K_m}{K_p}\left(\frac{K_p}{s_0}+\frac{p_0}{s_0}+Y_p\right)$	0	$\frac{K_m}{s_0}$
g	1	1	1	$1-(K_m+s_0)\frac{Y_p}{K_p}+\frac{p_0}{K_p}$	0	0
h	0	$1-\frac{K_mY_p}{K_b}$	$\frac{s_0}{K_i}$	$-\frac{Y_ps_0}{K_p}$	0	0
α_{crit}	$\frac{-fm+hk+\sqrt{(fm-hk)^2+g^2mk-gmfi-hlgk+hl^2f}}{gm-hl}$ (eq.16)					
	1	1	$\sqrt{\frac{l}{h}}=\frac{K_mK_i}{s_0^2}$	1	all α	1

In order to answer the question *if* an optimally designed series is worthwhile, equation [15] is substituted in equation [8] for N=2, and $\alpha_1 = \alpha_2 = \alpha_{crit}$ is applied. For this general case of 2 reactors in series this results in:

$$\alpha_{crit} = \frac{hk - fm + \sqrt{(hk-fm)^2 + g^2km - fgml - ghkl + fhl^2}}{gm - hl} \qquad [16]$$

Evaluation of equation 16 for the different types of kinetics (Table 4) shows that for Michaelis-Menten kinetics, unimolecular-equilibrium kinetics, product-inhibition kinetics, and first-order kinetics, an optimally designed series is superior to a single vessel at all effluent concentrations: the critical concentration

for these kinetics equals the system influent (i.e. α_{crit}=1). For substrate inhibited reactions however (Figure 5), the series configuration may be superior, but only if the desired effluent concentration α_N is below α_{crit} i.e. below $K_m K_i / s_0^2$ (Table 4). Evaluation of this latter expression shows that for substrate inhibition the feasibility range for the application of a cascade decreases with the severity of the inhibition: α_{crit} decreases with decreasing K_i values. For a zero-order reaction, there is no difference in performance between any cascade and a single-vessel system.

Interestingly, α_{crit} can be found in an alternative way by taking the first derivative of the reaction rate equation with respect to the substrate concentration. If a value of α exists at which this first derivative equals zero,

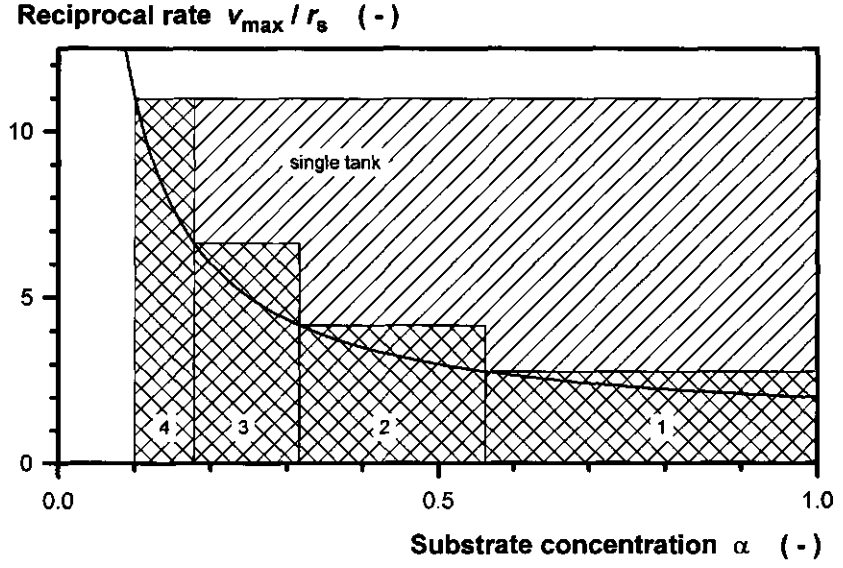


Figure 7. Overall residence times (shaded areas) for an enzymatic reaction with Michaelis-Menten kinetics in a single CSTR and in a cascade consisting of 4 CSTRs. For these kinetics, the curve decreases monotonically, and the cascade requires less holding time at all effluent concentrations.

then this value of α is α_{crit}. In other words, as long as the enzymatic reaction rate per volume is monotonically increasing with α, a series of bioreactors will allways be superior to a single vessel (Figure 7).

Now having the answer to the question *if* a series is worthwhile, that is after the determination of α_{crit} , the optimal design of the series according to equation [1] can generally be done in the same way as suggested by Hill & Robinson (1989) for autocatalytic processes. For the case of Michaelis-Menten kinetics, the final, surprisingly simple design equation following from equation [1] is (Luyben & Tramper, 1982) :

$$\alpha_i = \alpha_{i+1}^{i/(i+1)} \qquad [17]$$

Starting with the known α_N, the intermediate substrate concentrations can easily be calculated by equation [17], if the total number of reactors in the cascade is known. Optimum design results in a monotonically decreasing reactor volume along the series. The decrease in required total holding time is the largest when going from 1 to 2 bioreactors. Also, this decrease is larger at higher desired degrees of conversion. Note that for first order kinetics, exactly the same result is obtained.

Another way of assessing if a series of CSTR's is worthwhile is considering the difference between the residence time of a single CSTR and the residence time of a PFR. For Michaelis-Menten kinetics, the dimensionless residence time of a PFR can be described by (Luyben & Tramper, 1982) :

$$\tau_{pfr} = (\alpha_0 - \alpha_1) - \kappa \ln(\alpha_1) \qquad [18]$$

with κ being the dimensionless Michaelis-Menten constant (K_m / S_0). The maximum attainable decrease in dimensionless residence times then becomes (equations [4] and [18]):

$$\xi = \frac{\tau_{pfr}}{\tau_{cstr}} = \frac{1 - \frac{\kappa \ln(\alpha_1)}{1-\alpha_1}}{1 + \frac{\kappa}{\alpha_1}} \qquad [19]$$

This equation indeed shows that for high degrees of conversion (α_1 approaching zero), ξ approaches zero, or the residence time of a single CSTR is infinite times higher than that of a PFR. This is in accordance with the findings of Luyben & Tramper (1982). For the zero-order extreme of Michaelis-Menten kinetics (κ approaching zero) one can find that ξ equals one (no difference in residence times), and for the first order extreme it can be found that:

$$\lim_{\kappa \to \infty} \xi = \frac{-\alpha_1 \ln(\alpha_1)}{1-\alpha_1} \qquad [20]$$

illustrating that at high degrees of conversion series of CSTR's might be worthwhile: with α_1 = 0.1 and 0.01, an ξ of 0.26 respectively 0.05 can be found, indicating that a single CSTR would be 4 or 20 times the volume of a PFR, respectively.

Enzymatic decay

First-order decay of enzymes is described by several authors (Lopes & Malcata 1993, Paiva & Malcata 1993, Vos 1990, Yoon et al. 1989, Furusaki et al. 1980, Furusaki & Miyauchi 1977). Lopes & Malcata (1993) showed that as long as the time constant for decay is larger than the time constant for flow through the

series (V_{tot}/F), a simple relation can be found that allows a good estimate for the reactor sizes in series of 3 and 4 bioreactors. If non-isothermal operation is considered, completely different optima can be found (Paiva & Malcata, 1993).

Vos (1990) describes a reactor system for the production of High Fructose Corn Syrup with immobilized glucose-isomerase. His reactor is a multiple fluidized bed, where intermittently the flow is stopped and the biocatalyst is refreshed top-down, by simply allowing the biocatalyst beads to pass the holes in the sieve plates between the different compartments and removing the beads from the bottom compartment. He concludes that HFCS could be produced 20% cheaper in such a reactor than in a single CSTR.

Yoon et al. (1989) describe three other strategies to address enzymatic decay in continuously operated bioreactors:

i) change the feed rate for a constant degree of conversion which may affect mass-transfer properties in the bioreactor,

ii) accept a decreasing degree of conversion at a constant feed rate, or

iii) apply temperature control.

For the case of a multi-stage immobilized-glucose-isomerase reactor, it is described that at least 10% higher specific productivities (mol.kg^{-1} enzyme.s^{-1}) can be attained with optimal temperature control, and that three bioreactors in series perform better than two.

Dissolved enzymes

Dissolved enzymes are favourable if large througputs are involved, the enzyme costs are not too high (since it has to be constantly added to the reactor), and

residual enzymatic activity can be easily removed, e.g. by thermal treatment (Malcata & Cameron 1992, Paiva & Malcata 1993, Lopes & Malcata 1993).

Penicillin acylase shows substrate inhibition, non-competitive 6-APA inhibition and competitive phenyl acetic acid inhibition. Karanth (1979) showed that for the case of hydrolysis of penicillin-G to 6-APA and phenyl acetic acid, 2 CSTR's in series are to be favoured over one batch reactor. In this analysis, at a degree of conversion of 98%, the volume of 1 CSTR would be 8.6 times the value of a batch reactor, and 2 CSTR's would require 1.5 times the batch volume, if the batch would have a zero downtime. If the downtime for the batch reactor would be 1 h with a reaction time of 2 h, the series of CSTR's would show a higher volumetric productivity. These results were confirmed by Noworyta & Bryjak (1993), where the superiorety of a three-reactor series was shown over a single CSTR; the total residence time could be reduced by about 40%.

Malcata (1988, 1989) and Ong et al. (1986) extended the work of Luyben & Tramper (1982) with a description of the cost of scaleup by a power rule on the equipment capacity. The relation is only valid for a low number of reactors, and the extra costs (spare parts, cleaning) for differently sized bioreactors have to be carefully weighed. For a two-substrate reaction (ping-pong, obeying Michaelis-Menten kinetics) under application of the six-tenth-factor rule for capital investment, Malcata showed that the required volumes of the reactors in the series first decrease and after that increase again, and that never more than three reactors in the series are optimal with respect to reactor capital investment. This is in accordance with the findings of Blanch & Rogers (1972).

Using unimolecular equilibrium kinetics for the production of L-malic acid from fumaric acid, Malcata & Cameron (1992) showed that if the Monod

constant K_s is close to the product inhibition constant K_p (Table 4), the optimal reactor series consist of equal-sized bioreactors.

Immobilized enzymes

Compared to dissolved enzymes, only a few papers can be found that describe series of bioreactors with immobilized enzymes. As a logic continuation of the work of Luyben & Tramper (1982), De Gooijer et al. (1989) describe the optimum design of a series of CSTR's with invertase immobilized in alginate, obeying intrinsic Michaelis-Menten kinetics. The mathematical approach is the same as that of Luyben & Tramper (1982), except for the definition of the dimensionless holding time for each vessel: here the efficiency factor η appears in the denominator:

$$\tau_i = \frac{\eta_i V_i v_{max} e}{F S_0} \qquad [21]$$

Consequently, for optimal design the dimensionless concentrations can be calculated as if the enzyme were free (equation [17]), and after that for each intermediate substrate concentration along the series an effectiveness factor is determined. These effectiveness factors thus account for the extra volume required to compensate for both internal and external diffusion limitation caused by the immobilisation of the enzyme. Analogously, the same procedure can be applied for other kinetics. Hence, as for suspended enzymes, series of bioreactors are favourable for immobilized enzymes except for substrate inhibited kinetics. Note that for first-order kinetics, with negligible external diffusion limitation, there will be only a single internal effectiveness factor for all

bioreactors in the series, since the Thiele modulus for that case is independent of the substrate concentration (Van 't Riet & Tramper, 1991).

In 1994, Bakker et al. made the experimental comparison between a single vessel and a three-vessel reactor series. They observed improved sucrose conversion by immobilized invertase to 83% compared to 73% in the single vessel with the same overall residence time.

Concluding remarks

Theoretically, with respect to the overall residence time for a given degree of conversion, the use of more than one CSTR in series can be advantageous for any non-autocatalytic process that obeys non-zero order kinetics. For autocatalytic processes series of CSTR's can be favourable if a high product concentration combined with a high degree of conversion and an acceptable volumetric productivity is needed. However, one should always carefully weigh the practical and cost implications of more than one bioreactor to the possible advantages.

Few recent papers could be found that describe the use of series of bioreactors in industry. For an early review see Hospodka (1966), describing a few processes including the production of baker's yeast, ethanol (from sugar and starch), beer, and acetone/butanol. The largest scale recently described is of the pilot-plant type (Takahashi & Kyosai, 1991). This may of course be caused by the reluctance of industry to publish regarding their source of income, but it may also be explained by a certain kind of conservatism in the implementation of the results of scientific research at an early stage (Tramper, 1993).

VARIABLE STOICHIOMETRY PROCESSES

Processes with a variable stoichiometry need a physical separation into two or more reactors (either in space, or time) by their nature. Some examples of such processes are discussed below.

Insect cells

Insect-cell technology is a fast emerging tool for the expression of foreign genes. Also, due to the increase in strict regulations for chemical compounds for crop protection, the interest in the wild-type virus for use as a bioinsecticide emerges. Suspension cultures of insect cells can be generated by adapting cells of insects (e.g. *Spodoptera frugiperda*) via T-flasks to suspension cultures in spinner flasks or bioreactors. These cells are still susceptible to infection with the non-occluded form of a baculovirus (e.g. *Autographa californica* Nuclear Polyhedrosis Virus). After infection, cells will produce newly synthesized non-occluded viruses, and, subsequently, occluded viruses in the form of polyhedra, or recombinant proteins of foreign origin (Granados, 1976, 1980). After this, the cells lyse. Therefore, if continuous production is desired, a physical separation has to be introduced, as suggested by Tramper & Vlak (1986). Kompier et al. (1988) were the first to describe a successful experimental setup for such a process. They used a first CSTR to grow cells, followed by a second CSTR in series were infection takes place. Subsequently, Van Lier et al. (1990, 1992) investigated the optimization of the continuous production. It appeared that continuous production for prolonged periods of time is hampered by the occurrence of the so-called passage effect, which manifests itself as a reduction in the number of non-occluded viruses and a decrease in the infectivity of these viruses. Based

on an infection model, De Gooijer et al. (1992) suggested a pseudo-continuous mode of operation of the reactor series in the form of a repeated batch or fed-batch of the infection vessel, in order to reduce this passage-effect. The feasibility of such as process had already been demonstrated by Klöppinger et al. (1990) and was confirmed by Zhang et al. (1993).

Waste-water treatment

For waste-water treatment, reactor costs should be minimized under the constraint of high degrees of conversion. In the specialized journals in this field, numerous papers can be found describing reactor design. Some relevant studies are listed below.

Mitchell & Shuler (1978) studied the production of Single Cell Protein (SCP) for feedstuff purposes. Here, in a first vessel, carbohydrates and urea in poultry manure were converted. In a second stage, where the SCP was formed, glucose was added as additional carbon source, while the ammonia from urea was used as nitrogen source.

Aivisidis et al. (1989) described the anaerobic degradation of complex substrates to methane. In a first CSTR, acidogenic bacteria decomposed carbon sources into low-molecular-weight compounds with concommittant acidification of the waste-water to pH 3-4. In the second stage, for their case a fixed-bed loop reactor, methanogenic bacteria produced methane from these acids. This resulted in a more stable process with a lower occurrence of pathogens, but with a higher investment and a neccesarry pH control. A similar distribution of the subsequent steps in anaerobic degradation was found by Howgrave-Graham et al. (1994) for a three stage anaerobic digester with cellobiose as sole carbon source. Arora & Mino (1992) and Takahashi & Kyosai (1991) reported on the

use of a series of bioreactors for treating domestic wastewaters for standard COD removal. With 5 reactors in series at pilot-plant scale, a conversion degree of 90% could be reached producing less sludge and without the need of a final settler tank. Yang et al. (1993) reported on the development of a cascade of 5 ponds in series, consisting of empty oil barrels, for treating swine waste water in tropical areas. Over 90% of all pollutants were removed well in this ultimately cheap series of bioreactors.

(De)Nitrification

Due to the low specific growth rate of nitrifying bacteria, the nitrifying capacities of traditional waste-water plants is often poor (Barnes & Bliss, 1983). Taniguchi et al. (1988) described the use of two airlifts in series with sludge for simultaneous nitrification and denitrification, in which 80% if the ammonia was converted to nitrogen gas. Al-Haddad et al. (1991) used four aerated submerged fixed-film bioreactors in series to nitrify ammonia. Brauer & Annachhatre (1992a,b) used three reciprocating jet bioreactors in series to remove ammonia from real-life waste water. A reciprocating jet bioreactor consists of a cylindrical vessel containing an assembly of sieve plates attached to vertical rods, which is given a reciprocatory motion. The first reactor was used to remove the majority of the carbon, the second did the nitrification, and the third the denitrification. In this process, 90% of the carbon was removed, and 85-95% of the ammonia was converted into nitrogen gas. For the denitrifying bacteria, methanol was used as additional carbon source. A similar process was suggested by Santos et al. (1993) in a multiple airlift-loop reactor (see 'reactors').

Recombinant micro-organisms

Barbotin et al. (1990) and Berry et al. (1990) described the improvement of apparent plasmid stability by immobilization of recombinant *Escherichia coli* in a two-stage bioreactor series. In the first stage, cells, immobilized in carrageenan, were grown. Released cells were fed to a second stage, where a temperature shock was used for derepression(i.e. production). This resulted in a five-fold production rate of catechol 2,3-dioxygenase. Fu et al. (1993) and literature cited therein described the continuous production and excretion of ß-lactamase by genetically engineered *Escherichia coli* in suspension in a two stage-chemostat. The micro-organism was grown in the first chemostat, after which the expression of the protein was induced by isopropyl-β-D-thiogalactopyranoside in the second chemostat. Continuous production was possible for over 50 days, with the product accounting for 25% of the cellular protein. Due to cell death and the selection for *lac*$^-$ cells this process fails in a single chemostat.

Other products

Already in 1959, Pirt & Callow suggested the use of a series of CSTR's to produce penicillin. In the first vessel the mould would be grown at a pH below 7, in order to avoid the formation of aberrant hyphae, wheras in a second stage penicillin could be produced at pH 7.4 where the penicillin production is optimal.

Ricica (1964) showed that the biosynthesis of 6-azauracil (AzU) by *Escherichia coli* B was not feasible in a single CSTR since AzU inhibits cell growth. He successfully demonstrated the production in a series of four CSTR's, and also showed that the replacement of the last CSTR by a tubular reactor was not successful, which was attributed to a lack of oxygen in the latter reactor.

A rather important aspect of series was studied by Plevako (1964). Baker's yeast was grown in a first CSTR, after which in a smaller second stage, under the condition of slight aeration, the residual substances in the medium were utilized, thus allowing the stabilization of the cell's enzyme system in maturing cells. It was reported that a high-quality, stable product with good keeping conditions was obtained.

Lelieveld (1984) described the continuous production of yoghurt (two strains) and buttermilk (three strains) with mixed cultures in a two-stage cascade. It was reported that in the first stage the lactic acid bacteria multiply whereas in the second stage they produced most of the desired (flavour) metabolites. He addressed the risk of the product being affected by the selection of a faster-growing mutant strain. If it is assumed that 1 mutant organism, having a 10% higher specific growth rate, is present at the very start of the fermentation, he showed that a continuous process can be run for 3.3 weeks for buttermilk cultures and for 1.1 weeks for yoghurt cultures if it is acceptable that 1% of the final microbial population is the mutant. The results from several years of full-scale production supported this conclusion.

For the removal of hexose and pentose sugars from agricultural waste streams, Grootjen et al. (1991) concluded that a physical separation between the two yeast strains used (*Pichia stipitis* for pentose sugars and *Saccharomyces cerevisiae for hexose sugars*) was necessary, since otherwise the yeasts will compete for oxygen, resulting in a low conversion of xylose.

Pfaff et al. (1993) described the use of two CSTR's in series for the removal of trichloroethylene (TCE) from drinking water. *Pseudomonas putida* was grown in the first chemostat, with ethanol as carbon source and phenol to induce the toluene dioxygenase enzyme system. In the second reactor, TCE was added. By the use of this second stage, the competition between phenol

and TCE was minimised while the biomass, inactivated by toxic TCE oxidation products, was replenished.

Series of bioreactors can also function as a research tool in itself : Molly et al. (1993) used five CSTR's of different volumes to successfully mimick the human gastro-intestinal tract. The small intestine was simulated by a two-step "fill-and draw" series (not continuously operated), the large intestine consisted of a continuously operated series of three CSTR's with different residence times.

A last small-scale application is the use of two bioreactors in tandem in a continuous-flow / stopped-flow sample / reagent processing setup for the determination of alkaline phosphatase activity in serum, as described by Raba & Mottola (1994). With this analysis, co-immobilization of the enzymes involved (alkaline phosphatase and alcohol oxidase) would fail since the enzymes show mutual product inhibition, and buffer incompatability. By physically separating the two enzymes, a successful assay was reported.

Concluding remarks

Obviously, when the stoichiometry of the reaction changes with time in a batch process, a series of bioreactors is intrinsically favourable if a continuous process is aimed at. Some examples of such processes have been presented. The rules for deciding if a continuous process is more competitive than a classical (fed-) batch process, cannot be presented in a form analogous to the kinetically favourable series. Parameters involved in such a decision are the volumetric productivity, the stability of the organism, the acceptability of the risk of contamination, and the ease of construction and costs of a series compared to a single vessel.

REACTORS

The costs of series of conventional bioreactors form an important incentive to develop novel bioreactor types incorporating the principles of series. Many of those devices are patented. Only those patents that describe reactors that are relatively easy to scaleup are presented here. Almost all patents deal with cylindrical vessels in which compartments are formed by sieve plates (Lumb et al., 1970, Kitai et al., 1971, Blaß et al., 1979, Caro, 1987). Already in 1970, Lumb et al. patented a device consisting of five compartments for the production of neomycin with *Streptomyces fradiae*. Blaß et al. (1979) patented the sieve plate itself : the holes in the plate should not occupy more than 15% of the bubble column area. Caro (1987) patented a cylindrical vessel with concentric cylinders inside to produce biogas from organic wastes. In the inner cylinder hydrolysis takes place, the middle stages show acidification, and in the outer stage methanogenesis occurs. The patent of Aivasidis et al. (1987) describes a fixed bed column for anaerobic decomposition processes, in which two or more compartments are stacked with open plates in between and a separate gas outlet for each chamber. Grobicki & Stuckey (1991) describe the Anaerobic Baffled Reactor (ABR) for waste-water treatment. These rectangular boxes, with working volumes of 8-10 dm^3, are compartmentalized with alternately hanging and standing vertical baffles. ABR's with 4 to 8 compartments are decribed. As such, the ABR can be regarded as a series of upflow anaerobic sludge blanket reactors (Lettinga, 1980).

Recently, Bakker et al. (1993) presented results with a novel bioreactor (De Gooijer 1989) consisting of a series of concentrical airlift reactors with internal loop incorporated into one vessel. From the mixing behaviour it is shown that their prototype with three ALR's in series can be described by three ideal

mixers in series, thus approximating an aerated plug-flow reactor (Levenspiel, 1972).

CONCLUSIONS

In this paper a classification is presented to decide if and when a series of bioreactors can be advantageously used, for both catalytic and autocatalytic processes. The optimization criterion used is the total holding time of the series compared to a single CSTR, at a given substrate concentration in the last vessel.

For autocatalytic processes there is no type of kinetics where a series is always superior to a single CSTR. With the critical substrate concentration concept as introduced by Hill & Robinson (1989), the feasibility of a series can be predicted. The first step to take is to calculate a critical substrate concentration at which the single vessel and a two-reactor cascade are equivalent. At desired effluent concentrations $\alpha_N < \alpha_{crit}$, a cascade is superior, and at desired effluent concentrations $\alpha_{crit} < \alpha_N < 1$ the single vessel is to be preferred.

For catalytic processes based on enzymes, it is shown that a series of bioreactors is always superior to a single CSTR if the rate of reaction is monotonically increasing with the substrate concentration (Michaelis-Menten, first order, product inhibition, and unimolecular equilibrium kinetics). For substrate inhibition kinetics, a single vessel may be superior. This superiority can also be determined by applying the α_{crit} concept. If a series is superior, the second step is to calculate the intermediate substrate concentrations. For the case of free or immobilized enzymes following Michaelis-Menten or first-order

kinetics, these intermediary substrate concentrations can be calculated by a simple relation (equation [17]). Furthermore, from the work of Malcata (1988, 1989), who evaluated the capital investment for series of bioreactors for enzymatic processes, and from the work of Blanch & Rogers (1972) and Hill & Robinson (1989), who evaluated series for autocatalytic processes, it can be concluded that the maximum number of bioreactors, when a series is worthwhile, is equal to three.

From the examples of the application of series of bioreactors found in literature it is clear that the use of series on an industrial scale is limited, and that most applications for kinetically favourable series can be found in ethanol production. As illustrated in figure 1 and table 3, the combination of a high product concentration, a high degree of conversion, and a large volumetric productivity may well be attained in a series of bioreactors.

In the situation where use of a series is intrinsically favourable, that is virtually any process where the overall stoichiometry of the reaction changes with time, some interesting applications are presented, all on a laboratory scale.

The overview of the novel bioreactor types for series of CSTR's within one vessel shows that progress has been made in reactor development, which will lead to a possible reduction in cost for a series of reactors, and thus enhance the potential application of continuous processes on a larger scale.

APPENDIX A.

A general mass balance for biomass in an ideally mixed bioreactor is :

$$V\frac{\partial X}{\partial t} = FX_0 - FX + VX\mu \qquad [22]$$

which, under the assumption of steady state, and no biomass in the influent, reduces to :

$$\mu = \frac{F}{V} \qquad [23]$$

A general mass balance for substrate is :

$$V\frac{\partial S}{\partial t} = FS_0 - FS + Vr_s \qquad [24]$$

where r_s, the volumetric substrate uptake rate, is defined as :

$$r_s = \frac{\mu X}{Y_{xs}} \qquad [25]$$

Again under staedy state conditions, equation [24] can be simplified, and after combination with equation [23] this results in :

$$r_s = \mu(S_0 - S) \qquad [26]$$

Introducing Monod kinetics, and combining equation [26] with the Monod equation, the result is :

$$\frac{r_s}{\mu_m} = \frac{S(S_0 - S)}{K_s + S} \qquad [27]$$

which, with the introduction of the following dimensionless variables:

$$\rho = \frac{r_s}{\mu_m S_0},\ \kappa = \frac{K_s}{S_0},\ \alpha = \frac{S}{S_0} \qquad [28]$$

can be rewritten to:

$$\frac{1}{\rho} = \frac{\kappa + \alpha}{\alpha - \alpha^2} \qquad [29]$$

which is the equation that is depicted in figure 1.

NOMENCLATURE

α	dimensionless substrate concentration (S / S_0)	-
χ	dimensionless biomass concentration ($X/Y_{xs}S_0$)	-
τ	dimensionless residence time in enzyme reactor ($V_i\, v_{max}e / F\, S_0$)	-
	or in autocatalytic reactor ($V_i\, \mu_{max} / F$)	-
κ	dimensionless Michaelis constant (K_m/S_0)	-
ρ	dimensionless substrate consumption rate ($r_s/\mu_{max}S_o$)	-
ξ	ratio of the residence times in a PFR and a CSTR	-
μ	specific growth rate	s^{-1}
θ	residence time	s
D	dilution rate	s^{-1}
e	enzyme concentration	$kg.m^{-3}$
f	kinetic characteristic parameter (Table 1 and 2)	-
F	flow rate	$m^3.s^{-1}$
g	kinetic characteristic parameter (Table 1 and 2)	-
h	kinetic characteristic parameter (Table 1 and 2)	-
k	kinetic characteristic parameter (Table 2)	-
K	dimensionless monod constant ($K_s / (S_0 + x_0 / Y_{xs})$)	-
K_b	kinetic constant, equilibrium reaction backwards	$mol.m^{-3}$
K_i	substrate inhibition constant	$mol.m^{-3}$
K_m	Michaelis constant	$mol.m^{-3}$

K_p	product inhibition constant	$mol.m^{-3}$
K_s	Monod constant	$mol.m^{-3}$
l	kinetic characteristic parameter (Table 2)	-
m	kinetic characteristic parameter (Table 2)	-
N	number of vessels in a series	-
p	kinetic charateristic parameter (Table 1)	-
P	product concentration	$mol.m^{-3}$ or $kg.m^{-3}$
P_m	maximal product concentration	$mol.m^{-3}$ or $kg.m^{-3}$
q	kinetic charateristic parameter (Table 1)	-
r_s	volumetric reaction rate	$mol.m^{-3}.s^{-1}$
S	substrate concentration	$mol.m^{-3}$
V	reactor volume	m^3
v_{max}	maximum reaction rate per unit amount of enzyme	$mol.kg^{-1}.s^{-1}$
$v_{max,b}$	id., backward direction	$mol.kg^{-1}.s^{-1}$
X	biomass concentration	$kg.m^{-3}$
X_a	degree of conversion	-
Y_p	yield of product on substrate	$kg.mol^{-1}$
Y_{xs}	yield of biomass on substrate	$kg.mol^{-1}$

Subscripts

0	inlet of a series
crit	critical effluent concentration at which a single vessel and a two-reactor cascade are equivalent
i	i-th vessel in a series
j	j-th vessel in a series

max maximum

N last vessel in a series

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CHAPTER 2

OPTIMUM DESIGN FOR A SERIES OF CONTINUOUS STIRRED TANK REACTORS CONTAINING IMMOBILIZED BIOCATALYST BEADS OBEYING INTRINSIC MICHAELIS-MENTEN KINETICS

ABSTRACT

The optimum design of a series of mixed reactors containing immobilized biocatalysts is described. A detailed description is given of the modelling of internal diffusion and reaction in the beads, and external mass transfer resistance. The model is validated by experiments with cascades of two and three reactors, containing immobilized invertase. For that, invertase was first bound to DE-32 cellulose anion exchanger and then entrapped in calcium alginate.

INTRODUCTION

In a previous paper (Luyben & Tramper, 1982) an analytical expression was derived for the optimum design of a cascade of continuous stirred tank reactors in which a biocatalytic reaction following Michaelis-Menten kinetics is executed. The optimum was defined as the smallest total reactor volume needed to accomplish a specific conversion. Assuming that the volumetric activity of the biocatalyst remains constant in the reactors, the expression was derived.

In case of free biocatalyst special measures have to be taken to retain the biocatalyst in the reactors and thus keep the volumetric activity constant. Immobilization of the biocatalyst in a solid support can be such a measure. However, the consequence of this transition of homogeneous to heterogeneous biocatalysis can be a disguisement of intrinsic kinetics as a result of limitation of the reaction due to diffusion of substrate(s) and / or product(s). Only when mass transfer rate is fast in comparison to reaction rate this is not the case.

In general a concentration dependent effectiveness factor is introduced in the rate equation to account for this rate limitation by diffusion. Mostly, this is described for the extreme cases of first and zero order reaction kinetics, for which analytical expressions for the relation between the substrate concentration and the effectiveness factors can be derived. In a recent study, however, Manjon et al. (1987) described a design model for a plug flow reactor with immobilized naringinase, obeying reversible Michaelis-Menten kinetics.

In this paper we describe the derivation of the analytical expression for the dimensionless holding time of each reactor and the mathematical model used to calculate the effectiveness factor. The procedure is illustrated by a numerical example and verified experimentally by cascades of two and three reactors, using immobilized invertase as biocatalyst.

THEORY

Optimization

Consider a series of N continuous stirred tank reactors containing immobilized biocatalyst obeying intrinsic Michaelis-Menten kinetics. Introducing in the Michaelis-Menten equation an effectiveness factor dependent of substrate concentration (η_s) gives :

$$-r(s) = \frac{\eta_s V_m X \varepsilon S}{K_m + S} \qquad [1]$$

with $r(s)$ being the rate of substrate consumption, S the substrate concentration, X the concentration of biocatalyst in the gel, ε the holdup of gel in the reactor and V_m and K_m the Michaelis-Menten constants. A mass balance over the i-th reactor in the steady state gives :

$$\theta_i = \frac{V_i}{\phi_v} = \frac{(S_{i-1} - S_i)(K_m + S_i)}{\eta_{s,i} V_m X \varepsilon S_i} \qquad [2]$$

where θ_i is the holding time in reactor i, V_i the volume of reactor i and ϕ_v the volumetric flow rate. Writing equation [2] in dimensionless form by introducing the following variables :

$$\alpha_i = \frac{S_i}{S_0}, \quad \kappa = \frac{K_m}{S_0}, \quad \tau_i = \frac{\eta_i \theta_i V_m X \varepsilon}{S_0} \qquad [3]$$

with S_0 being the substrate concentration at the inlet of the first reactor, leads to:

$$\tau_i = \frac{(\alpha_{i-1} - \alpha_i)(\kappa + \alpha_i)}{\alpha_i} \qquad [4]$$

When optimum design is defined as the minimum total reactor volume (holding time) -realizing the limitations of this definition- the optimum can be found by :

$$\frac{\partial\left[\sum_{j=1}^{N}\tau_j\right]}{\partial\alpha_i}=0 \qquad i=1, 2, \ldots (N-1) \qquad [5]$$

which, analogous to Luyben & Tramper (1982), results in :

$$\alpha_i=\alpha_{i+1}^{i/(i+1)} \qquad i=1, 2, \ldots (N-1) \qquad [6]$$

By setting a desired degree of conversion ($1-\alpha_N$) and starting with the last reactor in the series, the dimensionless concentration α_i (equation [6]) and from this the dimensionless holding time τ_i (equation [4]) can be calculated for each reactor. In order to be able to then calculate the holding times θ_i, and thus the reactor volumes V_i, the effectiveness factors $\eta_{s,i}$ must be known (equations [2] and [3]).

Estimation of the effectiveness factor

A differential mass balance over a biocatalyst bead in which simultaneous diffusion and consumption of substrate occurs yields :

$$\frac{\partial S}{\partial t}+v_c\frac{\partial S}{\partial r}=D_e\left(\frac{1}{r^2}\frac{\partial}{\partial r}\left(r^2\frac{\partial S}{\partial r}\right)\right)-r'(S) \qquad [7]$$

where t is the time, r is the distance from the centre of the bead, v_c is the convection velocity, $r'(s)$ the rate of substrate consumption and D_e is the effective diffusion coefficient of substrate in the support material. Assuming that in the bead there is :

i) only a radial concentration gradient,

ii) a concentration independent diffusion coefficient,

iii) no convective transport, and

iv) a quasi steady state concentration profile,

equation [7] combined with Michaelis-Menten kinetics for steady state conditions reduces to :

$$D_e(\frac{1}{r^2}\frac{\partial}{\partial r}(r^2\frac{\partial S}{\partial r})) = \frac{V_m X S}{K_m + S} \qquad [8]$$

with the boundary conditions :

$S = S_s$ at $r = r_b$

and

$\frac{\partial S}{\partial r} = 0$ at $r = 0$ (or $r = r_f$),

where S_s is the substrate concentration at the surface of the bead, r_b is the radius of the bead, and r_f is the distance from the centre of the bead where the substrate concentration approaches zero. Partial differentiation yields :

$$\frac{\partial^2 S}{\partial r^2}_i = (\frac{V_m X S}{K_m + S})/D_e - \frac{2}{r}\frac{\partial S}{\partial r} \qquad [9]$$

Equation [9] can only be solved by numerical integration, finally resulting in a substrate concentration profile in the bead, a computed substrate concentration S_s and a (dS/dr) at $r = r_b$. In order to account for external mass transfer resistance, the latter can (optionally) be used for calculating a S_s' by means of the equation representing the film theory :

$$S_s' = S_b - \frac{D_e(\partial S/\partial r)_{r=r_b}}{k_l} \qquad [10]$$

with S_b the substrate concentration in the bulk of the solution, and k_l the substrate mass transfer coefficient in the film layer.

The numerical integration is performed by a computer program on a VAX-8600 computer (Digital Equipment Corporation), using the IMSL-routine DREBS, a Rebson algorithm with variable step size. This routine needs a starting point for correct integration. Initially, this point is set to the centre of the bead : $r_f = 10^{-7}$ m (not zero for numerical reasons) and $S_f = K_m / 10^5$, however, like r_f, S_f should not be smaller than 10^{-7} for numerical reasons.

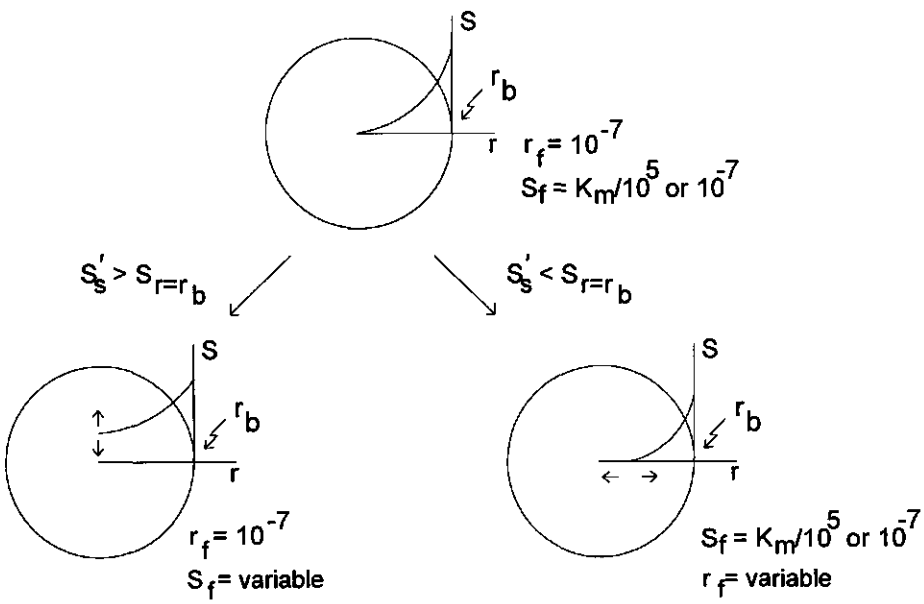

Figure 1. The calculation of a substrate concentration profile in a biocatalyst bead.

From this point the subroutine calculates the appropriate concentration profile in the bead, as illustrated in figure 1.

The obtained substrate concentration at the bead surface S_s is compared with the known concentration in the bulk, S_b, or with the computed S_s' when the film theory option has been invoked. If S_b (or S_s') > Ss then r_f is set to 10^{-7} and S_f is variable. On the other hand, if S_b (or S_s') < S_s then r_f will be variable and S_f is kept at the initial value. This process will iterate until S_s (or S_s') = S_b within a set limit, and the appropriate substrate concentration profile is plotted.

The internal effectiveness factor η_{int}, defined as the ratio of the macroscopic reaction rate and the rate at S_s, can be calculated using :

$$\eta_{int} = \frac{a_b D_e (\partial S / \partial r)_{r=r_b}}{V_m X_1 S_s / (K_m + S_s)} \qquad [11]$$

where a_b is the surface area of one bead, and X_1 is the amount of DE32-cellulose-invertase in one bead.

When the film theory option has been invoked, the external effectiveness factor η_{ext}, defined as the ratio of the rate at S_s and at S_b, is obtained from :

$$\eta_{ext} = \frac{S_s / (K_m + S_s)}{S_b / (K_m + S_b)} \qquad [12]$$

The overall effectiveness factor for the i-th reactor can then be calculated from :

$$\eta_{s,i} = \eta_{int,i}\, \eta_{ext,i} \qquad [13]$$

Figure 2 summarizes the computing scheme.

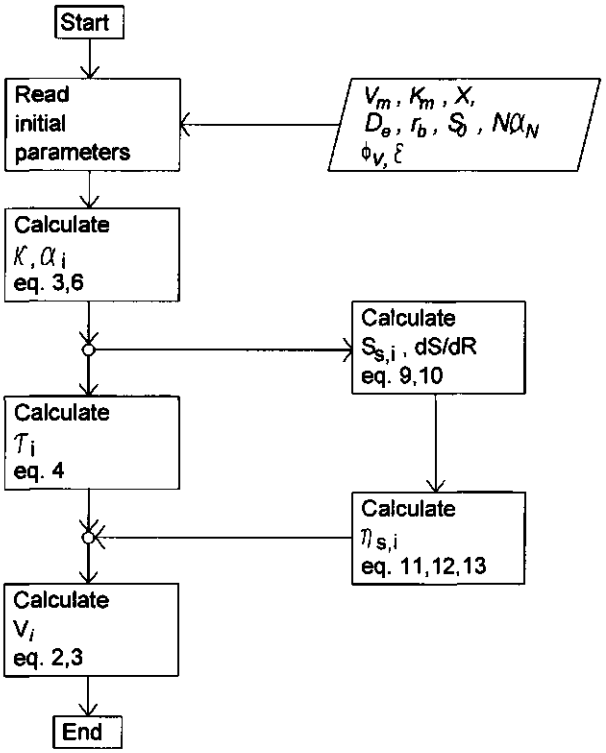

Figure 2. Computing scheme.

MATERIALS AND METHODS

Materials

Whatman DE32 Microgranular cellulose anion exchanger (rod shaped particles, 30 μm diameter, 110 μm length) was obtained from Whatman Chemical Separation Division, Maidstone, Kent (UK). Invertase solution (Maxinvert L10,000) was a kind gift of Gist-brocades Industrial Enzymes Division, Delft, the

Netherlands. Sodium Alginate (Manucol DM) was obtained from Kelco/Ail Int. Ltd, Girvan Agrshire, Scotland (UK). All other chemicals were of pure or analytical grade from Merck, Darmstadt, Germany. Solutions were made up in demineralized water.

Immobilization Procedure

As we found, in addition to the work of Klein et al. (1983), that a washout of invertase occurs when immobilized in 8% alginate without microcarrier, invertase was first coupled to DE32-cellulose before immobilization as described earlier by Woodward & Wiseman (1978). Half a gram of DE32-cellulose was equilibrated for 1-2 days in 100 cm^3 sodium phosphate buffer (0.01 M, pH 7.0). One hundred mm^3 of invertase solution was added to 10 cm^3 of this suspension and shaked for 1-2 hours at room temperature. The free invertase then was removed by washing three times with the equilibrating buffer and once with a sodium acetate buffer (0.01 M, pH 4.2). The latter liquid showed no enzymatic activity. The DE32-cellulose-invertase then was added to a 2% sodium alginate solution in acetate buffer, eventually resulting in a gel load of 6.15 g DE32-cellulose-invertase per dm^3 gel. The mixture was extruded dropwise through an 8 mm tube into a 0.2 M calcium chloride solution. After hardening for two hours, the beads were placed in a 0.05 M calcium chloride acetate buffer, and stored at 4 °C until needed.

Analysis

The concentration of glucose was quantitatively determined by the GOD-Perid Test Combination Glucose from Boehringer Mannheim GmbH Diagnostica, Germany. Before this, a filtration through a 0.2 μm membrane filter was performed after sampling. Each sample volume was 1 cm^3.

Activity Assays

DE32-cellulose-invertase (0.2 g) was kept at 30 °C in 80 cm^3 acetate buffer in a stirred reaction vessel. At t=0, 80 cm^3 of a 160 $g.dm^{-3}$ saccharose solution was added. Samples (0.5 cm^3) were taken each minute for thirty minutes. After sampling, the DE32-cellulose-invertase was removed immediately by filtrating through a 0.2 μm membrane filter. From these experimentally obtained concentration versus time curves the kinetic parameters were calculated by means of a computer program as described by Van den Tweel et al. (1987)

Diffusion Coefficient Assay

The effective diffusion coefficient of saccharose in calcium alginate at 30 °C was obtained as described by Tanaka et al. (1984). In 120 cm^3 of a 80 $g.dm^{-3}$ saccharose, 0.05 M calcium chloride acetate buffer solution, 60 cm^3 beads (not containing DE32-cellulose-invertase) were added at t=0. Samples were taken for 45 minutes each 30 seconds initially, and at greater intervals after 5 minutes. After complete conversion of the saccharose to glucose and fructose by adding invertase to the samples, the glucose concentration could be determined as

described. This experimentally obtained concentration versus time curve was evaluated by a computer program as described by Hulst et al. (1989), and the effective diffusion coefficient could be calculated.

Cascade Experiments

In order to check the validity of the model presented in this paper, it was tested with two cascades of two and three mixed reactors, respectively. With the computer program reactor volumes were calculated. Subsequently, the reactors were operated for one day in order to reach steady state, and the conversion rate, expressed as $1-\alpha_N$, was measured.

RESULTS AND DISCUSSION

Numerical Example

For a numerical example table 1 shows the results of five runs of the computer program. In these runs, and also in our experiments, external mass transfer resistance was neglected. An estimation of the Biot number, defined as the ratio of the mass transfer resistance in the solution and in the bead, resulted in a value of 115, so external mass transfer resistance is, for our case, negligible. Initial parameters used were : $V_m = 0.5$ mol.kg^{-1}.s^{-1}, $K_m = 200$ mol.m^{-3}, $X = 5$ kg DE32-cellulose-invertase.m^{-3} gel, $D_e = 0.4\ 10^{-9}$ m^2.s^{-1}, $r_b = 2.5$ mm, $S_0 = 400$ mol.m^{-3}, so $\kappa = 0.5$, $\phi_v = 0.5\ 10^{-6}$ m^3.s^{-1}, $S_N = 20$ mol.m^{-3} , so $\alpha_N = 0.05$, and $\varepsilon = 0.3$.

Table 1 clearly shows the considerable difference in total holding time going from one to two or more reactors. This effect becomes even more significant when considering equations [2] and [3], and calculating the reactor

Table 1 : Dimensionless concentrations (α_i), holding times (τ_i), effectiveness factors (η_i), and Reactor volumes (V_i) in cascades of CSTR's.

α_i							τ_i					
N		1	2	3	4	5	1	2	3	4	5	total
	1	0.05					10.45					10.45
	2	0.22	0.05				2.51	1.91				4.42
	3	0.37	0.14	0.05			1.49	1.09	0.94			3.52
	4	0.47	0.22	0.11	0.05		1.09	0.81	0.68	0.61		3.18
	5	0.55	0.3	0.17	0.09	0.05	0.86	0.66	0.55	0.49	0.45	3
η_i							V_i[dm^3]					
N		1	2	3	4	5	1	2	3	4	5	total
	1	0.21					13.17					13.17
	2	0.25	0.21				2.67	2.41				5.08
	3	0.28	0.23	0.21			1.42	1.26	1.19			3.87
	4	0.3	0.25	0.23	0.21		0.97	0.86	0.8	0.77		3.4
	5	0.31	0.27	0.24	0.22	0.21	0.74	0.66	0.61	0.59	0.57	3.16

volumes, as the effectiveness factor decreases with decreasing bulk concentration. This effect decreases with increasing *N*.

Determination of Diffusion Coefficient and Michaelis-Menten constants

Experiments were carried out at 30 °C as described in Materials and Methods. For the effective diffusion coefficient of sucrose in calcium alginate at 30 °C we

found D_e = 0.385 ± 0.015 10^{-9} $m^2.s^{-1}$, which is 63% of the diffusion coefficient in water (Weast, 1979; Perry & Chilton 1973). This agrees well with the work of Toda & Shoda (1975), who found for the diffusion coefficient of sucrose at 47.5 °C in 2% agar a value of 72% of the diffusion coefficient in water.

The Michaelis-Menten constants V_m and K_m for DE32-cellulose-invertase at 30 °C, determined as described, were found to be 0.346 $mol.kg^{-1}$ DE32-cellulose-invertase per second and 198.3 $mol.m^{-3}$, respectively, whereas for free invertase a value for K_m of 198.1 $mol.m^{-3}$ was found. These values were used as intrinsic parameters for DE32-cellulose-invertase immobilized in alginate in the reactor performance experiments.

Reactor Performance experiment

Two cascades of two and three reactors were tested as described in Materials and Methods. Initial and operational values for both the computer program and experiments were : V_m, K_m and D_e as described above, X = 6.15 g DE32-cellulose-invertase.dm^{-3} gel, r_b = 2.66 mm, S_0 = 233.7 $mol.m^{-3}$, and ε = 0.3. Other initial model parameters and experimental values for ϕ_v, V_{total} and α_N are presented in table 2.

Table 2 : Model input parameter values and experimental data for two cascades of two and three mixed reactors, respectively.

	N = 2		N = 3		
	Model	Experiment	Model	Experiment	Dimensions
ϕ_v	0.5	0.51	0.5	0.51	10^{-6} $m^3.s^{-1}$
V_{total}	1.51	1.5	1.37	1.39	10^{-3} m^3
α_N	0.7	0.73	0.7	0.69	-

Table 2 shows that the experimentally obtained conversion rates fit the initially set values well. Other tests with single mixed reactors showed relative errors in conversion rate of less than 6%.

In the reactors the overall effectiveness factors were calculated to be between 0.28 and 0.24, and the Thiele modulus varied between 3.21 and 3.84. This modulus for a spherical particle with a biocatalyst obeying Michaelis Menten kinetics (Froment & Bischof 1979, Aris 1975), is defined as the ratio of substrate transport by diffusion and the substrate consumption rate :

$$\Phi_m = \frac{r_b}{3}\sqrt{\frac{V_m X S_s l (K_m + S_s)}{2(K_m + S_s) D_e (1 + \frac{Km}{Ss} \ln(\frac{Km}{Km+Ss}))}} \qquad [14]$$

As an example, a concentration profile for a biocatalyst bead in the last reactor of both series (S_b = 70.1) is shown in figure 3. This clearly shows the diffusion limitated use of substrate in the bead.

CONCLUSIONS

The modelling of immobilized biocatalyst beads has been successfully carried out and implemented in a model for optimal design of continuous stirred tank reactors in series. It is shown that, for immobilized DE32-cellulose-invertase, internal mass transfer limitation is of much more importance than external mass transfer resistance, which is negligible in this study.

Additional work will be done on the modelling of the design of equal-sized mixed reactors in series containing immobilized catalysts, in order to improve the practical design purpose of the model.

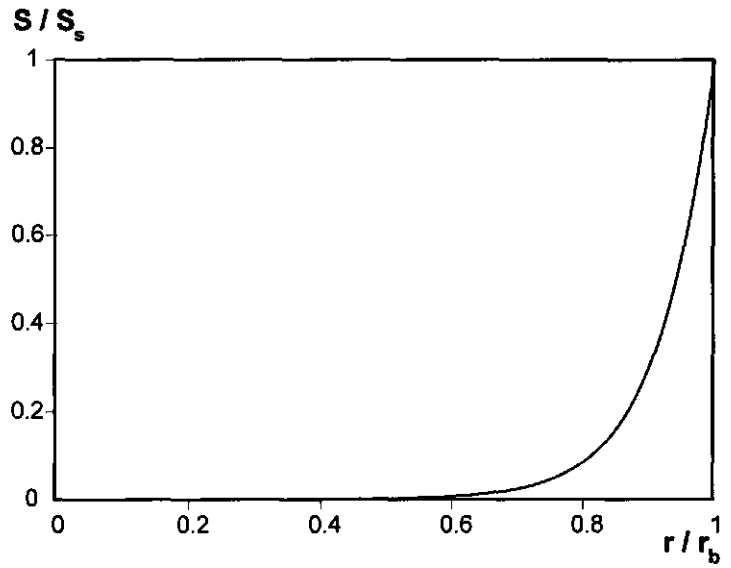

Figure 3. Substrate concentration profile in a biocatalyst bead : substrate concentration related to the substrate concentration at the surface of the bead versus the distance from the center of the bead related to the radius of the bead. (Parameters are referred to in text.)

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NOMENCLATURE

a_b	Surface area of one bead	m^2
D_e	Effective diffusion coefficient	$m^2.s^{-1}$
k_l	Substrate mass transfer resistance coefficient in the film layer	$m.s^{-1}$

K_m	Michaelis-Menten constant	$mol.m^{-3}$
N	Number of reactors in series	-
r	Distance from the centre of the bead	m
r_b	Radius of the bead	m
r_f	Distance from the centre of the bead where substrate concentration is negligible	m
$r(s)$	Reaction rate	$mol.m^{-3}.s^{-1}$
$r'(s)$	Reaction rate in the gel	$mol.m^{-3}.s^{-1}$
S	Substrate concentration	$mol.m^{-3}$
S_0	Substrate concentration at the inlet of the first reactor	$mol.m^{-3}$
S_b	Substrate concentration in the bulk	$mol.m^{-3}$
S_f	Substrate concentration in the centre of the bead	$mol.m^{-3}$
S_N	Substrate concentration in the last reactor of the series	$mol.m^{-3}$
S_s	Substrate concentration at the surface of the bead	$mol.m^{-3}$
S_s'	Substrate concentration at the surface of the bead, when external diffusion limitation is accounted for	$mol.m^{-3}$
t	Time	s
V_m	Maximum reaction rate	$mol.kg^{-1}.s^{-1}$
X	Gel load	$kg.m^{-3}$
X_1	Amount of DE32-cellulose-invertase in one bead	kg

Greek symbols :

α	Dimensionless concentration (S / S_0)	-
ε	Gel holdup in reactor	-
η_{ext}	External effectiveness factor	-
η_{int}	Internal effectiveness factor	-
η_s	Overall effectiveness factor	-

θ	Holding time (mean residence time)	s
κ	Dimensionless Michaelis-Menten constant (K_m/S_0)	-
τ	Dimensionless holding time ($\theta V_m \eta_s \varepsilon X / S_0$)	-
ϕ_v	Flow rate	$m^3.s^{-1}$
ϕ_m	Generalized Thiele modulus	-

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This chapter has been submitted for publication by the authors De Gooijer, C.D., Beeftink, H.H. and Tramper, J.

CHAPTER 3

OPTIMUM DESIGN OF A SERIES OF CONTINUOUS STIRRED TANK REACTORS CONTAINING IMMOBILIZED GROWING CELLS

ABSTRACT

The optimum design of a series of continuously operated stirred-tank reactors containing immobilized growing cells is described. Optimal design is defined as the minimal total holding time over the reactor series to achieve a certain degree of conversion. The analysis is made under the assumptions that there is a constant and equal concentration of immobilized biomass in all bioreactors along the series, no diffusion limitation takes place, all growth of the immobilized biomass will lead to an increase in suspended biomass, and that maintenance of the immobilized cells can be neglected. It is shown that the use of more than three bioreactors in series is likely to be obsolete.

INTRODUCTION

The optimal design for cascades of bioreactors has been investigated by many researchers (Luyben & Tramper 1982, Schügerl 1982, Malcata 1988, 1989, De Gooijer et al. 1989, 1995, Hill & Robinson 1989, Malcata & Cameron 1992, Lopes & Malcata 1993). Series of bioreactors are favourable if the combination of a high degree of conversion, a high product concentration and an acceptable volumetric productivity is aimed at (Levenspiel 1972, 1979, Schügerl 1982, Moser 1985, Godia et al. 1987, Shimizu & Matsubara 1987, Shama 1988, De Gooijer et al. 1995). In general, either enzymes or autocatalytic systems with a constant overall stoichiometry are considered. For the latter, only papers can be found that discuss the optimum design of a series of bioreactors with suspended cells (Schügerl 1982, Hill & Robinson 1989).

For immobilized cells only a strategy to design a single CSTR containing immobilized growing cells is described (Venkatasubramanian et al. 1983), whereby mass transport limitations were assumed to be negligible. In the present paper, using the same assumption, a straightforward approach for the optimal design of series of CSTR's containing immobilized growing cells will be presented.

THEORY

Optimal design can be defined as the minimal total holding time over the reactor series to achieve a certain degree of conversion, as introduced by Luyben & Tramper (1982) and as also used in previous papers (De Gooijer et al. 1989, 1995), and by Hill & Robinson (1989), fully realizing the limitation of this definition. Mathematically this leads to:

$$\frac{\partial \sum_{j=1}^{j=N} \tau_j}{\partial S_i} = 0 \qquad i = 1...N\text{-}1 \qquad [1]$$

with S the substrate concentration (mol.m^{-3}), τ the residence time (s) in a vessel in the series, and N the number of vessels in the series. A mass balance for substrate over the i-th reactor in the series yields :

$$\tau_i = \frac{Y_{xs}}{\mu_i} \frac{S_{i-1} - S_i}{X_i + X_{im}} \qquad [2]$$

where Y_{xs} is the yield of biomass on substrate (kg.mol^{-1}), μ is the specific growth rate (s^{-1}), X the biomass concentration (kg.m^{-3}), and subscripts i and im denote the i-th reactor in the series and the immobilized biomass concentration, respectively. In order to be able to execute the optimal design of a series of bioreactors, the following assumptions are made :

i) the concentration of immobilized biomass is equal and constant in all bioreactors along the series,

ii) no diffusion limitation takes place,

iii) all growth of the immobilized biomass will lead to an increase in suspended biomass, and

iv) maintenance of the immobilized cells is neglected.

Assumptions ii) and iii) were also used by Venkatasubramanian (1983). Assumption iv) is in fact a prerequisite to be able to do the optimal design, since the overall stoichiometry of the reaction has to be constant (De Gooijer, 1995), and all well-accepted mathematical formulations for maintenance change the overall reaction stoichiometry (Herbert 1959, Pirt 1965, Beeftink et al. 1990).

A mass balance for biomass over the i-th reactor in the series gives :

$$\tau_i = \frac{X_i - X_{i-1}}{\mu_i (X_i + X_{im})} \qquad [3]$$

A combination with equation 2, and addition over all vessels in the series up to the *i*-th reactor then yields the equation stating that along the series, substrate is converted into biomass :

$$X_i = Y_{xs}(S_0 - S_i) \qquad [4]$$

with subscript *0* denoting the inlet of the first reactor of the series. Combining equations [2] and [4] leads to :

$$\tau_i \mu_i = \frac{S_{i-1} - S_i}{S_0 - S_i + X_{im}/Y_{xs}} \qquad [5]$$

Introducing Monod growth kinetics, and the following dimensionless variables :

$$\theta_i = \tau_i \mu_{max},\ \alpha_i = \frac{S_i}{S_0},\ \kappa = \frac{K_s}{S_0},\ \gamma = \frac{X_{im}}{Y_{xs} S_0} \qquad [6]$$

with K_s the Monod constant (mol.m^{-3}), equation [5] can be rewritten as:

$$\theta_i = \frac{(\alpha_{i-1} - \alpha_i)(\kappa + \alpha_i)}{\alpha_i(1 + \gamma - \alpha_i)} \qquad [7]$$

With the dimensionless variables, the optimal design criterion (equation [1]) turns into :

$$\frac{\partial \sum_{j=1}^{j=N} \theta_j}{\partial \alpha_i} = 0 \qquad i = 1...N\text{-}1 \qquad [8]$$

As with Luyben & Tramper (1982), and Hill & Robinson (1989), only two terms in equation [8] contain an α_i. Hence, equation [8] simplifies to :

$$\frac{\partial}{\partial \alpha_i}\left(\frac{(\alpha_{i-1} - \alpha_i)(\kappa + \alpha_i)}{\alpha_i(1 + \gamma - \alpha_i)} + \frac{(\alpha_i - \alpha_{i+1})(\kappa + \alpha_{i+1})}{\alpha_{i+1}(1 + \gamma - \alpha_{i+1})}\right) = 0 \qquad i = 1..N\text{-}1 \qquad [9]$$

Taking the differentiation, it is found that

$$\frac{(\alpha_{i-1}-\alpha_i)-(\kappa+\alpha_i)}{\alpha_i(1+\gamma-\alpha_i)} - \frac{(1+\gamma-2\alpha_i)(\kappa+\alpha_i)(\alpha_{i-1}-\alpha_i)}{(\alpha_i(1+\gamma-\alpha_i))^2} + \frac{(\kappa+\alpha_{i+1})}{\alpha_{i+1}(1+\gamma-\alpha_{i+1})} = 0 \qquad i=1..N\text{-}1 \qquad [10]$$

Hill & Robinson (1989) report that it is possible to solve the set of equations [10] for all reactors in the series by means of a zero-finding routine on a PC. For the first vessel, α_{i-1} is known, α_i is estimated, and α_{i+1} is solved by the method of false position (Press et al., 1986). This is repeated for the subsequent vessels until α_N is calculated, which value then can be compared to the known α_N and a new value of α_1 is chosen, again by the method of false position. Where this method is valid for the three cases of Monod, Aiba and Haldane kinetics (Hill & Robinson, 1989), a closer inspection of equation [10] reveals, however, that for the pertinent case of Monod kinetics, equation [10] is quadratic in α_{i+1}, and hence :

$$\alpha_{i+1} = \frac{(Q+Q\gamma+1) \pm \sqrt{(Q+Q\gamma+1)^2+4Q\kappa}}{2Q} \qquad i=1..N\text{-}1 \qquad [11]$$

with

$$Q = \frac{(\alpha_{i-1}-\alpha_i)-(\kappa+\alpha_i)}{\alpha_i(1+\gamma-\alpha_i)} - \frac{(1+\gamma-2\alpha_i)(\kappa+\alpha_i)(\alpha_{i-1}-\alpha_i)}{(\alpha_i(1+\gamma-\alpha_i))^2} \qquad [12]$$

Here as well α_1 is used as estimator. Since for the first vessel α_{i-1} is known ($\alpha_{i-1} = \alpha_0 = 1$), α_i is estimated, and α_{i+1} can be calculated by equations [11] and [12]. This is repeated for all dimensionless substrate concentrations in the subsequent vessels until α_N is calculated. This value can then be compared with

the known α_N, after which α_1 can be changed, until a minimal error between the calculated and known α_N is found. This is done in two ways : for values of the dimensionless immobilized biomass concentration γ larger or equal to 1 the interval with possible values of α_1, i.e. [0..1], is constantly halved until the α_N that is calculated has a difference with the desired α_N of less than 10^{-6}. For values of the dimensionless immobilized biomass concentration γ smaller than 1, it was found that the case can arise where equation [11] has no roots (in other words : a negative discriminant), causing that there is no longer information available to decide how to halve the interval. Therefore, the interval in which the dimensionless substrate concentration in the first reactor of the series α_1 can lie, i.e. [0..1], is evaluated stepwise until a minimal difference between the calculated and desired α_N is found. This error was found to be in the range of 10^{-7}-10^{-8}.

After the calculation of the intermediate dimensionless substrate concentrations, the dimensionless residence times can easily be calculated by equation [7].

Hill & Robinson (1989) introduced the concept of α_{crit} with which the feasibility of optimal design of series of bioreactors can be evaluated : if the effluent concentration aimed for, α_N, is smaller than α_{crit}, series of bioreactors are superior to a single CSTR. For the pertinent case of Monod kinetics it can be derived that α_{crit} can be calculated according to (Hill & Robinson, 1989, De Gooijer et al. 1995):

$$\alpha_{crit} = \sqrt{\kappa^2 + \kappa + \kappa\gamma} - \kappa \qquad [13]$$

For all values of the parameters investigated here α_N was smaller than α_{crit}.

RESULTS AND DISCUSSION

For the pertinent case of Monod kinetics, three parameters are of interest : the dimensionless degree of conversion of the series α_N, the dimensionless Monod constant κ, and the dimensionless immobilized biomass concentration γ. Figures 1-3 show the normalized dimensionless total residence time of a series (that is the total dimensionless residence time of the series divided by the dimensionless residence time of a **single** vessel with the same α_N) versus the number of reactors *N* of the series for these three parameters.

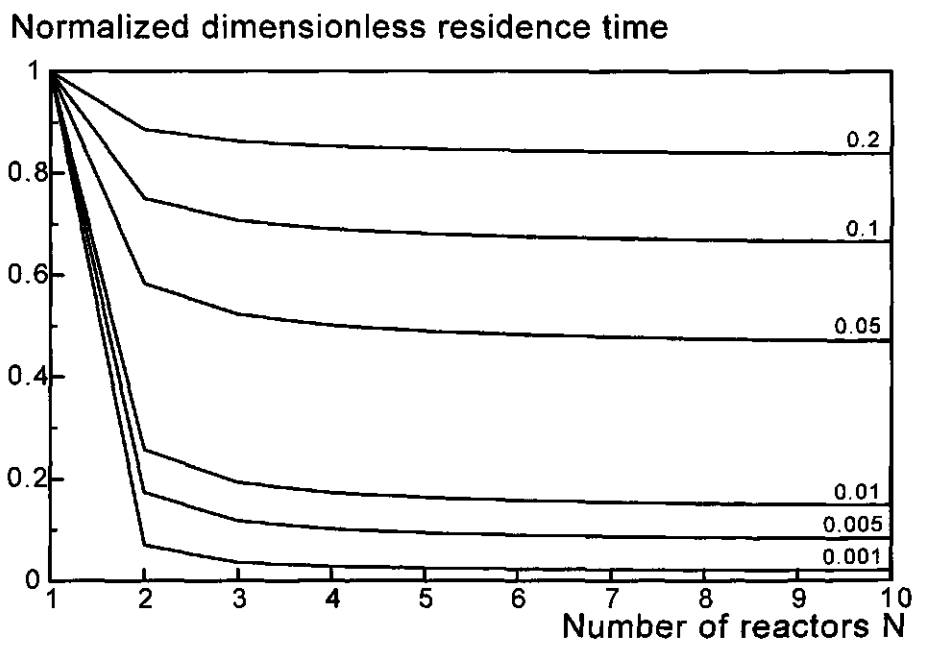

Figure 1 : Normalized dimensionless residence time ($\Sigma\theta_i / \theta_1$) versus the number of CSTR's in series. Parameter is the dimensionless substrate concentration at the exit of the series, α_N, with γ = 10 and κ = 0.1

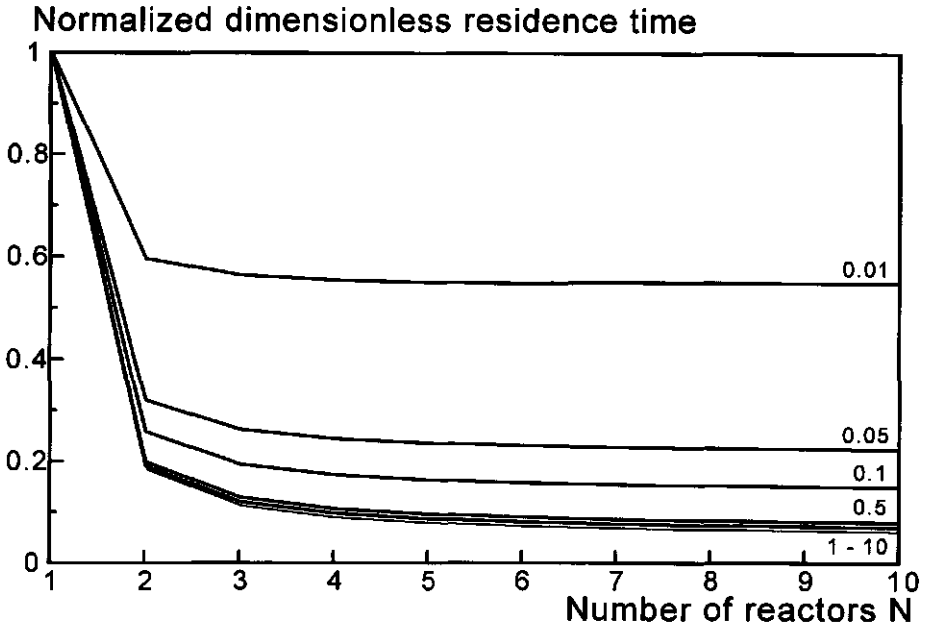

Figure 2A : Normalized dimensionless residence time ($\Sigma\theta_i / \theta_1$) versus the number of CSTR's in series. Parameter is the dimensionless Monod constant, κ, with $\gamma = 10$ and $\alpha_N = 0.01$

From figures 1-3 it is clear that the total residence time of the series is decreasing with an increasing number of reactors. However, the graphs also show that it is hardly worthwhile to consider the use of more than two or three reactors in series, as also stated by Hill & Robinson (1989).

Figure 1 shows that the total residence time of the series as compared to a single vessel increases with increasing outlet concentration α_N, which is consistent with experimental results (Bakker et al., 1995). By far the largest decrease in residence time by adding a bioreactor in the series is attained by using two reactors instead of one. The same is true for other values of the

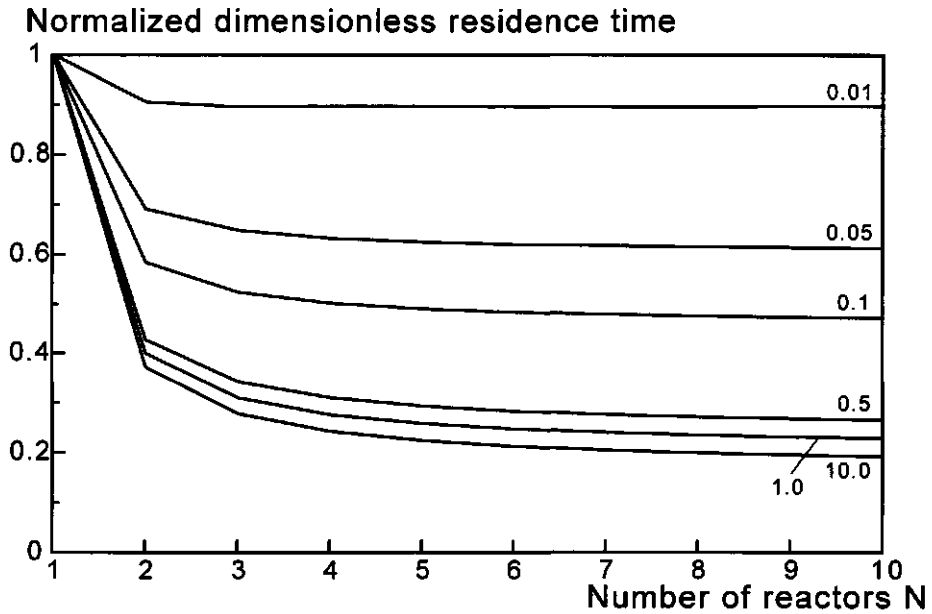

Figure 2B : Normalized dimensionless residence time ($\Sigma\theta_i / \theta_1$) versus the number of CSTR's in series. Parameter is the dimensionless Monod constant, κ, with $\gamma = 10$ and $\alpha_N = 0.05$

dimensionless Monod constant κ, e.g. 1 or 10, as shown in figures 2A and 2B for $\alpha_N = 0.01$ and 0.05, respectively.

From figure 2 it can be concluded that the decrease in total dimensionless residence time is larger at increasing κ, that is at higher Monod constants or lower substrate concentrations at the inlet of the series. Since a lower κ means approaching zero-order kinetics, this was to be expected. The decrease is more pronounced at higher degrees of conversion.

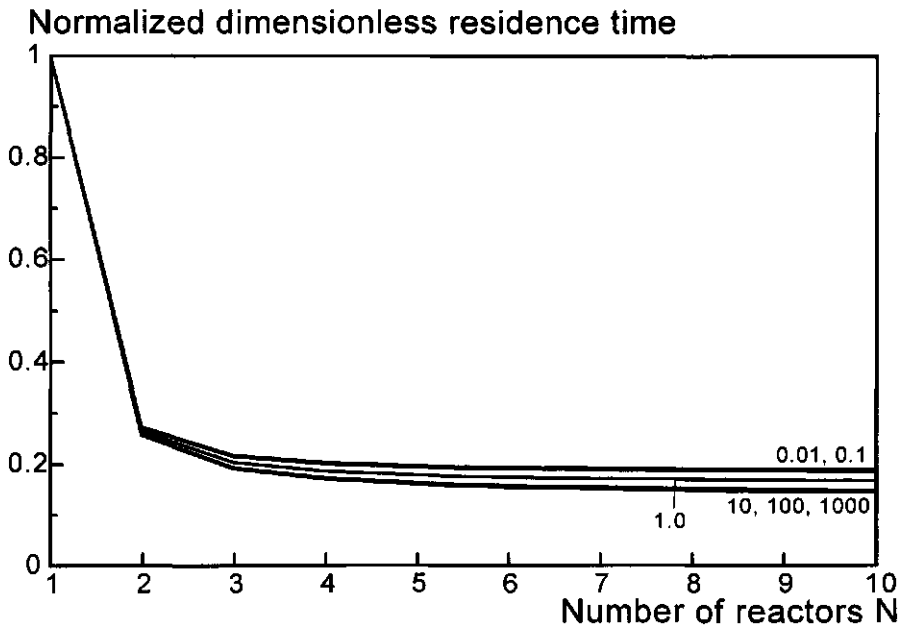

Figure 3A : Normalized dimensionless residence time ($\Sigma\theta_i / \theta_1$) versus the number of CSTR's in series. Parameter is the dimensionless immobilized biomass concentration, γ, with κ = 0.1 and α_N = 0.01

In figure 3A and 3B the effect of varying the dimensionless immobilized biomass concentration γ is depicted. Interestingly, both graphs indicate the existence of both a set of minimal and a set of maximal values of γ. For high values of γ, the contribution of suspended cells to the reaction is minimal and hence the reactor system approaches a catalytic system. For low values of γ, the contribution of the immobilized biomass is low, in other words, the situation of suspended cells is approached. For the latter case, Hill & Robinson (1989) presented the design procedure. The top lines in figures 3A and 3B are identical to their lines for suspended cells. Hill & Robinson (1989) also discuss the optimal design of a series of bioreactors with biomass in the influent, which is

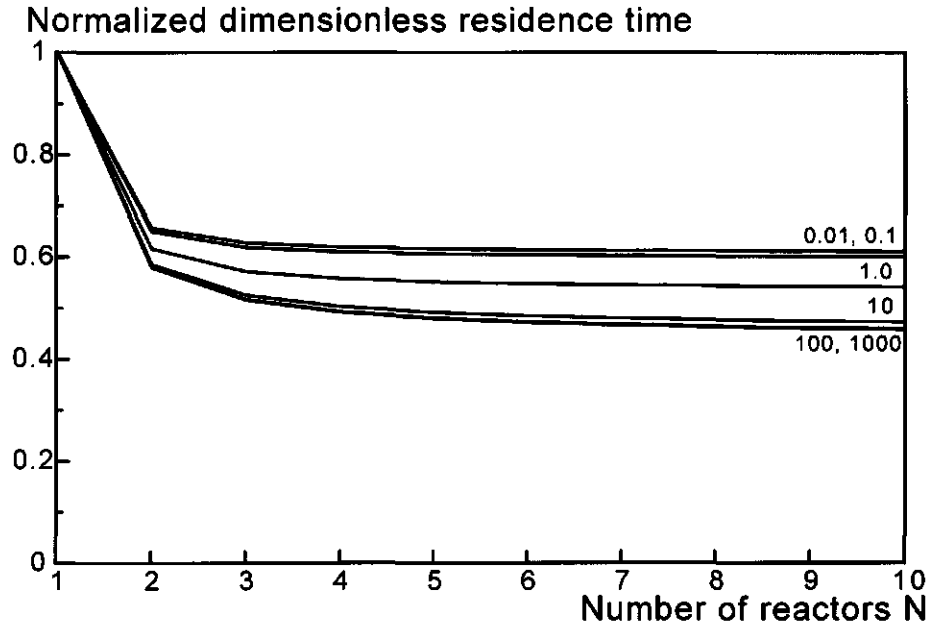

Figure 3B : Normalized dimensionless residence time ($\Sigma\theta_i / \theta_1$) versus the number of CSTR's in series. Parameter is the dimensionless immobilized biomass concentration, γ, with κ = 0.1 and α_N = 0.05

mathematically comparable to the case discussed here. The point in figure 3A where N=3 and γ = 1 matches exactly with their data, indicating that the design procedure and mathematical solution followed here are probably correct.

Figures 3A and 3B show that a change in dimensionless immobilized biomass concentration γ has not as large an influence on the ratio of the total dimensionless residence time of the series and the residence time of a single vessel as a change in dimensionless Monod constant κ (Figure 2A and 2B) or a change in dimensionless outlet concentration α_N (Figure 1) have.

In this paper only a steady-state situation is described. For a more thorough description of mass transfer and simultaneous substrate consumption

and growth of immobilized cells, a more detailed approach has to be used, as given by the dynamic models described by Nakasaki et al. (1989), Monbouquette et al. (1990), De Gooijer et al. (1991) and Wijffels et al. (1991). Whether these models, however, will result in a constant immobilized biomass concentration in each vessel along the series, as assumed in this paper, remains to be investigated.

CONCLUSIONS

For the case of immobilized growing cells, the largest decrease in residence time by adding a bioreactor in the series is attained by using two reactors instead of one reactor. The use of more than three bioreactors in the series has only a minor effect on the total dimensionless residence time.

The decrease in total dimensionless residence time is larger at higher dimensionless Monod constants κ. Also, the decrease is more pronounced at higher degrees of conversion. For low values of the dimensionless immobilized biomass concentration γ the situation of suspended cells is approached. The effect of γ on the normalized dimensionless residence time of the series is less pronounced than the effect of κ or α_N.

The optimal design for a series of CSTR's containing immobilized growing cells is shown to be possible, under the assumptions of a constant immobilized biomass concentration, no diffusion limitation, and negligible maintenance conditions.

NOMENCLATURE

α	dimensionless substrate concentration (S / S_0)	-
γ	dimensionless immobilized biomass concentration ($X_{im}/Y_{xs}S_0$)	-
θ	dimensionless residence time ($\mu_{max}\ \tau_i$)	-
κ	dimensionless Monod constant (K_s/S_0)	-
μ	specific growth rate	s^{-1}
τ	residence time	s
N	number of vessels in a series	-
S	substrate concentration	$mol.m^{-3}$
X	biomass concentration	$kg.m^{-3}$
Y_{xs}	yield of biomass on substrate	$kg.mol^{-1}$

Subscripts

0	inlet of a series
i	*i*-th vessel in a series
im	immobilized
max	maximum
N	last vessel in a series

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CHAPTER 4

GROWTH AND SUBSTRATE CONSUMPTION OF *NITROBACTER AGILIS* CELLS IMMOBILIZED IN CARRAGEENAN: PART 1. DYNAMIC MODELLING

ABSTRACT

The modelling of the growth of *Nitrobacter agilis* cells immobilized in κ-carrageenan is presented. A detailed description is given of the modelling of internal diffusion and growth of cells in the support matrix in addition to external mass transfer resistance. The model predicts the substrate and biomass profiles in the support as well as the macroscopic-oxygen consumption rate of the immobilized biocatalyst in time. The model is tested by experiments with continuously operated air-lift loop reactors, containing cells immobilized in κ-carrageenan. The model describes experimental data very well. It is clearly shown that external mass transfer may not be neglected. Furthermore, a sensitivity analysis of the parameters at their values during the experiments revealed that apart from the radius of the spheres and the substrate bulk

concentration, the external mass transfer resistance coefficient is the most sensitive parameter for our case.

INTRODUCTION

The application of immobilized, growing cell systems has become of increasing interest in the past few years. Examples of applications are immobilized yeast cells for ethanol production (Seki & Furusaki 1985, Godia et al. 1987, Nakasaki et al. 1989), and producing acrylamide from acrylonitril with *Corynebacterium* (Furusaki 1988). Traditionally, biofilms are used in wastewater treatment (Siegrist & Gujer 1987, Suidan et al. 1987, Canovaz-Diaz & Howell 1988, Saez & Rittmann 1988) and in this field the use of entrapped nitrifying bacteria is gaining importance (Wijffels & Tramper 1989).

Many researchers have reported the phenomenon of growth of cells near the surface of immobilized particles, whereas in the centre of these particles no growth, or even decay of cells occurred (Sato & Toda 1983, Toda & Sato 1985, Mahmoud & Rehm 1986, Karel & Robertson 1989, Wijffels & Tramper 1989). As a result of this non-homogeneous growth, the characteristics of the solid phase will alter, thereby affecting, among other things, the effective diffusion coefficient (Hiemstra et al. 1983, Monbouquette & Ollis 1986, Chen & Huang 1988, Gosmann & Rehm 1989, Scott et al. 1989).

The main feature of immobilized growing systems is the high attainable concentration of active biocatalyst in the solid phase, which, combined with a high reactor load, can lead to small reactor volumes compared to cell suspensions. A major drawback of these systems can be considered to be the rather troublesome mathematics involved with the simultaneous diffusion of substrate and/or product(s) and the growth of the immobilized cells. Steady-state

models for growing cells (Monbouquette & Ollis 1986, Suidan et al. 1987, Andrews 1988) can be quite satisfactory for design purposes but may fail to describe the start-up phase and the response to changing conditions in the reactor system. In recent work, Nakasaki et al. (1989) and Sayles & Ollis (1989) presented a dynamic model describing the transient growth of immobilized cells. However, in both studies external mass transfer resistance was neglected, whereas Nakasaki et al. (1989) also did not consider a biomass-dependent effective diffusion coefficient. Only recently, Monbouquette et al. (1990) presented a model that included both external mass transfer and a biomass-dependent diffusion coefficient.

In this article we describe the modelling of the growth of immobilized cells, accounting for external mass transfer resistance and simultaneous diffusion and consumption of substrate for respiration and growth, using a biomass concentration-dependent diffusion coefficient. The growth model used allows a negative net growth, and the biomass concentration is limited to a maximum value. The model has been tested by experiments in continuously operated air-lift loop reactors with *Nitrobacter agilis* cells immobilized in κ-carrageenan.

THEORY

General consideration

In many aerobic systems oxygen is the limiting substrate, due to the rather low solubility. For our case, oxygen transfer can be divided into four different regions, as illustrated in figure 1. Oxygen has to be transferred (1) from the (ideally mixed, with a negligible stagnant gas layer) gas phase through a stagnant liquid layer to the (ideally mixed) bulk phase, (2) from the bulk phase

through a stagnant layer adjacent to the solid phase, (3), in the solid phase to the cells, and (4) into the cells to the enzyme complex.

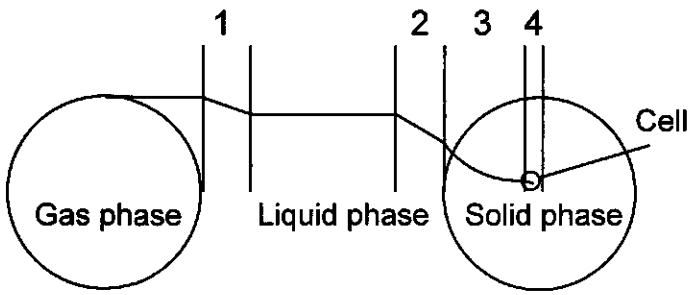

Figure 1. Oxygen transfer in an air-lift loop reactor with immobilized cells.

In order to define the rate-limiting steps, we calculated the characteristic times for each step, as presented in Table 1.

Table 1. Regime analysis for oxygen transfer.
***T* is the time constant (s), *a* the specific surface area (m^{-1}), $k_{l,s}$ and $k_{l,g}$ the mass transfer coefficients in the stagnant layers, D_e the effective diffusion coefficient in the gell and in the cell, r_b the radius of the biocatalyst beads, and r_c the radius of the cells.**

STEP	TIME CONSTANT (S)	CALCULATED FROM	REFERENCE
1	172	$T = \frac{1}{k_{l,g}a}$	Chang & Moo-Young 1988, Moser 1988; see also Appendix
2	35	$T = \frac{1}{k_{l,s}a}$	Sanger & Deckwer 1981, Moser 1988; see also Appendix
3	66	$\frac{D_e T}{r_b^2} = 0.1$	Furusaki 1989
4	10^{-4}	$\frac{D_e T}{r_b^2} = 0.1$	Furusaki 1989

Also, the possibility of exhaustion of oxygen from the gas bubble has to be considered. As for our case, the residence time of the air bubbles (1.2 s) is much lower than the characteristic time for exhaustion (35 s; Appendix) oxygen

depletion is quite unlikely. With scale-up, however, the air bubble exhaustion might become considerable (Oosterhuis 1984).

The diffusion of oxygen into the cells is fast in comparison to diffusion through the stagnant layers and in the solid phase. In our experimental set-up, the bulk concentration of substrate was kept constant. Therefore, in this study we do not consider mass transfer from the gas phase, and neglect diffusion within the cells. From table 1, it might be concluded that diffusion through the film layer can be neglected also, and the only limiting step would be diffusion within the solid phase. However, since the cells will grow in the outer shells of the bead, a biofilm develops, and the distance over which substrate is transported is smaller than the radius of the bead. Hence, the value for the radius used in table 1 should be decreased. If a radius of 100 μm, being a typical film thickness, is substituted, we find a characteristic time of 0.6 s. This indicates that, for our case, external diffusion limitation should be considered.

Growth model

Apart from the logistic growth model (Mulchandani et al. 1988), two models are often used to describe the growth of cells. The first model:

$$r_s = \frac{\mu}{Y_{xs}} X \qquad [1]$$

$$r_x = (\mu - mY_{xs})X \qquad [2]$$

is well-known as the Herbert model (Herbert, 1959), where r_s is the consumption rate of substrate (mol.kg^{-1}.s^{-1}), μ the specific growth rate (s^{-1}), Y_{xs} the yield coefficient (kg.mol^{-1}), X the biomass concentration (mol.m^{-3}), r_x the growth rate of

the biomass ($kg.m^{-3}s^{-1}$), and m the maintenance coefficient ($mol.kg^{-1}.s^{-1}$). The second model:

$$r_s = (\frac{\mu}{Y_{xs}}X + m)X \quad [3]$$

$$r_x = \mu X \quad [4]$$

is the often used Pirt model (Pirt, 1965). In both models the Monod equation is used :

$$\mu = \frac{\mu_{max} S}{K_s + S} \quad [5]$$

where μ_{max} is the maximum specific growth rate (s^{-1}), S the substrate concentration ($mol.m^{-3}$), and K_s the Monod constant ($mol.m^{-3}$).

Both models, however, have some major drawbacks, as stated by Beeftink et al. (1990): the Herbert model i) features a maximum specific growth rate that cannot be measured directly and ii) directs that cells cover their maintenance requirements from biomass, even in the presence of excess substrate, whereas the Pirt model shows i) a growth rate that is always positive or zero, and ii) a non-zero substrate consumption even if no substrate is available.

In the case of growing immobilized cells, substrate depletion near the centre of the biocatalyst bead may occur, whereas a high substrate concentration will be available near the surface. Hence, a growth model is needed that is capable of describing both situations, allowing a negative net growth at low substrate concentrations and an observable maximum growth rate at high substrate concentrations. As Pirt's extended model (Pirt, 1987) would introduce an extra variable, we use the growth model suggested by Beeftink et al. (1990):

$$r_s = \frac{\mu}{Y_{xs}} X + mX\left(\frac{S}{K_s + S}\right) \quad [6]$$

$$r_x = \mu X - mY_{xs}X\left(1 - \frac{S}{K_s + S}\right) \quad [7]$$

This is a combination of the models of Pirt and Herbert. At low substrate concentrations, this model will perform as the Herbert model, while at high substrate concentrations it acts as the Pirt model.

The model is based on the consumption of one limiting substrate only. For our case, nitrification, both nitrite and oxygen are substrates. Andrews (1988) showed that the component with the lowest product of diffusion coefficient, yield factor and concentration in the liquid phase is rate limiting. For our case, oxygen is always rate limiting (Wijffels et al. 1991).

Diffusion coefficient

As stated before, many researchers proved that the effective diffusion coefficient is affected by the concentration of biomass in the support material. The theoretically most correct way of describing this phenomenon is by:

$$D_e = D_0 \frac{\theta}{\tau} \quad [8]$$

where τ is the tortuosity (-), θ is the porosity (-) of the gel matrix, and D_0 is the diffusion coefficient in water ($m^2.s^{-1}$). This model, however, will introduce two, hardly determinable, variables. Therefore, a more convenient relation was proposed by Scott et al. (1989), using a simple second-order polynomial:

$$D_e = D_{e,g}(1 + bf + cf^2) \quad [9]$$

where f is the volumetric fraction of the entrapped cells on a wet basis (-), $D_{e,g}$ is the effective diffusion coefficient in the gel matrix without cells ($m^2.s^{-1}$), and b and c are appropriate constants. A more fundamental approach is given by Wakao & Smith (1962):

$$\frac{D_e}{D_0} = k_1(1 - k_2X)^2 \qquad [10]$$

where k_1 is the ratio of the diffusivities in the gel and in water (-), and k_2 corresponds to the specific cell volume ($m^3.kg^{-1}$). This model is also referred to as the random-pore model.

The latter two relations use the specific cell volume. Recent work of Stewart & Robertson (1989) and Karel & Robertson (1989) suggests that cells can grow under considerable pressure, thereby decreasing their specific cell volume. As in the support matrix the available space for growth will be limited, the specific cell volume may be altered, and therefore its accurate determination is troublesome.

The determination of the effective diffusion coefficient at high biomass concentrations is also difficult: a homogeneous high biomass concentration can hardly be reached, and for a correct determination the cells must be intact, and therefore a non-consumable compound with the same physical properties as the substrate must be found, which is difficult. Alternatively, using oxygen as diffusing compound, the respiration of the cells must be stopped, and then the properties of the cells, and with that the effective diffusion coefficient, may be altered. Even if a suitable compound can be found, the relative error in the determination of the diffusion coefficient in biocatalyst beads is considerable (Itamunoala 1989).

In order to account for the dependency of the effective diffusion coefficient on the biomass concentration, we have implemented a relationship that generates an effective diffusion coefficient that varies linearly with the biomass concentration between the diffusion coefficient in the gel and zero. The effective diffusion coefficient will become zero when all available space in the gel matrix is occupied by biomass, and if no biomass is present, the effective diffusion coefficient becomes equal to the diffusion coefficient in the gel. Mathematically this leads to:

$$D_e = D_{e,g}(1 - \frac{X}{X_{p,max}}) \qquad [11]$$

where $X_{p,max}$ is the maximum attainable biomass concentration, that would be reached if the cells could grow everywhere in the support matrix where space is not occupied by the gel material ($kg.m^{-3}$).

Calculations

From a differential mass balance over a biocatalyst bead in which simultaneous diffusion and consumption of substrate takes place, assuming that :

i) there is only a radial concentration gradient,

ii) no convective transport occurs,

iii) the effective diffusion coefficient is constant within one time step and within one step in the integrating algorithm,

the following equation can be found :

$$D_e(\frac{1}{r^2}\frac{\partial}{\partial r}(r^2\frac{\partial S}{\partial r})) = r'_s + \frac{\partial S}{\partial t} \qquad [12]$$

where t is the time (s), r'_s is the substrate consumption rate of the cells in the gel (mol.kg^{-1}.s^{-1}), and r is the distance from the centre of the bead (m). Boundary conditions for equation [12] are :

$$S = S_s \quad at\ r = r_b \qquad [13]$$

$$\frac{\partial S}{\partial r} = 0\ at\ r = 0\ or\ r = r_f \qquad [14]$$

where S_s is the substrate concentration at the surface of the bead (mol.m^{-3}), and r_f is the radial position where the substrate concentration approaches zero.

For the set of equations [5-7] and [12] no analytical solution can be found. In order to facilitate a numerical solution, we treat the biomass growth and substrate consumption process separately. This is a valid approach as long as the time scale for growth is larger then the time scale for consumption (Gujer & Boller 1989, Karel & Robertson 1989). For our case, the characteristic time for growth is 10^5 s (calculated from $1/\mu_{max}$), and for substrate consumption 66 s (from table 1; characteristic time for diffusion). Hence, if i) time is discretisized, ii) a pseudo-steady-state substrate distribution inside the support matrix is assumed within one time step, equation [12], after partial differentiation and substitution of equation [6] reduces to:

$$\frac{\partial^2 S}{\partial r^2} = \frac{\mu X(t)/Y_{xs} + mX(t)(S/K_s + S)}{D_e} - \frac{2}{r}\frac{\partial S}{\partial r} \qquad [15]$$

We have solved Equation [13] numerically using a second-order embedded Runge-Kutta algorithm (Hairer et al. 1986), with Newton's method to satisfy the boundary conditions. Starting with a small value for r_f and S_f this results in a substrate concentration profile in the bead and a computed substrate concentration S_s and a dS/dr at $r = r_b$.

In order to account for external mass transfer resistance, the latter can (optionally) be used for calculating a S'_s by means of the equation representing the film theory:

$$S'_s = S_b - \frac{D_e}{k_{l,s}} \frac{\partial S}{\partial r}_{r=r_b} \qquad [16]$$

with S_b the substrate concentration in the bulk of the solution (mol.m^{-3}). While varying either r_f or S_f, equations [13] and [14] are solved iteratively until the S_s and S'_s match, after which the new biomass distribution is calculated according to equation [7].

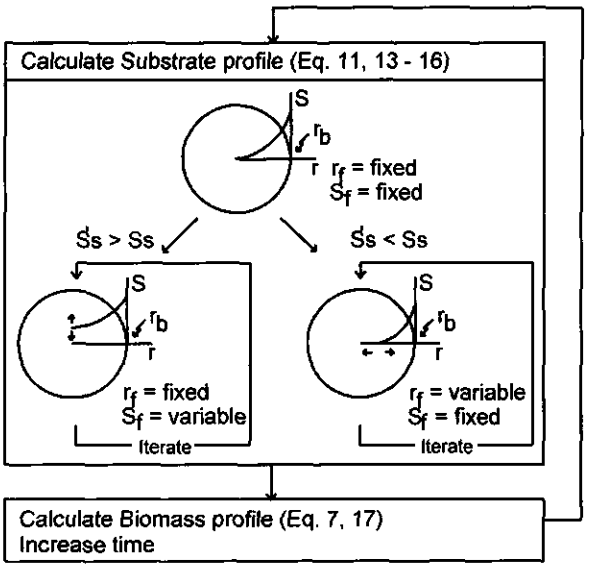

Figure 2. Calculation of substrate and biomass concentration profiles in a biocatalyst bead.

The calculation scheme, including the procedure used if the substrate concentration becomes essentially zero before the centre of the support matrix is reached, as presented earlier (De Gooijer et al. 1989), is illustrated in figure 2.

The software program incorporating the model presented here is written in Pascal on an HP vectra QS with 80387 mathematical coprocessor. An average run from time 0 to 1000 h requires about 4 minutes CPU time, which is more than thousand times faster than the program presented by Monbouquette (1990). This is probably caused by our much larger time steps, and the fact that we do not calculate biomass fluxes.

Maximum biomass concentration

A straightforward application of equation [7] would lead to infinitively high biomass concentrations near the surface of the bead. As this is impossible, a restriction is added to this equation:

$$X \leq X_{max} \qquad [17]$$

where X_{max} is the observable maximum biomass concentration inside the support matrix (which is not the same as $X_{p,max}$). This is, in a different notation, the same restriction as used by Toda and Sato (1985) and the same as used by Sayles & Ollis (1989) and Monbouquette et al. (1990). Nakasaki et al. (1989) did not explicitly consider this in their mathematical description, but mention the use of X_{max} in their text. As the substrate consumption does not decrease when X has reached the value of X_{max} according to equation [1], the substrate uptake by the cells may be used for a waste metabolism as suggested by McLaren (1970) or the cells may continue to grow and leak out of the support matrix as described

by Monbouquette & Ollis (1988), Cheng & Huang (1988), Sayles & Ollis (1989), and Monbouquette et al. (1990). In the latter case, Shiraishi et al. (1989) stated that the substrate consumption of free cells may be neglected if the dilution rate in the reactor is much larger than the dilution rate at washout conditions, as in our case. This has been experimentally confirmed (Wijffels et al. 1991).

RESULTS AND DISCUSSION

General Model

A typical result of the model is shown in figure 3. In the transient growth of immobilized cells, 4 phases can be recognized.

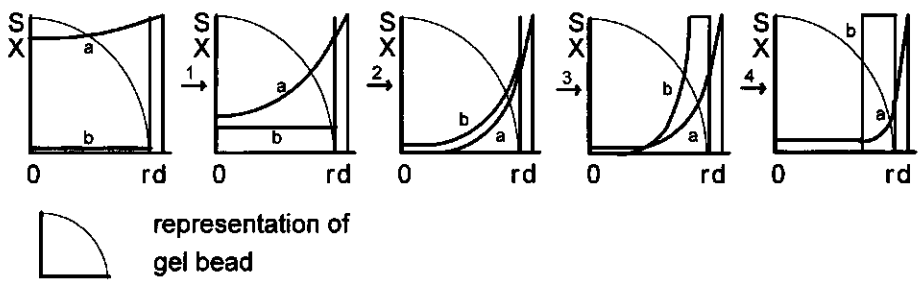

Figure 3. Typical model predictions for growth of immobilized cells. Substrate (a) and biomass concentrations (b) versus radial position *r* at various times. The external layer is represented by *d*.

In phase 1, there is hardly any diffusion limitation, and homogeneous growth occurs. Phase 2 shows inhomogeneous growth, as in the centre of the sphere substrate depletion aggravates. In phase 3, the substrate hardly reaches the core of the sphere, so biomass decays, whereas at the outer shell the biomass concentration reaches its maximum. In phase 4 this process of growth and

decay continues and a distinct biomass film develops, whereas the substrate concentration in the centre of the sphere increases. At the end time of the simulation a distinct biomass film near the surface of the bead has developed, whereas the substrate concentration inside the bead corresponds to maintenance level of zero net growth, which is the same result as reported by Sayles & Ollis (1989). By substituting $r(x) = 0$ in equation [7], this value can be calculated from:

$$S = \frac{mY_{xs}K_s}{\mu_{max}} \qquad [18]$$

Figure 3 clearly shows the increasing importance of external diffusion limitation in time. At the end time of the simulation, the concentration of substrate at the surface of the bead is only half the bulk concentration.

Model evaluation

The model was verified with *Nitrobacter agilis* cells immobilized in κ-carrageenan, as presented by Wijffels et al. (1991). Immobilized cells were kept in an air-lift loop reactor at sufficiently high nitrite concentrations and constant oxygen concentrations, so that oxygen was the limiting substrate. At three different oxygen concentrations the macroscopic oxygen consumption rates were determined in time by measuring the nitrite consumption rate, and compared to model predictions. Parameters used in the model are given in table 2, and results are shown in figure 4.

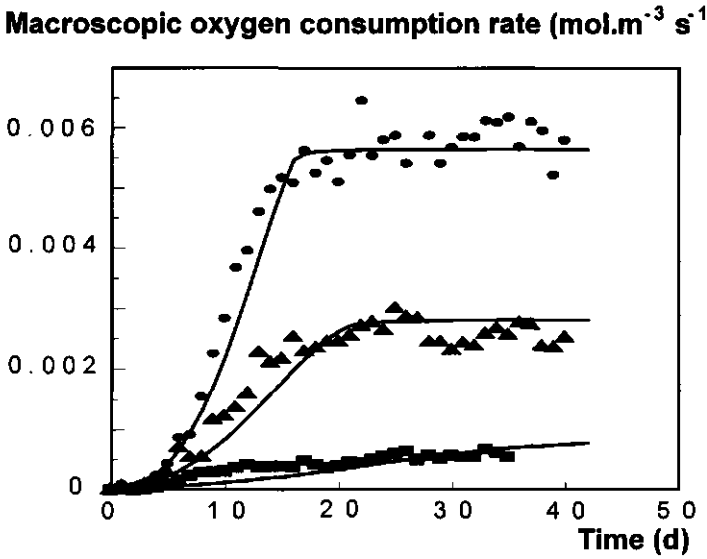

Figure 4. Experimental evaluation of the model (Wijffels et al. 1991). Macroscopic *consumption rates at three different oxygen concentrations versus time.* Lines are model predictions, markers are experimental data. Experiments were performed at oxygen concentrations of 0.012 mol.m^{-3} (■), 0.038 mol.m^{-3} (♦) and 0.08 mol.m^{-3} (●).

As shown in figure 4, the curves predicted by the model presented here match the experimental results very well.

Sensitivity analysis

To establish the sensitivity of the model for the different parameters, a sensitivity analysis was made around the set point values used in the evaluation (table 2). Each parameter was varied in the range 0.5-1.5 times the setpoint value, keeping all other variables constant. The predicted macroscopic consumption rate (mol.m$^{-3}_{bead}$.s^{-1}) at steady state was used to compare the predictions,

Table 2. Parameters used in the model evaluation.
Data are from Wijffels et al. (1991). Here, Δt is the time step (s) for the growth process (equation [7]), and X_0 is the initial biomass concentration (mol.m^{-3}).

PARAMETER	VALUE	UNITS
$k_{l,s}$	$3.7\ 10^{-5}$	$m.s^{-1}$
D_e	$1.58\ 10^{-9}$	$m^2.s^{-1}$
r_b	$1.02\ 10^{-3}$	m
X_0	$4.5\ 10^{-3}$	$kg.m^{-3}$
$X_{p,max}$	950	$kg.m^{-3}$
t_{end}	42	d
Δt	1	d
Y_{xs}	$1.16\ 10^{-3}$	$kg.mol^{-1}$
μ_{max}	$1.0\ 10^{-5}$	s^{-1}
K_s	$1.7\ 10^{-2}$	$mol.m^{-3}$
m	$1.1\ 10^{-3}$	$mol.kg^{-1}.s^{-1}$
X_{max}	11	$kg.m^{-3}$
S_b	0.012 / 0.038 / 0.08	$mol.m^{-3}$

realizing that other criteria can be used. Results are presented in figure 5. Figure 5A clearly shows the high sensitivity of the model for the radius of the beads and the substrate bulk concentration. At other oxygen bulk concentrations the same model behaviour was found (results not shown). The model showed no response at changing the time step size over the same range, which indicates that the assumption that the substrate consumption process and growth may be treated separately, was valid. Varying the maximum attainable biomass concentration $X_{p,max}$, which only appears in equation [3], did not result in a change in predicted substrate consumption rate, which can, for our case, be explained by the relatively low maximum biomass concentration X_{max}.

At all oxygen bulk concentrations, the model showed no response to a change in initial biomass concentration, indicating that at these set points, the model is capable of finding the steady state rather well.

Varying the end time of the simulation showed no response for the high bulk concentrations, whereas at the low oxygen concentration setpoint a 50 %

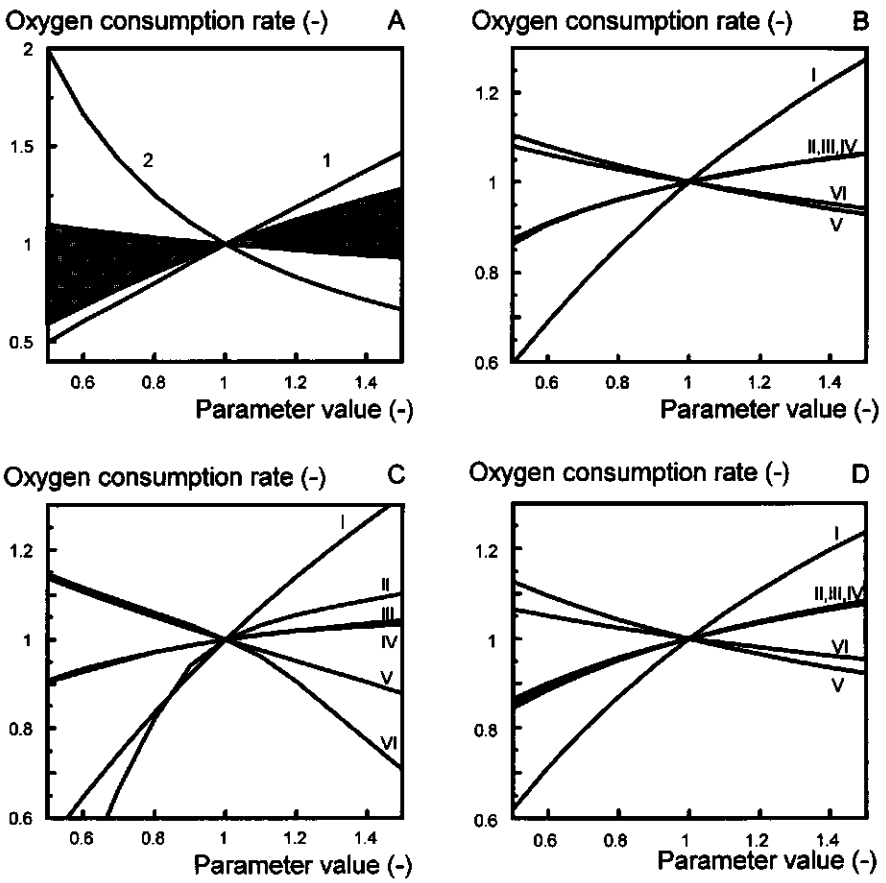

Figure 5. Sensitivity analysis of the model. The oxygen bulk concentration is 3.8 10^{-2} mol.m^{-3} in (A) and (B) and 1.2 10^{-2} and 8.0 10^{-2} mol.m^{-3} in (C) and (D), respectively. (A-D) Oxygen flux is plotted versus the dimensionless parameter value. In (A) line 1 is the bulk concentration, line 2 is the radius, and the shaded area represents all other parameters (B-D) : I is the mass transfer coefficient in the film, II is the maximum specific growth rate, III is the diffusion coefficient, IV is the maximum biomass concentration in the gel, V is the yield coefficient, and VI is the Monod constant.

smaller flux was predicted at the lowest end time, and from the set point to a higher end time no change occurred, indicating that at low bulk concentrations, it takes longer to reach the steady state, probably due to the low growth rate. Varying the maintenance coefficient at high bulk concentrations influenced the substrate flux less than 1 %, whereas at a low bulk concentration the predicted flux decreased by 10 % at a 50 % higher maintenance coefficient. This is a result of the use of equation [6], where the second term will become more important compared with the first term at concentrations in the same magnitude as K_s, i.e., at the low oxygen bulk concentration.

The similarity between figures 5B and 5D indicates that at higher substrate bulk concentrations, the entire process becomes diffusion controlled, whereas at low substrate bulk (figure 5C) concentrations the kinetics gain importance. From figure 5 it is clear that apart from the radius and the bulk concentration, the most important parameter is the mass transfer coefficient in the stagnant layer, which indicates the importance of external mass transfer resistance.

The model was also tested without external diffusion limitation. Steady-state oxygen consumption rates are given in table 3 where it is clearly shown that for our case, external mass transfer resistance is not negligible.

Table 3. Oxygen consumption rates with and without external diffusion limitation.

Oxygen bulk concentration ($mol.m^{-3}$)	$1.2\ 10^{-2}$	$3.8\ 10^{-2}$	$8.0\ 10^{-2}$
Oxygen consumption rate with film theory ($mol.m^{-3}.s^{-1}$)	$8.1\ 10^{-4}$	$2.8\ 10^{-3}$	$5.6\ 10^{-3}$
Oxygen consumption rate without film theory ($mol.m^{-3}.s^{-1}$)	$2.7\ 10^{-3}$	$7.0\ 10^{-3}$	$1.2\ 10^{-2}$
Ratio (dimensionless)	3.3	2.5	2.1

CONCLUSIONS

The modelling of immobilized *Nitrobacter agilis* cells was successfully carried out. Measured macroscopic oxygen consumption rates matched well with model predictions at three different oxygen concentrations. The model shows, at the values of the parameters used in this case, the highest sensitivity for the substrate bulk concentration, the radius of the biocatalyst beads, and the external mass transfer resistance coefficient. More research is under way to verify the model prediction of the substrate concentration profile as proposed by De Beer et al. (1988) and to extend the model to an overall reactor model.

APPENDIX: CALCULATION OF THE CHARACTERISTIC TIMES FOR AIR BUBBLE EXHAUSTION AND OXYGEN TRANSFER

As from our experiments the oxygen consumption rates are known at three different oxygen bulk concentrations, k_la values for mass transfer from the gas bubbles can be calculated, since in steady state, the oxygen consumption rate must equal oxygen transfer:

$$r_s = k_{l,g} a_l (S_b^* - S_b) \qquad [19]$$

with:

$$a_l = \frac{6}{d_b} \frac{\varepsilon}{1-\varepsilon} \qquad [20]$$

The characteristic time for oxygen transfer from the gas bubbles to the liquid phase can be calculated from (Oosterhuis 1984, Seki & Furusaki 1985):

$$T_{transfer} = \frac{1}{k_{l,g} a_l} \quad [21]$$

The characteristic time for exhaustion of the gas bubble can be calculated from (Oosterhuis 1984, Seki & Furusaki 1985):

$$T_{exhaustion} = \frac{H}{k_{l,g} a_g} \quad [23]$$

with:

$$a_g = \frac{6}{d_b} \quad [22]$$

Characteristic times calculated from our experiments, using H = 40 $m^3.m^{-3}$, ε = 0.5 %, and d_b = 0.5 mm are given in table 4.

Table 4. Characteristic times for oxygen transfer and exhaustion at three different bulk concentrations.

S_b	$1.2\ 10^{-2}$	$3.8\ 10^{-2}$	$8.0\ 10^{-2}$	$mol.m^{-3}$
r_s	$1.3\ 10^{-4}$	$4.4\ 10^{-4}$	$8.8\ 10^{-4}$	$mol.m^{-3}.s^{-1}$
S^*_b	$3.6\ 10^{-2}$	$1.2\ 10^{-1}$	$2.1\ 10^{-1}$	$mol.m^{-3}$
$T_{exhaustion}$	37	37	30	s
$T_{transfer}$	185	181	151	s

As can be seen from Table 4, the characteristic time for exhaustion of the gas bubbles can be estimated to be on average 35 s, and for oxygen transfer this is on average 172 s. These values lie well within the range of values that can be found using correlations for k_l reported in literature (Shah et al. 1982).

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NOMENCLATURE

a	specific surface area of a sphere	m^{-1}
a_g	specific surface area of the gas phase	m^{-1}
a_l	specific surface area of the liquid phase	m^{-1}
b	constant in equation [9]	-
c	constant in equation [9]	-
d_b	diameter of air bubbles	m
D_o	diffusion coefficient in water	$m^2.s^{-1}$
D_e	effective diffusion coefficient	$m^2.s^{-1}$
$D_{e,g}$	effective diffusion coefficient in gel	$m^2.s^{-1}$
f	volumetric cell fraction	-
H	Henry coefficient	$m^3.m^{-3}$
k_1	ratio of the diffusivities in gel and in water	-
k_2	specific cell volume	$m^3.kg^{-1}$
$k_{l,g}$	substrate mass transfer resistance coefficient in the film layer near the gas phase	$m.s^{-1}$
$k_{l,s}$	substrate mass transfer resistance coefficient in the film layer near the solid phase	$m.s^{-1}$
K_s	Monod constant	$mol.m^{-3}$
m	maintenance coefficient	$mol.kg^{-1}.s^{-1}$

r	distance from the centre of the bead	m
r_b	radius of the bead	m
r_c	radius of the cell	m
r_f	distance from the centre of the bead where substrate concentration is negligible	m
r_s	macroscopic consumption rate of substrate	$mol.m^{-3}.s^{-1}$
r'_s	substrate consumption rate in the gel	$mol.m^{-3}.s^{-1}$
r_x	biomass growth rate	$kg.m^{-3}.s^{-1}$
S	substrate concentration	$mol.m^{-3}$
S_b	substrate concentration in the bulk	$mol.m^{-3}$
S^*_b	saturation concentration (of oxygen) in the liquid	$mol.m^{-3}$
S_f	substrate concentration in the centre of the bead	$mol.m^{-3}$
S_s	substrate concentration at the surface of the bead	$mol.m^{-3}$
S'_s	substrate concentration at the surface of the bead, when external diffusion limitation is accounted for	$mol.m^{-3}$
t	time	s
t_{end}	end time of the simulation	s
X	biomass concentration	$kg.m^{-3}$
X_0	biomass concentration at t = 0	$kg.m^{-3}$
X_{max}	observable maximum biomass concentration	$kg.m^{-3}$
$X_{p,max}$	maximum attainable biomass concentration	$kg.m^{-3}$
Y_{xs}	yield coefficient	$kg.mol^{-1}$
ε	gas hold-up	-
μ	specific growth rate	s^{-1}
μ_{max}	maximum specific growth rate	s^{-1}
θ	porosity	-
τ	tortuosity	-

T	characteristic time	s
Δt	time step size for biomass growth (Equation [7])	s

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CHAPTER 5

GROWTH AND SUBSTRATE CONSUMPTION OF *NITROBACTER AGILIS* CELLS IMMOBILIZED IN CARRAGEENAN: PART 2. MODEL EVALUATION

ABSTRACT

A dynamic model, which predicts substrate and biomass concentration profiles across gel beads and from that the overall substrate consumption rate by the gel beads containing growing cells, was evaluated with immobilized Nitrobacter agilis cells in an air-lift loop reactor with oxygen as the limiting substrate. The model predictions agreed well with the observed oxygen consumption rates at three different liquid phase oxygen concentrations.

Image analysis showed that 90 % of the immobilized cells after 42 days of cultivation was situated in the outer shells in a film of 140 μm, while the bead radius was about 1 mm. The maximum biomass concentration in the outmost film of 56 μm was 11 $kg.m^{-3}$ gel.

INTRODUCTION

Nitrification is a problematic process within waste water treatment due to the slow growth rate of the nitrifying organisms. The main species within this group are *Nitrosomonas* and *Nitrobacter*, which successively oxidize ammonia to nitrite and nitrate.

In previous studies we have shown that nitrifying bacteria can be retained in continuous-flow bioreactors by immobilization in a gel with maintenance of their nitrifying capacities (Van Ginkel et al. 1983, Tramper et al. 1985, Tramper 1985, Tramper & De Man 1986, Tramper & Grootjen 1986, Tramper 1987, Wijffels & Tramper 1989). It was demonstrated that diffusion limitation increased with increasing amounts of immobilized cells (Van Ginkel et al. 1983, Tramper & De Man 1986). It was also shown that both *Nitrosomonas europaea* (Wijffels & Tramper 1989) and *Nitrobacter agilis* (Tramper & Grootjen 1986) can grow within the gel. For immobilized *Nitrosomonas europaea*, the effect of diffusion limitation on growth was described qualitatively (Wijffels & Tramper 1989). Initially, the cells were growing homogeneously across the beads, but as growth proceeded, a biomass density gradient developed, eventually resulting in a biofilm just below the surface of the gel beads. Accordingly, the reaction rate, which was originally limited by the kinetics of the cells, became diffusion limited.

For non-growing systems, substrate profiles across the beads and substrate fluxes into the beads were modelled and validated by De Gooijer et al. (1989). A homogeneous distribution of enzyme across the beads was assumed. A differential mass balance with simultaneous diffusion and substrate consumption was made and integrated numerically across the biocatalyst bead. External mass transport was also incorporated by using the film theory. This model has been extended to growing systems by coupling substrate consumption to growth via a yield coefficient. A negative net growth was allowed

at low substrate concentrations and the local biomass concentration was limited to a maximum (De Gooijer et al. 1991). It predicts the overall substrate consumption rate in addition to biomass and substrate concentration profiles across the beads.

This article describes the experimental evaluation of the latter model with immobilized *Nitrobacter agilis*. Immobilized cells were cultivated in continuous-flow air-lift loop reactors with oxygen as the limiting substrate. Andrews (1988) defined the limiting substrate as the component whose concentration reaches zero first; this is the component with the lowest value for the product of effective diffusion coefficient, yield factor and the pertinent component concentration. In our case this value was always lower for oxygen than for nitrite. For that, as the nitrification capacity of the system increased, the nitrite concentration of the influent was increased in such a way that nitrite in the reactor never was limiting. The oxygen concentration in the liquid phase was kept at a constant level. The oxygen consumption rates at various constant oxygen concentrations were determined and compared with model predictions. Biomass concentration profiles across the gel beads were determined by image analysis to estimate the local maximum immobilized biomass concentration.

MATERIALS AND METHODS

Organism and culture conditions

Nitrobacter agilis (ATCC 14123) was cultivated in a 5-dm^3 batch culture. The composition of the medium was based on studies on nutrient requirements (Aleem & Alexander 1960, Boon & Laudelot 1962, Van Droogenbroeck & Laudelot 1967, Painter 1970, Sharma & Ahlert 1970, Belser 1984). The medium

contained per m^3 of demineralized water: 14.5 mol $NaNO_2$; 0.21 mol $MgSO_4$; 5 mol KH_2PO_4; 5 mol Na_2HPO_4; 1 mmol Na_2MoO_4; 0.015 mmol $ZnSO_4$; 0.016 mmol $CuSO_4$ and 5 mmol $CaCl_2$. Per mole nitrite 0.02 mol of $NaHCO_3$ was added. The pH was adjusted to 7.8 with 2 *N* KOH. The medium was inoculated with about 10% (v/v) of cell culture. Every 3 days 72.5 mmol of $NaNO_2$ was added to obtain dense cultures. After 9 days the culture was harvested. Cultivation was executed at 30°C in the dark to prevent light inhibition (Müller-Neuglück & Engel 1961, Bock 1965, Yoshioka & Saijo 1984).

Immobilization procedure

The cell suspension was centrifuged during 10 minutes at 16,300 *g* and 5 °C. The cells were washed with 15.4 m*M* NaCl solution.

A 3% κ-carrageenan solution (Genugel X0828, A/S Kobenhavns Pektinfabrik, DK Lille Skensved) was mixed gently with the washed suspension such that a 2.6 % carrageenan solution was obtained. The carrageenan solution and the cell suspension were kept at 35 °C.

Immobilization was performed with a resonance nozzle (Hulst et al. 1985) at 35 °C. Drops were collected in 0.75 *M* KCl at 5 °C. To obtain perfect spheres a decane layer was brought upon the KCl solution as proposed by Buitelaar et al. (1989). The decane and the KCl solution were kept at 5 °C in order to initiate the gelation. Decane is non-toxic due to its high log *P* value (Laane et al. 1987).

The average bead diameter was 2.04 mm (± 0.22 mm) and the initial biomass concentration in the beads was 4.5 10^{-3} $kg.m^{-3}$.

Immobilized cell cultivation

Immobilized cells were cultivated in continuous-flow air-lift loop reactors (two of 3.40 dm^3 and one of 2.35 dm^3). The experimental set up is shown schematically in figure 1.

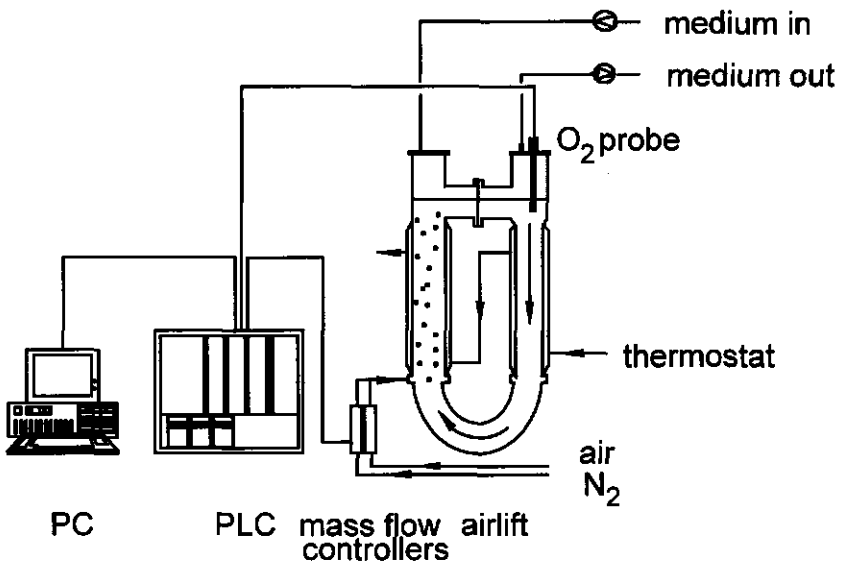

Figure 1. Experimental set up of the immobilized cell cultivation.

The reactors were coupled to a Programmable Logic Controller (PLC Melsec-G, Mitsubishi G62P). Communication with the PLC was accomplished via a personal computer. The oxygen concentration in each reactor was monitored (WTW Oxy 219/90R) and the signal sent to the PLC. Depending on the difference between the measured value and the set point, the PLC adjusted 2 mass flow controllers (Brooks instruments 5850 TR); one for air and one for nitrogen. The total gas flow into the reactors was thus kept constant (5.8 $cm^3.s^{-1}$

for the 3.4 dm^3 reactors and 4.2 $cm^3.s^{-1}$ for the 2.35 dm^3 reactor) in order to maintain constant mass transfer properties across the stagnant layer surrounding the beads.

In three experiments, the bulk oxygen concentration was kept at 0.012 (smallest reactor), 0.038 and 0.080 $mol.m^{-3}$, respectively. The influent nitrite concentration was adjusted daily so as to keep the reactor concentration between 3 and 15 $mol.m^{-3}$. In this way oxygen is the limiting substrate and there are no toxicity problems of nitrite.

The medium contained per m^3 of demineralized water: a variable amount of KNO_2; 0.21 mol $MgSO_4$; 5 mol KH_2PO_4; 5 mol K_2HPO_4; 1 mmol Na_2MoO_4; 0.015 mmol $ZnSO_4$; 0.16 mmol $CuSO_4$; 5 mmol $CaCl_2$ and 5 mol of KCl. Per mole nitrite 0.02 mol of $KHCO_3$ was added. The pH was adjusted to 7.8 with 2 *N* KOH. Cultivation was executed at 30°C in the dark to prevent effects of light inhibition.

In the reactor operated at 0.012 mol $O_2.m^{-3}$, the dilution rate was 4.6 10^{-5} s^{-1} and the reactor gel load 25 % (v/v). For the reactors operated at 0.038 and 0.080 mol $O_2.m^{-3}$, the dilution rate based on the liquid-phase volume was 4.0 10^{-5} s^{-1} and the reactor gel load was 15 % (v/v). In preliminary runs it appeared that the oxygen transfer rate from the gas phase to the liquid phase at a gel load of 25 % (v/v) was too low to keep the liquid phase oxygen concentration at 0.038 and 0.080 $mol.m^{-3}$, respectively, during the entire experiment. Therefore the gel load was lowered to 15 % (v/v) for these two cases.

Oxygen consumption rate

Nitrite oxidation by *Nitrobacter* requires stoichiometric amounts of oxygen (Lees & Simpson 1957, Aleem & Alexander 1958, Laudelot & Van Tichelen

1960, Silver 1961). Thus to estimate the oxygen consumption rate, the nitrite consumption rate per amount of gel was determined regularly. Reactor nitrite concentrations were determined at least 24 hours after increasing the influent nitrite concentration, such that refreshment of the medium had occurred four times and a nitrogen balance across the reactor could be made.

Nitrite and nitrate concentrations in influent and effluent were determined (Greenberg et al. 1985) using a continuous-flow analysis system (Technicon Auto Analyser 2). For nitrate analysis the samples first passed through a copper-coated cadmium column in order to reduce the nitrate to nitrite. By addition of α-naphtylamide in acid medium, the nitrite forms a red diazo compound, that was measured at 550 nm.

Biomass concentration

Overall viable biomass estimates were made by activity assays in a biological oxygen monitor as described before (Van Ginkel et al. 1983). In a reaction cuvette 50-200 beads and 4 cm^3 potassium phosphate buffer (0.1 $mol.dm^{-3}$, pH 7.8) were suspended and saturated with air. If the activity exceeded 0.002 $mol.m^{-3}gel.s^{-1}$, pure oxygen was used to prevent mass transfer limitations. Through a small opening a concentrated KNO_2 solution was injected to a final concentration of 20 $mol.m^{-3}$ and the decrease in oxygen concentration at 30 °C was recorded.

The activity per amount of gel was converted to a biomass dry weight concentration by dividing by the specific activity. The specific activity of *Nitrobacter agilis* is 7 10^{-3} $mol.kg^{-1}.s^{-1}$ at a nitrite concentration of 20 $mol.m^{-3}$ (Boon & Laudelot 1962, Tramper & Grootjen 1986).

random there. In thin slices the areal density is equal to the volumetric density according to the principle of Delesse (Weibel 1979).

As the observed areas are projections of the observed colonies situated in slices with a thickness L (m), it may be necessary to correct for over-estimations as more images are projected for thicker slices (the Holmes effect) and under-estimations as images can overlap (Hennig 1969, Underwood 1972, Weibel 1979, 1980).

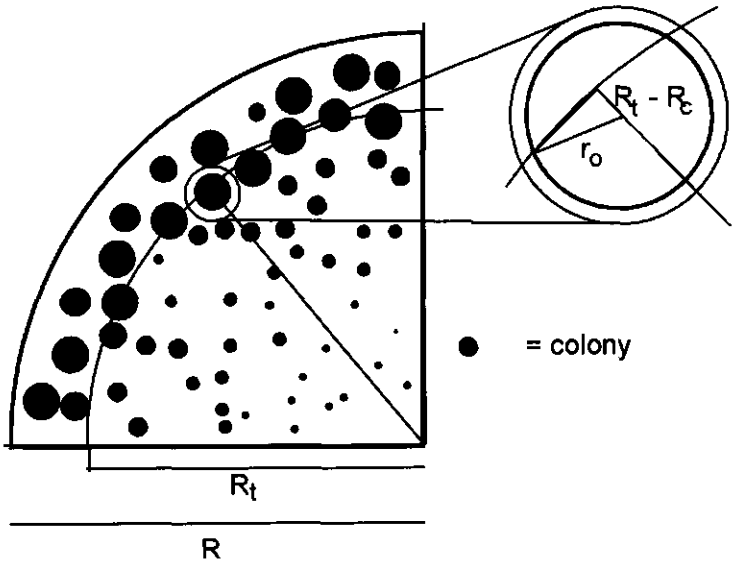

Figure 2. Procedure for determination of the volumetric fraction of biomass: r_o, observed colony radius (m); R_c, distance between centre of the colony and the centre of the bead (m); R, bead radius (m); R_t, radius test line (m).

As the slices are thin compared to the colony radii, overlap may be neglected (Weibel 1980). For the Holmes effect, the observed radii can be corrected by a factor C defined as (Weibel 1980):

$$C = \frac{\pi}{\pi + 2L/r_c} \quad [1]$$

As the observed colony radii are expectations of the colony radii ($E(\underline{D})$), the colony radii can be obtained from:

$$E(\underline{D}) = 2r_c \frac{1 + \pi r_c/2L}{1 + 2r_c/L} \quad [2]$$

and the observed projections can be corrected individually.

Starvation under oxygen depletion

For determination of maintenance energy *Nitrobacter agilis* was cultivated batch wise in a stirred fermenter (at 450 rpm). Culture conditions were as described above. After one week of cultivation the oxygen supply was stopped and nitrogen gas was supplied instead. The decrease in potential activity was taken as a measure for the specific biomass degradation rate, as can be obtained directly from the relation of Herbert (1959) if the substrate concentration is zero:

$$\ln \frac{X_t}{X_0} = -mY_{xs}t \quad [3]$$

with X_t and X_o the biomass concentrations at time t (s) and time 0, respectively ($mol.m^{-3}$), m the maintenance coefficient ($mol.kg^{-1}.s^{-1}$), and Y_{xs} the yield factor ($kg.mol^{-1}$).

Data for the molar substrate yield were obtained from the literature.

Activity was determined as described before, except that it was directly measured in the culture medium.

RESULTS AND DISCUSSION

Input parameters

All input parameters used in the dynamic mathematical model were obtained from the literature or determined in separate experiments and are listed in table 1.

Table 1. Input parameters (references are given in text).

Effective diffusion coefficient		
$D_{e,g}$	$1.58\ 10^{-9}$	$m^2.s^{-1}$
Liquid-solid mass transfer coefficient		
$k_{l,s}$	$3.7\ 10^{-5}$	$m.s^{-1}$
Kinetic parameters		
μ_{max}	$1.0\ 10^{-5}$	s^{-1}
K_s	0.02	$mol.m^{-3}$
Y_{xs}	$1.16\ 10^{-3}$	$kg.mol^{-1}$
m	$1.1\ 10^{-3}$	$mol.kg^{-1}.s^{-1}$
Biomass concentrations		
X_0	$4.5\ 10^{-3}$	$kg.m^{-3}$
X_{max}	11	$kg.m^{-3}$
$X_{p,max}$	950	$kg.m^{-3}$

Effective diffusion coefficient ($D_{e,g}$)

The effective diffusion coefficient for oxygen in κ-carrageenan beads was determined by Hulst et al. (1989). Up to a polymer concentration of 3 % a constant effective diffusivity of $1.58\ 10^{-9}$ $m^2.s^{-1}$ was found. This value was used in the present work for a zero immobilized biomass concentration (Table 1), since a carrageenan concentration of 2.6 % was used.

The effective diffusion coefficient is affected by the concentration of biomass in the support material (Hiemstra et al. 1983, Monbouquette & Ollis 1986, Chen & Huang 1988, Gosmann & Rehm 1988, Scott et al. 1989). As stated by De Gooijer et al. (1991), the effective diffusion coefficient will be zero if all pores in the gel are completely filled by bacterial cells, with no intercellular space left. At the gel concentration used, the available pore volume will be at least 95 % of the total gel volume and the pores will be completely filled if the biomass concentration is about 950 $kg.m^{-3}$. This value is defined as the maximum physically attainable biomass concentration $X_{p,max}$.

Liquid-solid mass transfer coefficient ($k_{l,s}$)

From the Biot number it can be estimated whether external mass transfer has to be taken into consideration. If *Bi* approaches zero, all resistance is situated in the stagnant layer, and if it reaches infinite values the resistance across the stagnant layer may be neglected. The Biot number was estimated to be 25 in our case. The value will be much lower when only the active layer is considered. This means that external mass transfer certainly may not be neglected, such that $k_{l,s}$ is an important factor.

For calculation of the liquid/solid mass transfer coefficient ($k_{l,s}$) the relation of Ranz and Marshall (1952) was used:

$$k_{l,s} = \frac{D_l(2 + 0.6Sc^{1/3}Re^{1/2})}{d_p} \qquad [4]$$

The Reynolds number was calculated from the Galileo number. Relations between *Re* and *Ga* can be derived easily for the Stokes, Newton and intermediate regimes (Aleem & Alexander 1958). It was assumed that the

particles move with the rate of free fall. *Re* thus calculated is 58, which means that Ranz and Marshall is applicable (Monbouquette & Ollis, 1981). Equation [4] gives with *Re* = 58 a mass-transfer coefficient of 3.7 10^{-5} $m.s^{-1}$ (Table 1), which gives good predictions of the overall oxygen consumption rates in all experiments as will be discussed.

In first instance the method of Kolmogoroff as used by Sänger & Deckwer (1981) for a bubble column and extended for an air-lift loop reactor by Wagner & Hempel (1988) seemed more appropriate to us. Kolmogoroff gave a relation between the energy dissipation and the Sherwood number. However, poor model predictions were obtained if those relations were used. This can be explained by differences in superficial gas velocities (u_g), which were minimal double of what we used (0.0018 $m.s^{-1}$). When the energy dissipations are compared, the differences are even more substantial. They found an energy dissipation (ε) between 0.25 and 0.52 $m^2.s^{-3}$, while we calculated for our experiments 0.011 $m^2.s^{-3}$, which makes it inappropriate to apply their equations in our regime.

Maximum specific growth rate (μ_{max})

Literature data for maximum specific growth rates range from 0.98 10^{-5} to 2.3 10^{-5} s^{-1} (Gould & Lees 1960, Rennie & Schmidt 1977, Gay & Corman 1981, Helder & De Vries 1983, Keen & Prosser 1987). In our experiments μ_{max} was taken to be 1.0 10^{-5} s^{-1}, a value that was found by Tramper & Grootjen (1986), who used the same strain (Table 1).

Monod constant (K_s)

Most kinetic studies with nitrifying bacteria were executed with the nitrogenous compound as the limiting substrate. The few data known for oxygen as limiting substrate were obtained from activity assays instead of growth experiments (Boon & Laudelot 1962, Peeters et al. 1969, O'Kelly et al. 1970, Williamson & McCarthy 1975, Stenstrom & Poduska 1980). An overview of the effect of the dissolved oxygen concentration on nitrification has been given by Stenstrom & Poduska (1980). The general range for K_s appears to be 0.009-0.026 mol.m^{-3} (Müller-Neuglück & Engel 1961, Boon & Laudelot 1962, Williamson & McCarthy 1975). Sensitivity analysis demonstrated that within this range there was little effect on the predicted oxygen consumption rate (De Gooijer et al. 1991). Therefore, an average K_s-value of 0.017 mol.m^{-3} was chosen (Table 1).

Yield (Y_{xs}) and maintenance (m) coefficients

In the present model maintenance energy plays an important role, because this factor determines the negative growth of cells that live under oxygen depletion. Almost no values are available for maintenance coefficients (m). Apart from that, in studies where maintenance was determined, nitrite was taken as the limiting substrate. The same holds for the yield coefficients (Y_{xs}). Available data were recalculated to oxygen yields and maintenance coefficients according to the reaction stoichiometry. As general composition formula for biomass the relation as given by Roels (1983) $CH_{1.8}O_{0.5}N_{0.2}$, and the cell weight as given by Lees & Simpson (1957), 10^{-10} mg.cell^{-1}, were used.

Keen & Prosser (1987) found yield coefficients of 19.6 10^{-3} and 3.92 10^{-3} kg.mol^{-1} and maintenance coefficients of 5.6 10^{-4} and 11.0 10^{-4} mol.kg^{-1}.s^{-1}. The differences in their results were caused by the fact that two different calculation procedures were used for the same set of data. The first values were calculated by an iterative procedure and the latter by linear regression analysis of the double reciprocal plot. There is doubt about their results obtained by iteration, because the product of this growth yield (Y_{xs}) and maintenance coefficient (m), i.e., the specific biomass degradation rate as defined by Herbert (1959), is higher than the maximum specific growth rate, or in other words, even at a maximum specific growth rate the biomass decay rate was higher than the growth rate.

Helder & De Vries (1983) and Belser (1984) found values for the yield coefficient of 0.98 10^{-3} and 1.16 10^{-3} kg.mol^{-1}, respectively. As Belser (1984) studied in our opinion yield most extensively, this value has been taken as input parameter (Table 1).

From the work of Chiang (1969) as reported by Belser (1984) a maintenance of 2.78 10^{-3} mol.kg^{-1}.s^{-1} can be estimated. When a yield factor of 1.16 10^{-3} kg.mol^{-1} is assumed and with the maintenance coefficient given by Belser (1984), the specific biomass degradation rate is 32 % of the maximum specific growth rate. When the regression results of Keen & Prosser (1987) apply, this ratio is 12.8 %. Laudelout et al. (1968) found a very broad range between 11 and 53 %. Because no more values were found in the literature, this parameter has been estimated in a separate experiment. Our model (De Gooijer et al. 1991) involves a combination of the models of Pirt (1966) and Herbert (1959), as proposed by Beeftink et al. (1990). Determination of maintenance energy was done with the bacteria under oxygen depletion. In that region the

model of Herbert (1959) is applicable and the specific biomass degradation rate can directly be obtained from the observed decay rate (equation [3]).

The decrease in activity under oxygen depletion is shown in figure 3. The observed specific degradation rate was 12.8 % of the maximum specific growth rate, which means that the maintenance coefficient of 1.1 10^{-3} $mol.kg^{-1}.s^{-1}$ as given by Keen & Prosser (1987) is appropriate as input parameter (Table 1).

With the chosen set of values model predictions were obtained which compared well to experimental results as will be shown. It was also shown that in the applied regimes the predicted oxygen consumption rates were relatively

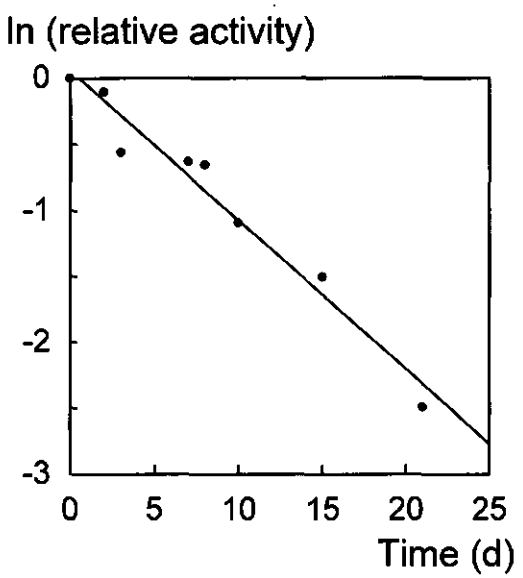

Figure 3. Activity decrease of *Nitrobacter agilis* under oxygen depletion.

insensitive to changes in values of the yield and maintenance coefficients (De Gooijer et al. 1991).

Performance immobilized cell reactor

Oxygen consumption rate

In three separate experiments the cells were cultivated in continuous-flow air-lift loop reactors. Daily results of the experiment with the highest oxygen concentration (0.080 mol.m^{-3}) are given in figure 4.

The influent nitrite concentration was 4 mol.m^{-3} at start-up. After 4 days, the effluent nitrite concentration decreased, and the influent concentration was increased to 10 mol.m^{-3}. Subsequently, it was adjusted every day to keep the

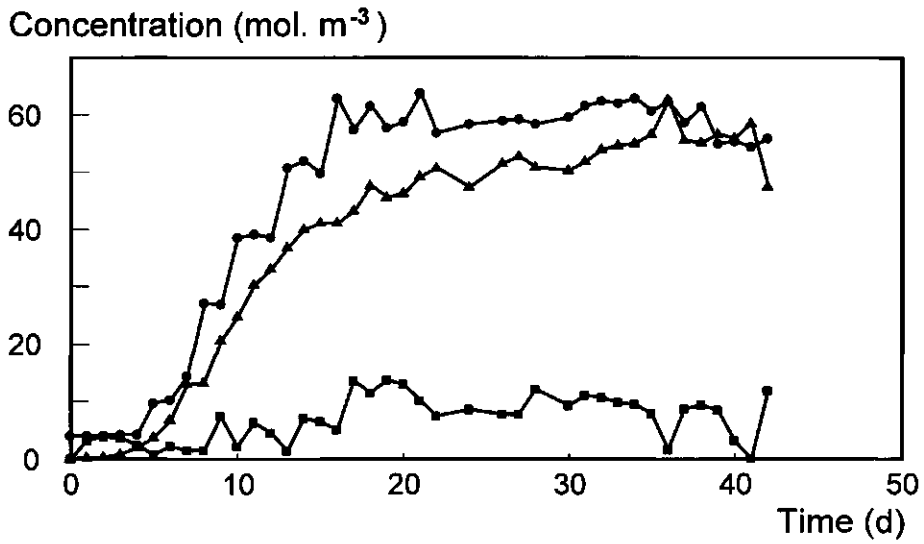

Figure 4. Influent nitrite (●) and effluent nitrite (■) and nitrate (◆) concentrations in a continuous flow experiment at a liquid phase oxygen concentration of 0.08 mol.m^{-3}.

effluent nitrite concentration above 3 mol.m^{-3}. As shown, in only a few days this concentration was lower. The influent nitrite concentration was raised almost linearly until 17 days after start-up. From that day on the influent nitrite

concentration could be kept at 60 mol.m^{-3} as no further increase in substrate consumption was observed.

Two additional experiments were executed with bulk oxygen concentrations of 0.012 and 0.038 mol.m^{-3}, and a similar reactor performance was observed. At these oxygen concentrations the influent nitrite concentration could be kept constant after a while at levels of about 10 and 30 mol.m^{-3}, respectively.

From the influent and effluent nitrite concentrations, the dilution rate and the amount of gel, the overall nitrite consumption rates of the beads were calculated.

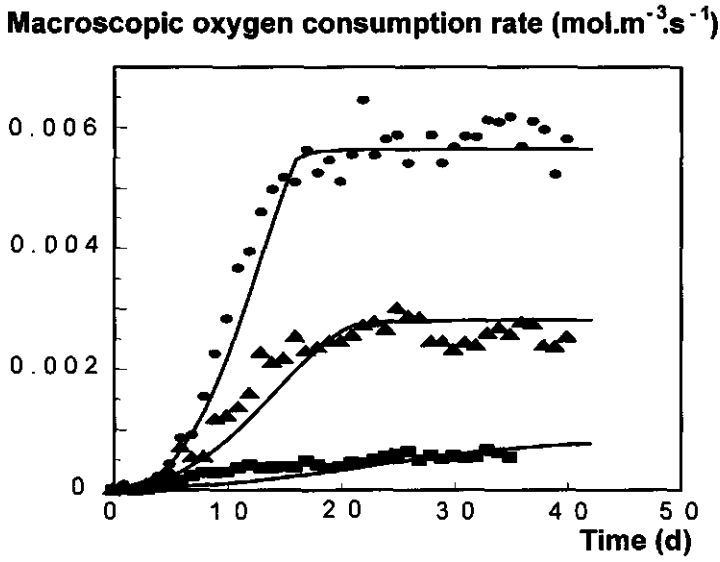

Figure 5. Observed (markers) and predicted (lines) overall consumption rates in the biocatalyst beads at different liquid phase oxygen concentrations: (■) 0.012 mol.m^{-3}, (◆) 0.038 mol.m^{-3} and (●) 0.08 mol.m^{-3}.

With that, the overall oxygen consumption rate of the beads was estimated. These rates are given for the three experiments in figure 5, which shows that the

experimental results and the model predictions as described by De Gooijer et al. (1991) compare very well.

It is also clearly shown that at higher oxygen concentrations the reactor capacity increased.

Biomass profiles and maximum biomass concentration (X_{max})

The overall viable biomass concentrations were estimated by activity assays. The development of the potential activity for all experiments is shown in figure 6.

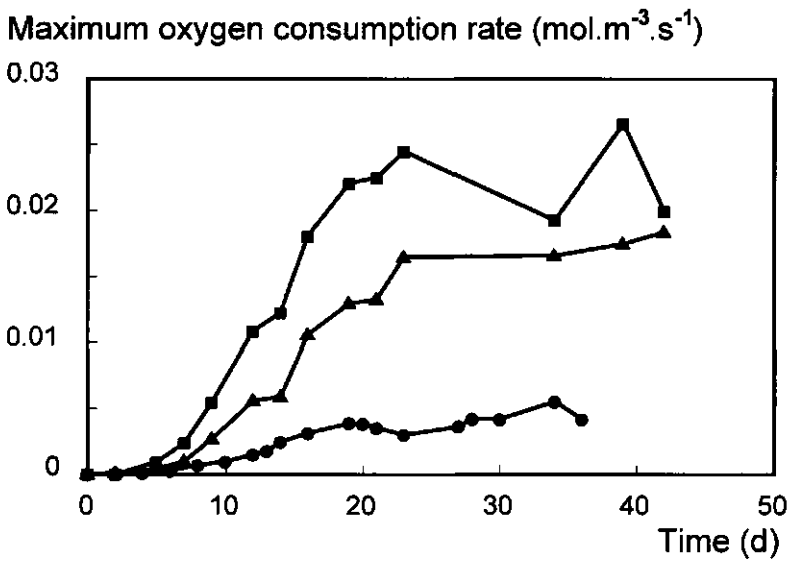

Figure 6. Overall viable biomass concentration versus time in the air-lift loop reactors with bulk oxygen concentrations of 0.012 (●), 0.038 (◆) and 0.080 (■) mol.m^{-3}, measured as potential activity.

Beads obtained at the end of the experiment with the highest liquid phase oxygen concentration (0.080 mol.m^{-3}) were used to determine the biomass profile and the maximum biomass concentration. The overall average biomass concentration in these beads at that time was 3.7 kg.m^{-3}.

Sections with a thickness of 3 and 4 μm, respectively, were analyzed. Figure 7 shows one of the two results directly obtained from image analysis. The apparent colony radii are given as a function of the radial position within the bead. Those results were converted into relative biomass concentrations as shown in figure 8 for both samples. It is shown that 90 % of the immobilized biomass was situated in an outmost shell of about 140 μm. The maximum, with a thickness of about 20 μm, was reached between a relative radius of 0.94 and 0.96. At a relative radius exceeding 0.96, the biomass concentration was

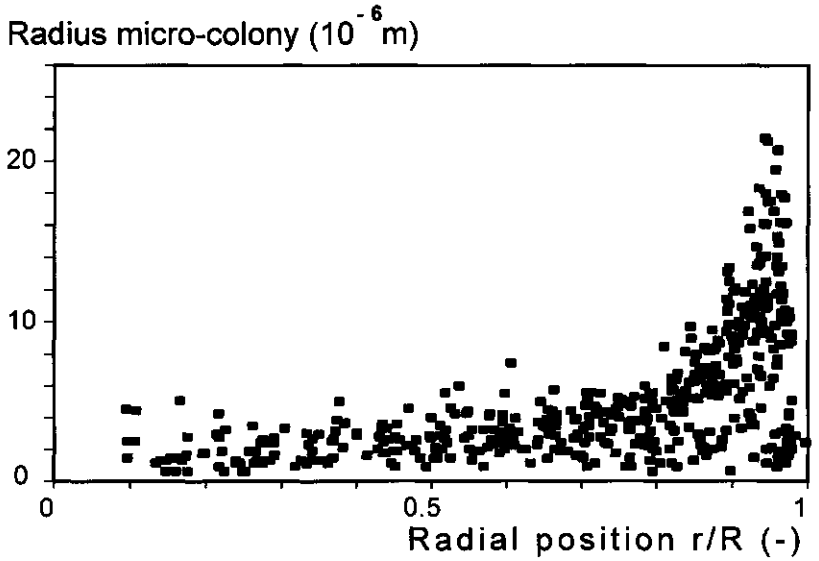

Figure 7. Measured colony radii as a function of radial position within the beads.

decreasing. This phenomenon was also observed by Salmon (1989). In the model (De Gooijer et al. 1991), however, we assumed that the biomass concentration in the outer shells reached a constant maximum. From the sections maximum biomass concentrations were calculated, which were used as an input parameter in our model (De Gooijer et al. 1991). The concentration under the peaks were 17.8 and 17.3 $kg.m^{-3}$, respectively. When the concentration was averaged from the maximum to the bead surface (between a relative bead radius of 0.94 and 1.0), the biomass concentration was in both cases 11 $kg.m^{-3}$ in a shell of 56 μm. The latter value was used as input parameter, and gave acceptable model predictions of the oxygen consumption rates. This value compares well with model predictions of Gujer & Boller (1989), who reported values up to 14 $kg.m^{-3}$. For the conversion of the apparent colony radii to a relative biomass concentration, the following remarks apply.

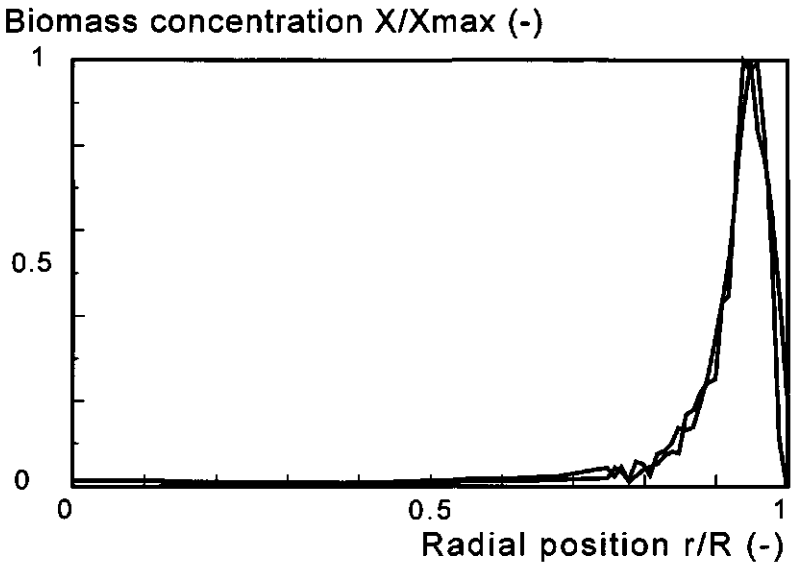

Figure 8. Biomass profile as a function of radial position within the beads.

As the sections were not infinitely thin, the principle of Delesse was not directly applicable. Correction for the Holmes effect did lead to a 17 % lower volumetric fraction at a relative radius higher than 0.85. At a radius lower than 0.85, correction did lead to 44 % reduction. In the latter part of the bead however, only 10 % of the biomass was situated. During preparation of the beads shrinkage occurred. The radius decreased 16-19 %. We have assumed that there was no difference in shrinkage between colonies and beads. No protrusions were formed as the result of the fact that gel shrinkage was higher than colony shrinkage. There were no non-filled holes observed from which colonies had disappeared due to the fact that colony shrinkage was higher than gel shrinkage.

A possible explanation for a decreasing biomass concentration in the most outer shell can be given by the fact that the colonies will grow out of the gel beads. In the immobilization procedure the bacterial cells are distributed homogeneously across the gel and are closely surrounded by the polymer matrix. Because of growth, single cells will form expanding colonies. The formed micro-colonies thus provide for their own space in the gel, at the expense of pressure increase (Stewart & Robertson 1989). Near the gel surface colony discharge due to this pressure build-up may have contributed to the decrease in peripheral biomass concentration. In future research, the present model will be extended and a biomass release term will be included.

Biomass release influences the reactor nitrite consumption. This effect has been considered for steady states (Venkatasubramanian et al. 1983, Black 1986). At high dilution rates, however, the contribution of suspended biomass is negligible. This was checked in our experiments. The discharged effluent did not show activity. At lower dilution rates this effect can be considerable. This effect will also be added to the model in order to come to an overall dynamic reactor model for all regimes.

APPENDIX: DIMENSIONLESS NUMBERS

Biot :

$$Bi = \frac{k_{l,s} 0.5 d_p}{D_{e,g}} \quad [5]$$

Galileo :

$$Ga = d_p^3 g \frac{\rho_p - \rho_l}{\eta^2} \quad [6]$$

Reynolds :

$$Re = \frac{\rho_l u d_p}{\eta} \quad [7]$$

Schmidt :

$$Sc = \frac{\nu}{D_l} \quad [8]$$

ACKNOWLEDGEMENTS

We wish to thank Mr. H. Heij for nitrite and nitrate analysis, Drs. M. Smit for assistance in sectioning of the beads, Mr. D. Schoonderbeek for assistance with the image-analysis equipment, Mr. H. Meijer for performing the experiment starvation of cells under oxygen depletion and Dr. M. van Montfort for discussions concerning the interpretations of the results from image analysis.

NOMENCLATURE

Bi	Biot number	-
c	correction factor for the Holmes effect	-
D_L	diffusion coefficient liquid	$m^2.s^{-1}$
D_e	effective diffusion coefficient	$m^2.s^{-1}$
$D_{e,g}$	effective diffusion coefficient in gel	$m^2.s^{-1}$
d_p	particle diameter	m
$E(\underline{D})$	expectation of observed diameter	m
Ga	Galileo number	-
g	gravitational acceleration	$m.s^{-2}$
$k_{l,s}$	liquid-solid mass transfer coefficient	$m.s^{-1}$
K_s	Monod constant	$mol.m^{-3}$
L	section thickness	m
m	maintenance coefficient	$mol.kg^{-1}.s^{-1}$
r_c	colony radius	m
r_o	observed colony radius	m
R	bead radius	m
R_c	distance centre colony to centre of the bead	m
R_t	radius test line	m
Re	Reynolds number	-
S_b	substrate concentration in the bulk	$mol.m^{-3}$
Sc	Schmidt number	-
t	time	s
u_g	superficial gas velocity	$m.s^{-1}$
X	biomass concentration	$kg.m^{-3}$
X_0	biomass concentration at $t = 0$	$kg.m^{-3}$

X_{max}	maximum biomass concentration	$kg.m^{-3}$
X_{pmax}	maximum physically attainable biomass concentration	$kg.m^{-3}$
X_t	biomass concentration at t = t	$kg.m^{-3}$
Y_{xs}	molar substrate yield	$kg.mol^{-1}$
ε	energy dissipation rate	$m^2.s^{-3}$
μ_{max}	maximum specific growth rate	s^{-1}
ν	kinematic viscosity	$m^2.s^{-1}$
η	dynamic viscosity	$kg.m^{-1}.s^{-1}$
ρ_l	density liquid phase	$kg.m^{-3}$
ρ_p	density particles	$kg.m^{-3}$

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CHAPTER 6

A MODEL FOR BACULOVIRUS PRODUCTION WITH CONTINUOUS INSECT-CELL CULTURES

ABSTRACT

A model, suitable for reactor-design purposes, is presented for the infection process of insect cells with baculovirus in a continuous bioreactor system. The infection process can be described by a first-order reaction mechanism if the viable, non-infected, insect cells are regarded as substrate, and the resulting polyhedra as product. Experimental results from continuous systems, consisting of a series of mixed reactors in which growth of *Spodoptera frugiperda* cells takes place in the first reactor and infection with *Autographa californica* nuclear polyhedrosis virus in the other reactors in the series, show typical values for the reaction rate constant of 0.8 - 1.1 10^{-5} s^{-1}.

INTRODUCTION

Baculoviruses, causative agents of fatal diseases in insects, are not only of interest because of their application as control agents of insect pests in agriculture (Martignoni 1984), but have increased importance since they are used as expression vectors for the production of heterologous proteins of pro- and eukaryotic origin (Luckow et al. 1988). The latter include proteins of medical, pharmaceutical and veterinary importance, a HIV (AIDS) subunit vaccine being a prominent example. Production of genetically engineered proteins using a baculovirus expression vector requires a suitable and efficient insect-cell culture system, preferably in a continuous fashion, in order to obtain large quantities of proteins in a high concentration. Development of suitable bioreactors is therefore of prime importance.

Usually, with mammalian cell systems, batch-type fermenters are used. The transient character of such systems imposes certain disadvantages, such as a more difficult process optimization and control, and batch-like downstream processing. Therefore, in our laboratory, we introduced continuous operated fermenters, both to grow insect cells and to perform infection with virus (Tramper et al. 1986, Kompier et al. 1988). Baculoviruses have a unique, bi-phasic replication cycle (Faulkner 1981, Kelly 1982), as illustrated in figure 1. After infection of insect cells, non-occluded virus (NOV) particles are produced and secreted into the medium by budding through the cell membrane (step 1).

Later in the infection cycle this process switches to the occlusion of the virus particles in newly-synthesized polyhedra in the cell nucleus. Finally, the infected cell desintegrates, releasing the polyhedra (step 2). Polyhedra are infectious for insects, whereas NOVs are infectious for insect cells. In expression vectors the gene coding for polyhedrin, the major component of the polyhedra, has been replaced by the gene of choice (Luckow et al. 1988).

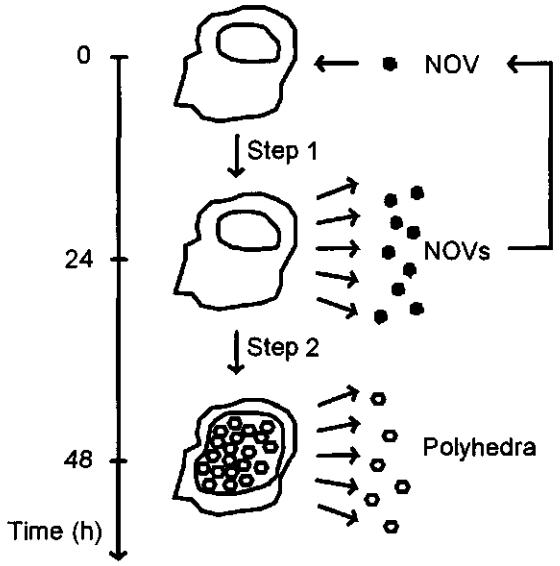

Figure 1. Replication cycle of baculovirus in insect cells.

For a quantitative description of the infection process, needed for reactor design purposes, it is important to describe the effect of crucial reactor operation parameters, such as pH, temperature and growth medium (Vaughn 1976, Weiss et al. 1986). However, this process is yet poorly understood and experimental data on these parameters are scarce (Tramper et al. 1986). In this paper we present a model for the continuous viral infection process, abstracting from all possible influences imposed by reactor operating parameters, and using the well-known concept of mass balances. The model is validated with experimental data, using the *Autographa californica* nuclear polyhedrosis virus (AcNPV)-*Spodoptera frugiperda* cell system (Kompier et al. 1988, Van Lier et al. 1990).

THEORY

Two products are generated in the viral infection process : NOVs and polyhedra. Experiments carried out in our laboratories showed the number of NOVs produced during one cycle of virus replication, as illustrated in figure 2. Here, anchored cells were inoculated with an initial multiplicity of infection (MOI, the number of NOVs per cell) of 20. The yield of infectious NOVs, expressed as $TCID_{50}$ units, was determined as described by Vlak (1979). From this figure it can be calculated that per insect cell, about 200 NOV particles are produced.

If we regard the viable, non-infected, insect cells and NOVs as substrates, and both the NOVs and the polyhedra (P) as products resulting from the infection 'reaction', the following reaction equation can be derived :

$$\text{insect cell} + 20\ \text{NOV} \rightarrow 200\ \text{NOV} + n\text{P} \qquad [1]$$

In equation [1], the denominator 20 denotes the MOI. In our laboratory we found for n, the number of polyhedra released per insect cell, a value of 25, but this may vary with reactor operating parameters (Kompier et al. 1988). Equation [1] shows the large amount of NOVs produced in the infection process, relatively to the number of insect cells. This value, determined from anchored cell cultures (Figure 2), may differ under varying reaction conditions.

In order to be able to obtain a mass balance for the viable, non-infected, insect cells for each mixed vessel in the infection reactor series, the next assumptions are made :

i) The growth rate of the cells is constant both in time and place,

ii) Cell growth is a first order process,

iii) The NOVs are, in steady state, available in excess and therefore equation [1] can be described by a first-order reaction rate with respect to the cell concentration.

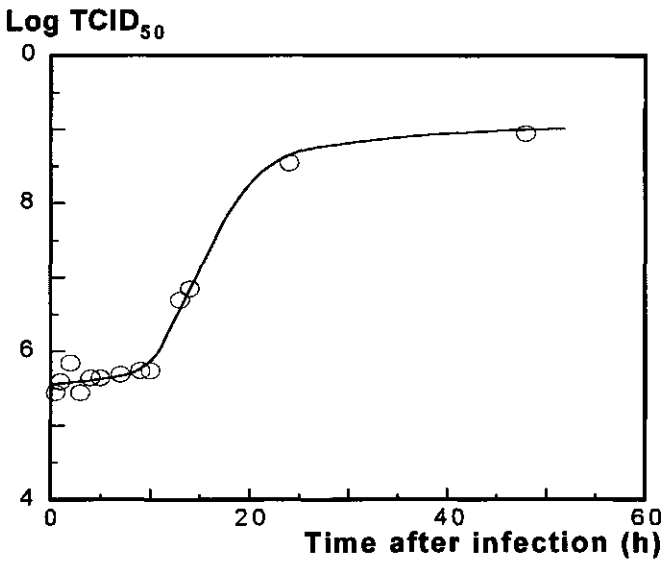

Figure 2. Production of AcNPV non-occluded virus per 5.10^6 anchored *Spodoptera frugiperda* cells (MOI = 20).

Considering these assumptions, the following equations can be derived :

$$r_s = k_r C_j \quad [2]$$

$$r_g = k_g C_j \quad [3]$$

Equation 2 shows the first-order infection rate (r_s) with k_r the reaction rate constant (s^{-1}), and C_j the concentration of viable, non-infected cells ($cell.cm^{-3}$). Equation 3 describes the growth of insect cells (r_g) in any reactor, with k_g the cell

growth-rate constant (s^{-1}). The subscript j denotes the reactor number in the series, (j=1,2,3...), with C_0 the concentration in the growth vessel (Figure 3).

A mass balance for viable, non-infected cells for the j^{th} reactor in steady state gives:

$$\text{In- Out - Infection + Growth = 0} \quad [4]$$

or :

$$\phi_v(C_{j-1} - C_j) - r_s V_j + r_g V_j = 0 \quad [5]$$

Here, V is the reactor volume (m^3) and ϕ_v is the flow rate in the reactor series ($m^3.s^{-1}$). Introducing the mean residence time $\tau = V/\phi_v$ (s), substituting equations [2] and [3] and rearranging gives :

$$k_r = \frac{C_{j-1} + (k_g\tau_j - 1)C_j}{C_j\tau_j} \quad [6]$$

or:

$$C_j = \frac{C_{j-1}}{1 + \tau_j(k_r - k_g)} \quad [7]$$

A special case arises if the series of reactors contains one reactor for growth followed by one reactor for infection, with both volumes equal, so that $\tau_0 = \tau_1$. Then, since the growth rate of the cells adapts to the mean residence time in the growth vessel, i.e. $k_g = 1/\tau_0$, equation [6] reduces to:

$$k_r = \frac{C_0}{C_1\tau_1} \quad [8]$$

All values for the parameters in equations [6] to [8] can be measured easily, except for the reaction rate constant k_r.

MATERIALS AND METHODS

Spodoptera frugiperda cells (Sf-AE-21) (Vaughn et al. 1977) were maintained on solid supports (Costar tissue culture flasks), containing TNM-FH medium (Hink 1970) without egg ultrafiltrate and supplemented with 10% fetal calf serum. When cells were grown in suspension, 0.1% (w/v) methylcellulose was added to the medium.

The virus used was the E2-strain of *Autographa californica* nuclear polyhedrosis virus (Smith et al. 1978), and the infectivity of NOVs was measured using an end-point dilution assay (Vlak 1979). The stock solution contained approximately 10^8 $TCID_{50}$ units per cm^3 medium.

For continuous cultivation of cells 1 dm^3 round-bottomed and 2 dm^3 flat-bottomed fermenters (Applikon), equipped with marine impellers, were used. Air was passed through a 0.2 μm filter (Millipore) into the headspace of the reactors at about 10 $dm^3.h^{-1}$. The mean residence time in a cell growth reactor and in the subsequent virus production system was set to 2.15 10^5 s. The temperature in the fermenters was kept at 28 °C.

Both experimental, continuously-operated, reactor configurations are illustrated in figure 3. Configuration A consisted of one reactor where insect cells were grown, followed by two reactors in series where the infection process took place (Van Lier et al. 1990).

A second experimental reactor configuration (B), also operated continuously, consisted of one reactor where the insect cells were grown, followed by only one equally sized vessel where infection took place (Kompier et al. 1988).

The number of cells was measured microscopically using an Improved Neubauer Brightline hemocytometer. Cell viability was determined using the

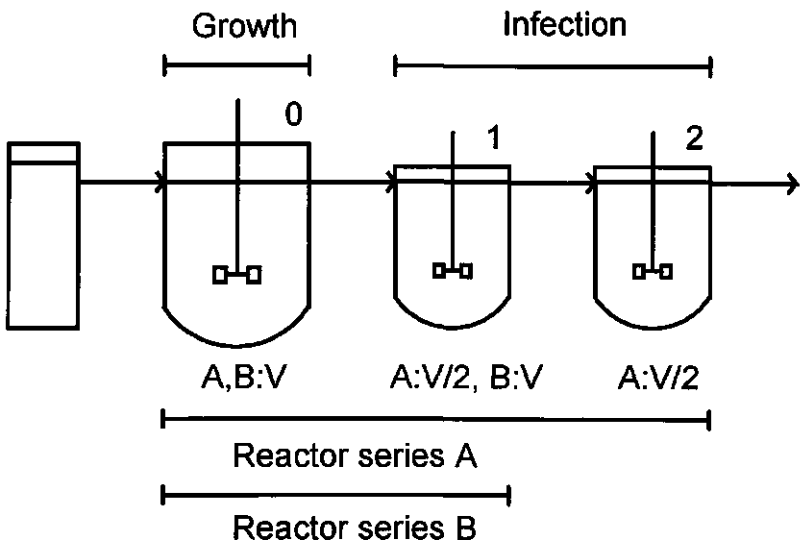

Figure 3. Experimental reactor configurations for the continuous production of insect cells and baculoviruses.

trypan blue dye exclusion method. Cells were considered to be infected when polyhedra could be observed microscopically inside the cell nucleus.

RESULTS AND DISCUSSION

Both reactor series used in our experiments were operated continuously until a steady state was reached. At this point, both the number of viable, non-infected cells and the number of infected cells were determined at all reactor outlets in the series.

For the reactor series with two infection vessels, the viable, non-infected cell concentration in the first infection vessel (Table 1) was used (equation [6]) to calculate the reaction rate constant in the first infection vessel. If the presented

first-order reaction kinetics model is valid, the same value for the reaction rate constant should apply for the second infection vessel, and therefore the viable cell concentration in the second infection vessel can be predicted (equation [7]). Moreover, using the same cell line and virus in the other reactor configuration, the observed reaction rate constant (equation [8]) has to be of the same range. Experimental results, taken from Kompier et al. (1988) and Van Lier et al. (1990), and calculated reaction rates are listed in table 1.

Table 1. Results with two reactor configurations. A and B are referred to in text. Numbers between parenthesis denote the number of continuous operated infection vessels.

PARAMETERS		DIMENSION	A (2)	B (1)
C_0	Cell concentration at inlet of reactor 1	cell.cm^{-3}	8.1 10^5	7.8 10^5
C_1	Viable cell concentration in reactor 1	cell.cm^{-3}	4.9 10^5	4.2 10^5
τ_1	Residence time in reactor 1	s	1.08 10^5	2.16 10^5
I_1	Infected fraction in reactor 1	% of cells	31	55
k_r	Reaction rate constant	s^{-1}	1.07 10^{-5}	0.86 10^{-5}
C_{2p}	Predicted viable cell concentration in reactor 2	cell.cm^{-3}	3.0 10^5	-
C_{2m}	Measured viable cell concentration in reactor 2	cell.cm^{-3}	2.1 10^5	-
I_2	Infected fraction in reactor 2	% of cells	59	-

Due to the increase in residence time of the first infection vessel going from reactor series A to reactor series B, an increase in the infected fraction in the first reactor can be expected, as is clearly shown by our results in table 1. Moreover, as stated by Kompier et al. (1988), the infected rate in the second vessel of reactor series A should be higher than in the infection vessel of series B. We indeed could observe this effect, however not as strongly as theoretically could be expected.

In reactor series A, one growth vessel followed by two infection vessels, the reaction rate constant in the first infection vessel is 0.93 10^{-5} s^{-1}. With this

reaction rate, the concentration of viable, non-infected cells in the second infection vessel can be calculated to be $3.0\ 10^5$ cells.cm^{-3}. The actual concentration observed was $2.1\ 10^5$ cells.cm^{-3}. For reactor series B, one growth vessel followed by one infection vessel, a value for the reaction rate constant of $0.72\ 10^{-5}$ s^{-1} can be calculated.

The results match our theory rather well, considering that the infected fraction is determined by microscopically observing formed polyhedra in the cell nucleus only. This has two consequences for the observed reaction rate constant and the infected fraction.

First, as we frequently observed free polyhedra in the medium, it can be concluded that a number of cells lysed. Therefore, the infected fraction must be higher than observed, and the reaction rate constant thus also must be higher. Given this, the predicted viable cell concentration in the second infection vessel becomes lower. This effect is even larger if we consider the effect of cell lysis on the infected fraction in the second vessel as well. Considering cell lysis in comparing the reaction rate constants of reactor series A and B, the fact that the reactor volume of the first infection vessel, and with that the mean residence time and thus the number of lysed cells, of series B is higher, imposes that the real reaction rate constant in series B will show a higher increase than in reactor series A. Therefore these values in reality will be even closer to each other.

Second, an insect cell can be infected with virus, but will not yet show polyhedra in the cell nucleus (Figure 1). This effect also will lead to a higher infected fraction than observed, and, from that, a higher reaction rate constant. For reactor series A this will lead to a lower predicted viable cell concentration in the second infection vessel, resulting in an even better prediction.

The high abstraction level of our model imposes some constraints on its practical use. First, the reproduction process of virus has a discrete character

(Figure 1), but is described as a (continuous) steady state. Therefore, a minimum mean residence time of the infected cells should be regarded. This residence time should not be smaller than the time needed for all virus to replicate. In our case (Figure 2) this minimum is about 24 hours. Second, reaction rate constants observed in a laboratory-scale experiment might differ from values found on a larger scale, due to different (bio-)chemical influences or possible physical damage to infected cells caused by higher shear stresses usually occurring as the reactor volume of stirred vessels increases (Katinger et al. 1985). This effect might not affect the 'substrate' consumption rate, but will probably have an impact on the product formation rate, i.e. a lower number of polyhedra per insect cell, due to premature cell disruption.

CONCLUSIONS

The predicted concentration of viable, non-infected cells in the second infection vessel matches well the experimentally obtained value. The observed reaction rate constants in both reactor configurations A and B do not differ significantly, considering cell-lysis effects and the fact that both cells and viruses present in reactor configuration A are of higher age. Yet, in order to completely validate our model, more research is under way.

The influence of crucial reactor operation parameters on both growth and infection of insect cells are not incorporated in our model. Additional experimental data concerning these parameters, the virus concentration at reactor start up, and the importance of the age of both insect cells and viruses are needed to optimize design and operating time of these bioreactors.

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NOMENCLATURE

C	Cell Concentration	$cells.cm^{-3}$
I	Infected fraction	% of cells
k_g	First-order growth rate constant	s^{-1}
k_r	First-order infection rate constant	s^{-1}
r_s	Infection reaction rate	$cell.cm^{-3}.s^{-1}$
r_g	Cell growth rate	$cell.cm^{-3}.s^{-1}$
V	Reactor volume	m^3
ϕ_v	Volumetric flow rate	$m^3.s^{-1}$
τ	Residence time	s
j	Reactor number in a series	-

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CHAPTER 7

A STRUCTURED DYNAMIC MODEL FOR THE BACULOVIRUS INFECTION PROCESS IN INSECT-CELL REACTOR CONFIGURATIONS

ABSTRACT

A mathematical description of the infection of insect cells with baculovirus in a continuously-operated reactor configuration is presented. The reactor configuration consists of one bioreactor in which insect cells (*Spodoptera frugiperda*) are grown, followed by one (Kompier et al. 1988) or two (van Lier et al. 1990) bioreactors in which cells are infected by a baculovirus (*Autographa californica* nuclear polyhedrosis virus). It was demonstrated that the so-called passage effect is responsible for the observed difference in run time between a configuration with one or with two infection vessels. Furthermore, a model is presented based on the hypothesis that the limited run time of series of

continuously-operated bioreactors is associated with the occurrence of a virus particle (so-called virion) that is defective and has interfering properties (Kool et al. 1991). With the assumption that not all non-occluded virions are capable of establishing a correct infection leading to new virus production, infection levels in continuously-operated reactor configurations could be described well with the model.

INTRODUCTION

Baculoviruses are attractive biological agents for control of insect pests in agriculture. In addition, these viruses can be reprogrammed for the production of recombinant proteins by genetic engineering (Luckow & Summers, 1988; Vlak & Keus, 1989). To obtain commercially-attractive levels of productivity and at the same time meet regulatory requirements, the production of baculoviruses or recombinant proteins can best be achieved in insect-cell bioreactors, preferably operated in a continuous fashion (Tramper & Vlak, 1986). Two phenotypically different forms of a baculovirus exist. The occluded form (polyhedra) is infectious for insect larvae, whereas the non-occluded virus form (NOV) is infectious for insect cells in culture. Kompier et al. (1988) and Van Lier et al. (1990) showed that polyhedra of *Autographa californica* nuclear polyhedrosis virus (AcNPV) could be produced over long periods of time in a system consisting of an upstream bioreactor in which insect cells are cultured, followed by one or two bioreactors where infection with the non-occluded form of AcNPV takes place (Figure 1). The continuous production could be maintained up to about four weeks, after which production declined. The level of infection could be increased by increasing the number of infection reactors as shown by Van Lier et al. (1990) for reactor configuration A. However, the time that this system could be

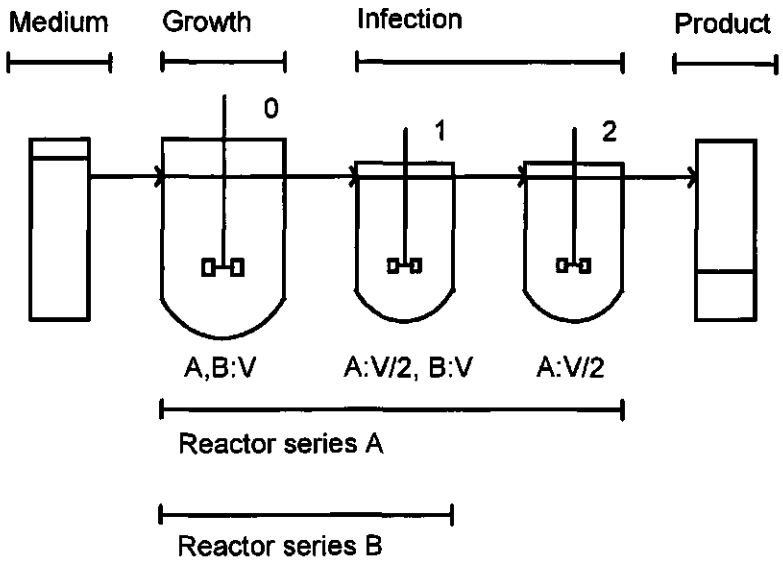


Figure 1. Schematic representation of the experimental continuously-operated reactor configurations as used by Van Lier et al. (1990) (series A) and Kompier et al. (1988) (series B). The total residence time in both configurations is the same.

operated at this enhanced level of infection decreased significantly to less than three weeks (Figure 2).

Kool et al. (1990, 1991) showed that the reduction in productivity was due to the occurrence of mutant virions present in the infection reactor(s) that interfered with the replication of intact virus and affected productivity. This defective interference phenomenon has been confirmed by Wickham et al. (1991).

This paper presents a structured model for the infection of insect cells with NOV particles, capable of dynamically describing the infection process in continuous, batch and fed-batch bioreactors. This model forms the theoretical foundation explaining that the decrease in the levels of polyhedra-producing cells in continuously-operated vessels is the result of the so-called

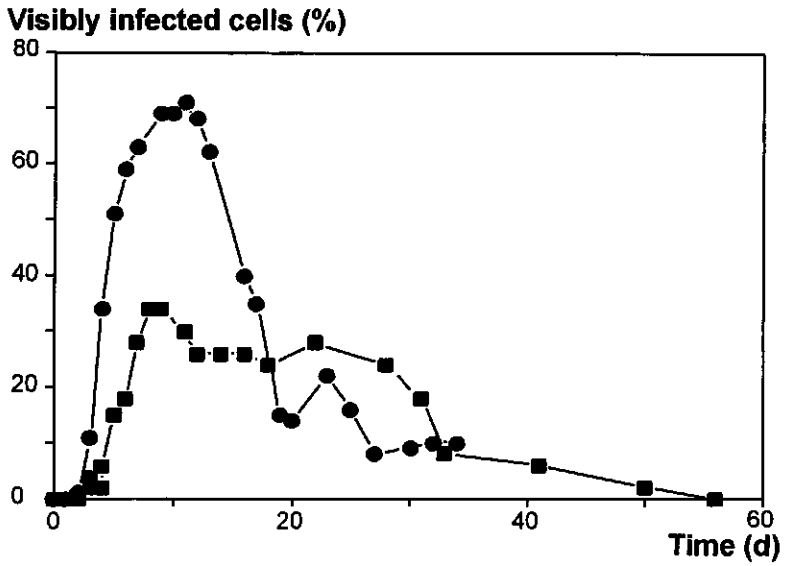


Figure 2. Percentage of cells visibly containing polyhedra present in reactor 2 of configuration A (●) or in reactor 1 of configuration B (■) versus time. Pseudo-steady-state levels of infection in the continuously-operated infection vessels of about 68% and 26% were reached, respectively.

passage effect, and that this decrease can be quantitatively explained by the occurrence of a high number of so-called defective interfering virions (D-NOV).

THEORY

The passage effect, as described by Faulkner (1981), manifests itself as a decrease in the production of polyhedra at higher passages of non-occluded baculovirus preparations. This effect is also reflected by a decrease in the number of infectious NOVs (I-NOVs) that is produced per insect cell. One passage is defined as the process of an I-NOV entering an insect cell, transport of the genetic information into the cell nucleus, production of new I-NOVs,

transport to and budding through the cell membrane, and secretion of the virions to the extracellular fluid. After completion of this process the passage number is increased by one. Schematically this is illustrated in figure 3.

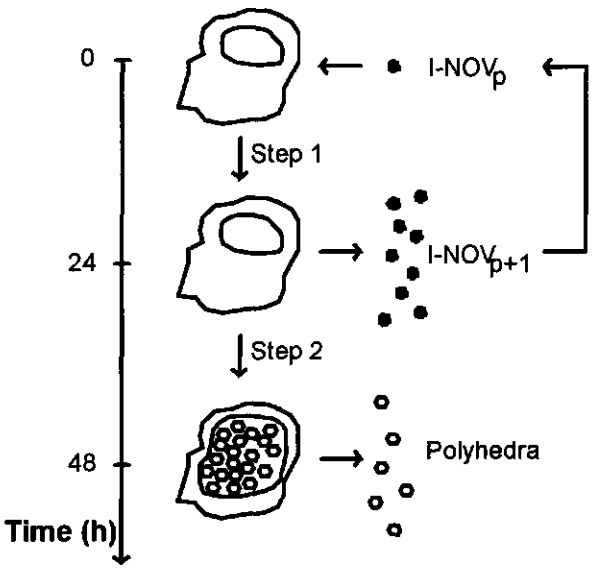


Figure 3. Schematic representation of the replication cycle of baculoviruses in insect cells (adapted from de Gooijer et al. (1989)). *p* and p+1 denote passage numbers. See text for more details.

Upon entry in the infection vessel, a cell will be infected with a virion of a certain passage *p*. After some time the cell will start to produce virions of passage *p + 1* (Figure 3). These virions in turn will maintain the infection process in the continuously-operated infection vessels. Apart from this infectious I-NOV, two additional types of virions are introduced in our model. The first additional type of virion was described by Kool et al. (1990, 1991). They showed the existence and established some of the properties of a defective interfering virion, present in the medium of the reactor during continuous runs. This D-NOV lacked

about 44% of the viral genome, among which the polyhedrin gene and the gene coding for DNA-polymerase. Due to this deletion the D-NOV needs the intracellular presence of an intact I-NOV as a helper for multiplication. Since their genome is smaller, D-NOVs are likely to be reproduced faster than I-NOVs.

For both reactor configurations the theoretical level of infection can be calculated from a residence-time distribution of the insect cells in the infection vessels. Considering the fact that cells were denoted as visibly infected when polyhedra were observed, only the cells with a residence time of more than 24 hours in the bioreactor with infected cells are of interest (Figure 3). For our experiments, with a total residence time of 60 hours, it could be calculated that 68% of the cells had a residence time of more than 24 hours and therefore should have been visibly infected in the case of one infection reactor, whereas this is 82% in the second reactor for two infection vessels with the same total residence time (Levenspiel, 1972) (Configurations B and A in figure 1, respectively). This theoretical value, however, could never be reached in our laboratory (Figure 2). As we also did not reach a 100% infection in batch cultures (this paper), we assume that a second additional virion type is present which is characterized by its inability to complete a replication cycle as discussed above. These virions are of normal size and for example may attach to the cell surface but fail to form an endosome, or may attach, form an endosome, but fail to leave the endosome (Seth et al. 1985). In these mechanisms no reproduction takes place, whilst cell receptors are being occupied. To handle this assumption mathematically, we therefore included a NOV leading to an abortive infection (A-NOV) in our model. An A-NOV does occupy an entry site, but will not lead to any reproduction. Volkman et al. (1976) reported a ratio I-NOV/A-NOV of less than 1% for AcNPV produced in *Trichoplusia ni* cells. In our laboratory, approximately 1 g of viral DNA could be isolated from a sample containing 10^8

median tissue culture infective dose ($TCID_{50}$) units of extracellular AcNPV. With a molecular weight of the baculovirus of about 10^8 ($g.mol^{-1}$) and Avogadro's number of $6\ 10^{23}$ ($molecules.mol^{-1}$) a ratio of 1 I-NOV to 60 A-NOVs could be calculated.

Reactor model

A general mass balance of virions or cells over one reactor vessel can be described as:

$$\frac{\partial x}{\partial t} = (r_{x,in} - r_{x,out}) + r_{x,prod} - r_{x,use} \qquad [1]$$

where t is the time (h), x is a compound (number of cells or virions), $r_{x,in}$ and $r_{x,out}$ are the in and outgoing flows of x (h^{-1}), $r_{x,prod}$ is the production rate of the compound (h^{-1}), and $r_{x,use}$ is the consumption rate of x (h^{-1}). This mass balance was applied to virions and insect cells.

Virions

Within the general mass balance for virions a simple production term $r_{x,prod}$ can be defined by the assumptions that enough virions are available to infect all insect cells, that all cells entering the infection reactor are infected immediately, and that after 16 hours the cells start to produce new virions. As we are interested in the passage effect this is done for each passage p. Hence,

$$r_{x,prod,p} = a\, f_{p-1,\, t-16} \qquad [2]$$

with f being a fraction of I-NOV of a certain passage, that is the total amount of I-NOV$_p$ divided by the total amount of all I-NOV, t is the time (h), a is a production constant formed by the number of I-NOVs produced per cell, multiplied by the number of cells flowing into the reactor per unit of time (h^{-1}), and subscript p denotes the passage number.

The fraction $f_{p,t}$ of a certain passage at a certain time is determined not only by the fractions of the other passages at that time, but also by the fractions of the passages before that time. These passages determined how many insect cells are infected with a certain passage and with that how many virions of a higher passage will be produced 16 hours later. With increasing time, the number of passages increases, and with that the set of equations arising from equation [1] that has to be solved augments also. As with increasing time this exercise will become quite complicated, equation [1] is made discrete :

$$x_{t+\Delta t} = x_t + ((r_{x,in} - r_{x,out}) + r_{x,prod} - r_{x,use})\,\Delta t \qquad [3]$$

with Δt being one time step (h).

Equation [3] can be applied to all types of NOV as well as insect cells. For the I-NOV this can be done for each virion passage. This leads to:

$$v_{i,m,p,t+\Delta t} = (1 - D_m \Delta t)(v_{i,m,p,t} + v_{i,in,m,p,t}) + (R_{i,m,p} - I_{i,m,p})\Delta t \qquad [4]$$

where D is the dilution rate in a reactor (h^{-1}), I is the virion consumption rate used for infection (h^{-1}), R is the rate of virion release by the cells (h^{-1}), v is the amount of virions, subscript m denotes the reactor number in the series, and subscript i denotes the infectious I-NOV.

For D-NOVs and A-NOVs it is not necessary to calculate with separate passages, so equation [3] is simplified to :

$$V_{(d\vee a),m,t+\Delta t} = (1 - D_m\Delta t)(V_{(d\vee a),m,t} + V_{(d\vee a),in,m,t}) + (R_{(d\vee a),m} - I_{(d\vee a),m})\Delta t \quad [5]$$

where *d* denotes the defective interfering virion (D-NOV), and *a* denotes the virion leading to an abortive infection (A-NOV).

The dilution rate D_m in reactor *m* will be zero as long as this reactor is in batch or fed-batch mode, which will be the case in the start-up phase of the reactor configuration. Due to the fact that in the cell growth vessel (m=0) in the series no virion production takes place, v_0 is equal to zero for all NOV types.

The total amount of I-NOV in vessel *m* can be calculated from:

$$V_{i,m,tot,t+\Delta t} = \sum_{p=0}^{p=p_{max}} V_{i,m,p,t+\Delta t} \quad [6]$$

where p_{max} is the maximum number of passages at time $t+\Delta t$, and subscript *tot* denotes the total number of I-NOVs.

The amount of virion release by the insect cells is controlled by the number of infected cells some time before. As shown previously (de Gooijer et al. 1989), the virions are not released at once. Therefore, a time distribution of the I-NOV and A-NOV release is introduced in the model, as illustrated in table 1.

Table 1. Distribution of virion release from infected insect cells (adapted from De Gooijer et al. (1989)).

TIME (H)	% RELEASE	% RELEASE (TOTAL)
16	7	7
18	13	20
20	20	40
22	26	66
24	17	83
26	15	98
28	2	100

Kool et al. (1990, 1991) showed that D-NOVs have a 44% smaller genome than I-NOVs. If D-NOV DNA and I-NOV DNA are replicated at the same speed, the replication cycle of a D-NOV will be finished sooner. Since the process of transport to and from the cell nucleus may also be of importance, the D-NOVs are arbitrarily assumed to have the same time distribution but moved forward in time by two hours.

To facilitate the calculation of the I-NOV balance for each passage, $R_{i,m,p}\Delta t$ is calculated in advance, that is as the number of virions that will be released by the cells after some time. Therefore the number of infected cells and $I_{i,m,p}\Delta t$ are evaluated in each time step. In the software these data are then stored in an array. Each time step the index of the array is increased, and in this way the virion amounts are available for calculation of $R_{i,m,p}\Delta t$ at the appropriate time.

Insect cells

Equation [3] can also be applied to viable insect cells. With a similar derivation as for equation [4], for the first infection vessel in the series, equation [3] turns into:

$$C_{m,t+\Delta t} = (1 - D_m \Delta t)(C_{m,t} + D_{m-1}\Delta t C_{m-1,t}) + k_g(C_{m,t} - G_m)\Delta t \qquad [7]$$

where C is the number of non-infected insect cells, G is the number of cells that are infected in one time step, and k_g is the first-order cell growth constant (h^{-1}). Note that C_0 is the number of cells in the growth vessel. Cells that are not infected can grow in infection vessels, with the same speed as in the cell growth vessel. Infection of insect cells with baculovirus will annihilate cell division (Faulkner, 1981).

Infection model

With the three virion types (I-NOV, A-NOV, and D-NOV), three important modes of infection can be distinguished, as illustrated in figure 4.

The first mode is formed by correct infections, being an insect cell infected with at least one I-NOV, and not infected by any D-NOV. Such cells will produce I-NOV, A-NOV, and a small amount of D-NOVs. The second mode consists of defective infections, where an insect cell is infected with at least one I-NOV and at least one D-NOV. Such an insect cell will produce I-NOV, A-NOV, and a large quantity of D-NOVs. The third mode is formed by an abortive infection, defined by an insect cell that is infected with an A-NOV and/or D-NOV, but without an infection with infectious I-NOV. Insect cells infected this way will not produce

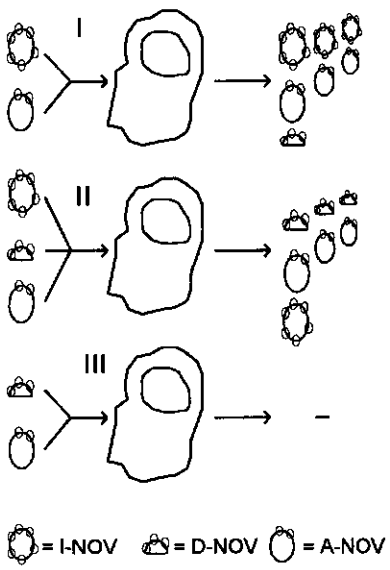


Figure 4. Schematic representation of three important modes of infection. See text for more details.

any virions. For both the first and second mode of infection the ratio of the numbers of virions produced is constant with time.

To be able to calculate the fractions of each mode of infection, the following assumptions were made :

i) Each insect cell has an equal amount of 'entry sites' for NOVs. This number is much less than the number of receptors. Wickham et al. (1990) reported a number of 10^5 AcNPV receptors per cell in the case of *Spodoptera frugiperda* cells. As production expressed as number of NOVs per cell is considerably less than this amount (this paper), it would be difficult to perform continuous runs if all NOVs would attach to cells and each virion - receptor interaction leads to an infection. Moreover, it is most likely that the entry of 10^5 NOVs in an insect cell will lead to a considerable overload of the cellular machinery, or cause cytotoxic effects.

ii) The number of entry sites is constant in time for one experimental run.

iii) All entry sites are equal.

iv) All NOVs have identical binding sites. Hence, the probability that an NOV attaches is the same for all types of NOV.

v) Attachment of an NOV to a cell is irreversible.

vi) The three NOV types cannot change into alternate types spontaneously.

vii) Binding of NOVs to cells will take place within one time step.

With these assumptions the fractions of the different modes of infection can be calculated, for each time step, if the probabilities for the different modes of infection are known.

This problem, where a known number of three types of virions can occupy a known number of entry sites, is the same as the problem where red, white and blue balls are to be dispended over a number of small boxes. It is known from the latter that the probability of a box remaining empty has a Poisson

distribution. Therefore, it is assumed that the probability (P_o) of an entry site remaining empty is having a Poisson distribution :

$$P_0 = e^{-v_{tot}/BC} \quad [8]$$

where B is the number of entry sites on a cell, and subscript *tot* denotes the total number of all virions.

From that, the probability (P_i) of an entry site being infected with an I-NOV is :

$$P_i = \frac{v_i(1 - P_0)}{v_{tot}} \quad [9]$$

and the probability (P_d) that an entry site is infected with a D-NOV :

$$P_d = \frac{v_d(1 - P_0)}{v_{tot}} \quad [10]$$

and finally the probability (P_a) that an entry site is infected with an A-NOV :

$$P_a = \frac{v_a(1 - P_0)}{v_{tot}} \quad [11]$$

The sum of these probabilities equals unity :

$$P_o + P_i + P_d + P_a = 1 \quad [12]$$

With these probabilities the fractions of the various modes of infection of the cells can be calculated. The fraction of the cells that are not infected (F_o) then is :

$$F_0 = F(B_a = 0 \wedge B_i = 0 \wedge B_d = 0) = P_0^B \quad [13]$$

The fraction of the cells that are abortively infected (F_a) then is :

$$F_a = F(B_i = 0 \wedge (B_a + B_d > 0)) = \sum_{\alpha \geq 0, (\beta+\lambda)>0}^{\alpha+\beta+\lambda=B} \frac{B!}{\alpha!\beta!\lambda!} P_0^{\alpha} P_a^{\beta} P_d^{\lambda} \quad [14]$$

where α is the number of entry sites per cell that are not occupied by any virion, β is the number of entry sites per cell that are occupied by A-NOVs, and λ is the number of entry sites per cell that are occupied by D-NOVs.

The fraction of cells that have a correct infection (F_i) is calculated from:

$$F_i = F(B_i > 0 \wedge B_d = 0) = \sum_{\kappa>0, \alpha, \beta \geq 0}^{\alpha+\beta+\kappa=B} \frac{B!}{\alpha!\beta!\kappa!} P_0^{\alpha} P_a^{\beta} P_i^{\kappa} \quad [15]$$

where κ is the number of entry sites per cell that is occupied by I-NOVs.

The fraction of cells with a defective infection (F_d) is calculated from :

$$F_d = F(B_i > 0 \wedge B_d > 0) = \sum_{\kappa, \lambda>0, \alpha, \beta \geq 0}^{\alpha+\beta+\lambda+\kappa=B} \frac{B!}{\alpha!\beta!\lambda!\kappa!} P_0^{\alpha} P_a^{\beta} P_d^{\lambda} P_i^{\kappa} \quad [16]$$

As the sum of all fractions is equal to one :

$$F_o + F_i + F_d + F_a = 1 \quad [17]$$

it is also possible to calculate F_d with equation [17]. The actual amounts of cells and virions, as required in the equations [4], [5], and [7] can easily be calculated from these fractions. Note that the implementation of equations [14], [15], and [16] also limit the theoretical number of entry sites that can be handled in the model for numerical reasons. The maximum number in our software is 1546. With the above equations the concentration in time for each of the virion types can be calculated. With these concentrations the decline in infection level in continuously-operated bioreactors can be described.

MATERIALS AND METHODS

Cells and viruses

Spodoptera frugiperda cells (Vaughn et al. 1977) were maintained in TNM-FH medium (Hink 1970) without egg ultrafiltrate, but supplemented with 10% fetal calf serum. For suspension cultures 0.1% (w/v) methylcellulose was added. The E2-strain of *Autographa californica* nuclear polyhedrosis virus (AcNPV) (Smith & Summers 1978) was used. The stock solution of the virus contained 10^8 $TCID_{50}$ units per cm^3.

Reactor configuration

The continuously-operated reactor configuration with one infection vessel has been described by Kompier et al. (1988), and the configuration with two infection vessels by van Lier et al. (1990).

Assays

In order to determine the infectivity of the virus, 4-cm^3 aliquots of the sample were centrifuged (1600 *g* for 15 min). The supernatant was filtered (0.45 μm), and the infectivity was measured using the end-point dilution method as described by Vlak (1979). The presence of polyhedra in the cell nucleus was determined using an inverted microscope (magnification 400x). The cell concentration was measured using a Neubauer hemocytometer.

Batch infection

Cells from a continuous culture were pumped into a round-bottom bioreactor (working volume: 0.6 dm^3, Applikon, Schiedam, the Netherlands). The cells were infected with 15 cm^3 containing 10^8 $TCID_{50}$ units of third passage AcNPV per cm^3, resulting in a multiplicity of infection of 2 and 3 $TCID_{50}$ units per cell in two separate experiments. Samples were taken to assess the number of infected cells and the cell viability with time.

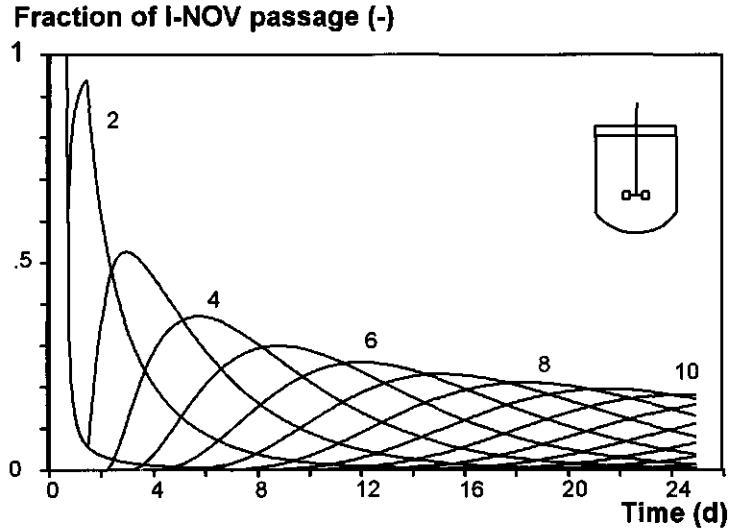
Serial passages

Cells were grown in 100 cm^3 Erlenmeyer flasks in 20 cm^3 of medium until the cell density exceeded $1.5x10^6$ cells.cm^{-3}. To maintain the cells in an exponential growth phase, cells were diluted to a concentration of $5x10^4$ cells.cm^{-3} and further incubated at 27 °C. From these suspensions a volume equivalent to $2x10^6$ cells was taken and centrifuged (225 *g*, 5 min). A volume containing $2x10^7$ $TCID_{50}$ units of a single passage *p* was added to the cell pellet, thus resulting in a Multiplicity of Infection of 10. Medium was added to a volume of 20 cm^3 giving a cell concentration of 10^5 cells.cm^{-3} and a multiplicity of infection of 10 $TCID_{50}$ units per cell. The Erlenmeyer flask was then further incubated for three hours at 27 °C. The medium, and, with that, the non-bound I-NOVs were removed by centrifugation (225 g, 5 min). After resuspension in 20 cm^3 fresh medium the cells were incubated for 21 hours at 27 °C. The extracellular fluid of this sample then contained almost exclusively I-NOVs of a higher passage *p* + 1. After centrifugation the infectivity of the supernatant was assayed as described before. Hereafter, a new experiment was started.

RESULTS AND DISCUSSION

Passage-time distribution of I-NOVs

With the model discussed above, the effects of different reactor configurations were studied. For one set of parameters, the total residence time in the series was kept constant by varying the reactor volumes for each reactor at a constant flow. The fractions of infectious NOV of each passage in time were calculated for reactor configurations with one, two and three infection reactors, with the infection reactor volume being 1.2×10^{-3}, 0.6×10^{-3}, and 0.4×10^{-3} m^3, respectively. Results are shown in figure 5. This figure clearly shows that higher passages occur sooner if the number of vessels in the series of infection reactors is increased, i.e. if plug flow is more closely approximated.



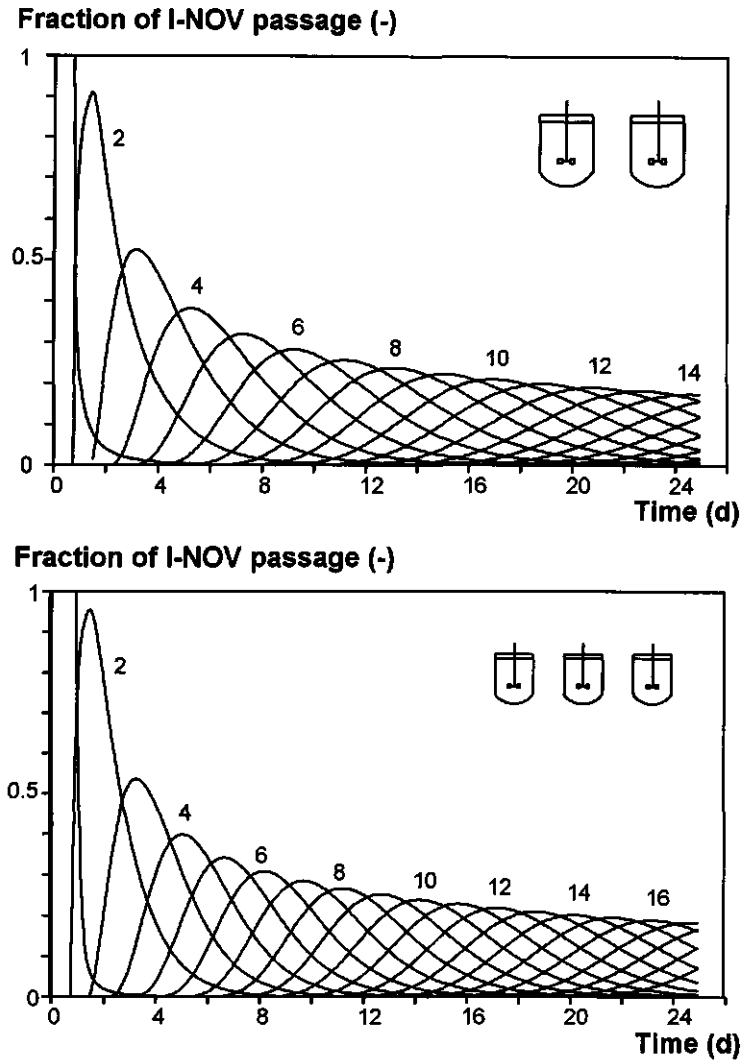


Figure 5. Passage distribution of I-NOV, depicted as a fraction ($I\text{-}NOV_p$/ $I\text{-}NOV_{tot}$) occurring in time in the last vessel for three different continuously-operated reactor configurations with one, two and three infection vessels. The total residence time is kept constant for all configurations.

The model was used to test the hypothesis whether the passage effect is a viable explanation for the decrease in the number of cells producing polyhedra in the experiments with different reactor configurations as mentioned in the introduction section. Therefore, at the calculated time ($t_{1/2}$) where the level of infection was only half of the pseudo-steady-state level as shown in figure 2, the distribution of the virion passages was calculated with the program. Results for the configurations with one and two infection vessels are shown in figure 6.

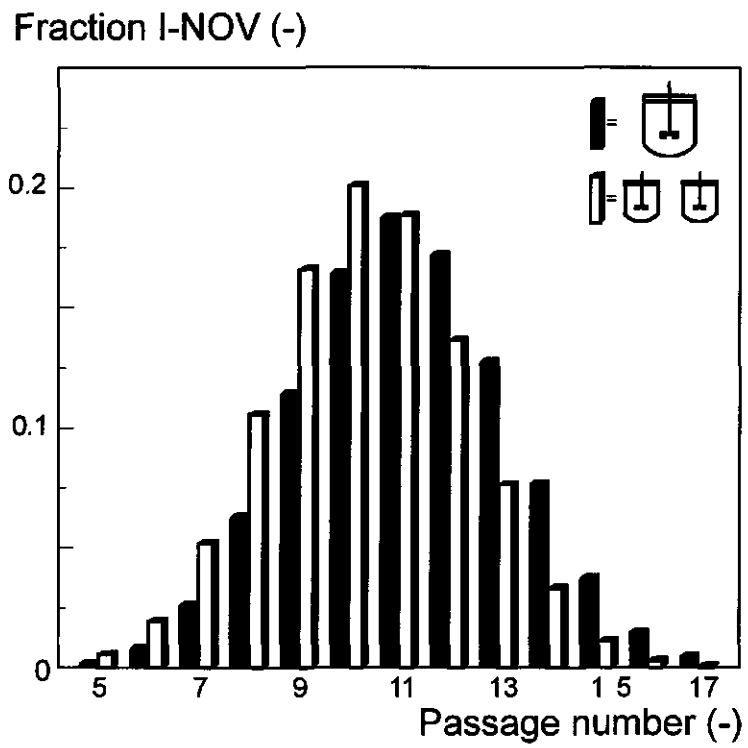


Figure 6. Calculated passage distributions of I-NOV for two continuously-operated reactor configurations at the time the infection level is half of the pseudo-steady-state level. The I-NOV fraction of a certain passage (I-NOV$_p$/I-NOVtot) versus the passage number *p*. The distribution for one infection reactor is in black, and for the second reactor of a series of two infection reactors the bars are white.

As both passage distributions in figure 6 showed a remarkably good resemblance, it can be concluded that the decline of polyhedra production after a certain time may indeed be attributed to the passage effect.

To assess this in more detail, virions were serially passaged in two independent series of batch experiments and the amount of NOV produced in each passage was determined. The results are shown in figure 7.

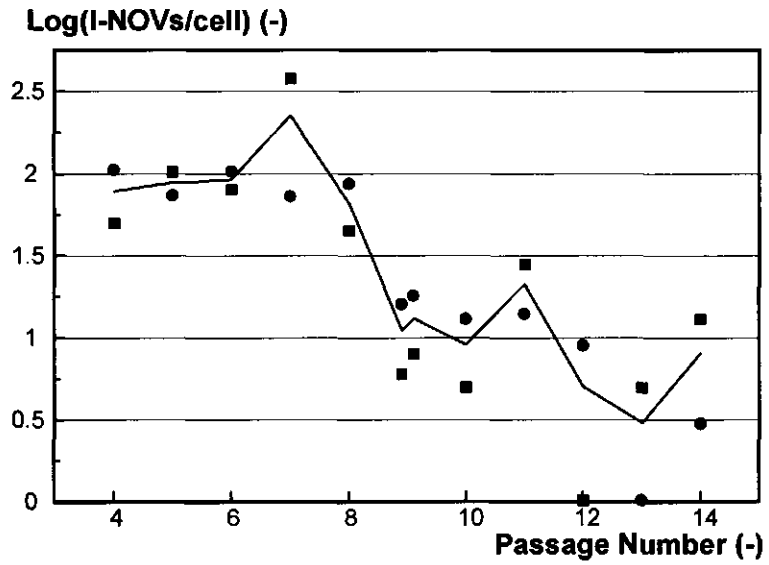


Figure 7. Prolonged passages of I-NOV : the logarithm of the averaged number of produced I-NOVs per cell as a function of the passage number. (●, ■) are the results of two duplicate experimental series.

At the ninth passage a sharp drop in the number of I-NOVs produced per cell was observed. At higher passages, a further decrease in the number of polyhedra per cell was found. Furthermore, the polyhedra showed morphological aberrations, which is a known consequence of the passage effect (Faulkner, 1981).

The combination of figures 6 and 7 shows that at the time at which the infection level is half of the pseudo-steady-state level, the preponderance of the virions present in the two reactor configurations has a passage number higher than 8. Moreover, with virions of these passage numbers not enough virions is produced to maintain the continuous infection process at the pseudo-steady-state level.

To further elucidate the phenomenon of the passage effect two other virion types A-NOV and D-NOV as described before were introduced into the infection model.

Infection process

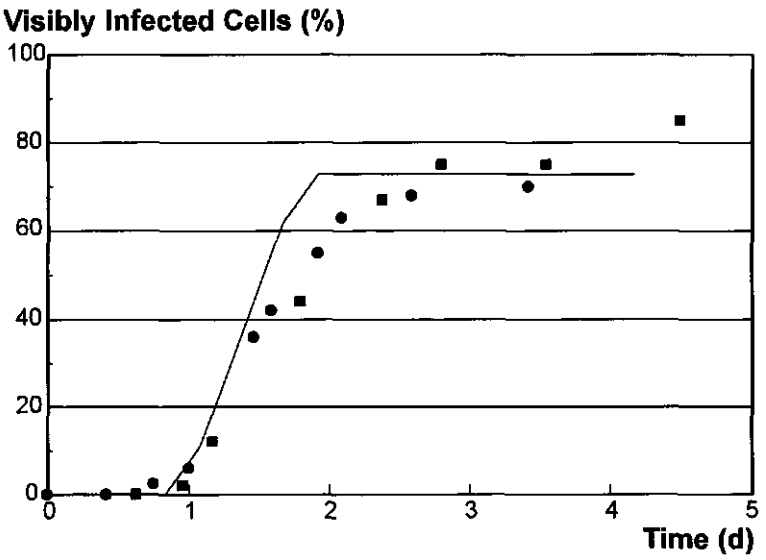


Figure 8. Fit (line) of two batch infection runs (●, ■) with the model. The percentage of visibly infected cells versus time. Parameters are as in table 2 under A.

The assumption of the A-NOV type facilitated the description of our data on batch infections, as shown in figure 8. Here, two independent runs were fitted by the model. The fit of the model was reached on the basis of visibly infected cells. We assumed that of the synchronously-infected cells, the first ones become visibly infected after 20 hours, and the last after 44 hours. Visibly infected cells remain visible for 60 hours, then they lyse.

After including the three NOV types into the program all our experimental continuous runs could easily be described with the model. Input variables are given in table 2.

Table 2. Input variables for the infection model. Values A,...,E are used in figures 8-12, respectively. Values under D are also used in table 3.

VARIABLE	A	B	C	D	E	UNIT
Reactor working volume	600	800	2 x 600	2 x 600	800	cm^3
Reactor startup volume	600	400	2 x 300	2 x 300	200	cm^3
Flow	0	$1.33\ 10^{-2}$	$2\ 10^{-2}$	$2\ 10^{-2}$	$8.3\ 10^{-4}$	$dm^3.h^{-1}$
Number of I-NOVs at startup	$1.5\ 10^{9}$	$1.6\ 10^{3}$	$7.1\ 10^{6}$	$1.5\ 10^{9}$	$8\ 10^{7}$	-
Number of A-NOVs at startup	$2.9\ 10^{10}$	$6.2\ 10^{4}$	$1.1\ 10^{8}$	$2.9\ 10^{10}$	$1.3\ 10^{9}$	-
Number of D-NOVs at startup	0	0	0	0	0	-
Cell concentration in growth vessel (C_0)	$1\ 10^{6}$	$9\ 10^{5}$	$9.5\ 10^{5}$	$1\ 10^{6}$	$8\ 10^{5}$	cm^{-3}
Time step (Δt)	2	2	2	2	1	h
Number of entry sites B	33	33	40	33	33	$cell^{-1}$
Number of virions produced at a correct infection	3,340	4,000	3,340	3,340	3,340	$cell^{-1}$
id., of which A-NOVs	95	97.5	94	95	94	%
id., of which D-NOVs	10^{-6}	10^{-6}	10^{-6}	10^{-6}	10^{-6}	%
Number of virions produced at a defective infection	1,600	1,600	1,600	2,000	1,600	$cell^{-1}$
id., of which A-NOVs	71	77	71	53	71	%
id., of which D-NOVs	25.5	22.9	25.5	46.95	25.5	%

Parameters in table 2 that were either measured or set by experimental conditions were the reactor working and start-up volume, the flow through the series, the number of I-NOVs at start-up, the cell concentration in the growth vessel, the number of I-NOVs produced per cell, and the time step. Fitted parameters were the ratio between I-NOVs and A-NOVs both at start-up and with virion production, the number of entry sites, the D-NOV production rate at correct infections, and the number and ratio of virions produced at a defective infection. The ratio of I-NOV to A-NOV was in the same range as discussed earlier.

Two examples of fits on the data of Kompier et al. (1988) and van Lier et al. (1990) are given in figures 9 and 10.

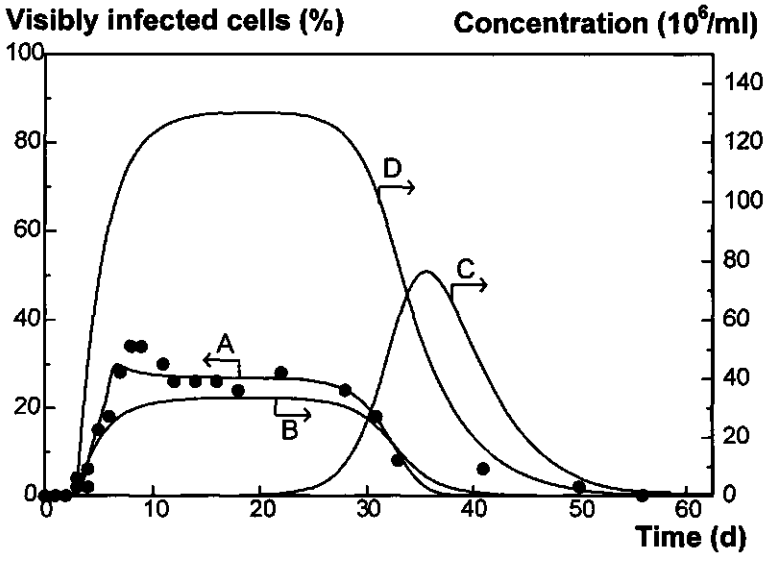


Figure 9. Fit of the model on the percentage of visibly infected cells in one continuously-operated infection reactor. Parameters are in table 2 under B. (●) = experimental data, Line A is the model description of the percentage of visibly *infected cells. Lines B and C are model descriptions of the I-NOV and D-NOV* concentration, respectively, and line D is the A-NOV concentration divided by 10.

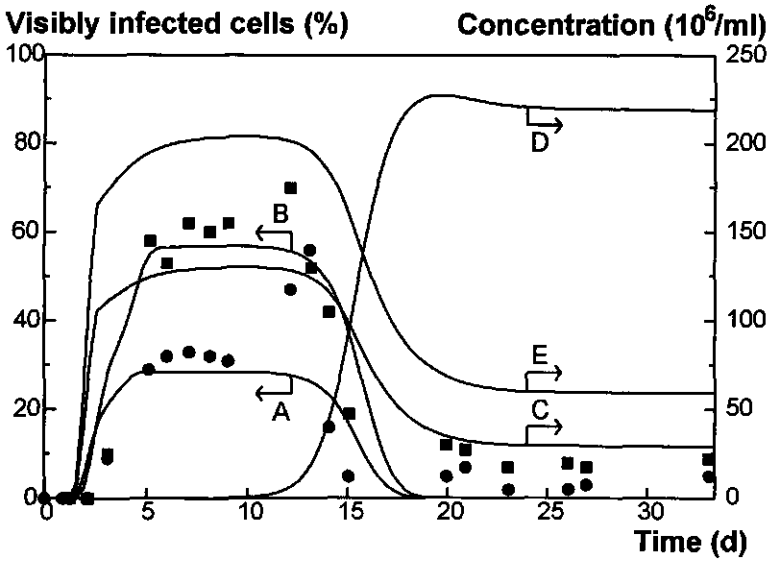


Figure 10. Fit of the model on the experimental (●, ■) percentage of visibly infected cells in the first (line A) and second (line B) reactor of a series of two continuously-operated infection reactors with the model. Lines C and D are I-NOV and D-NOV concentrations in the second vessel, respectively. Line E is the A-NOV concentration divided by 10. Parameters are in table 2 under C.

From these figures it can be seen that at the time at which the decrease in I-NOV concentration occurred, the number of D-NOVs increased. The model behaved this way with all experimental runs analyzed so far. Note that in figure 10, the concentration of virions does not decrease to zero. This was experimentally observed, and can be predicted by the model assuming that the cells that are either infected with I-NOV and D-NOV and/or A-NOV will still produce some I-NOVs.

Kool et al. (1990, 1991) showed with a recombinant virus that D-NOVs are generated during continuous runs at each point in time, with a clear increase at the end of the run. Apparently, after a period of low occurrence, D-NOVs are produced in large amounts due to their faster rate of synthesis. After the

exponential increase in the amount of D-NOVs, there are too few correct infections to produce enough I-NOVs to support D-NOV reproduction (helper function), and the infection process ceases. This seems a valid explanation for the passage effect.

As reported by Von Magnus (1959), prolonging the serial passages will eventually result in an increase in the number of I-NOVs again. Since D-NOVs need I-NOV to be reproduced, the number of D-NOVs will drop when there is too little I-NOV. After this phase, at low multiplicities of infection of the three virion types the chance increases that correct infections will occur, thereby starting the I-NOV production again. In a continuous culture high passages occur soon, and eventually D-NOV production will decrease and subsequently the D-NOVs will be washed out of the reactor. Then, a low multiplicity of infection is reached and

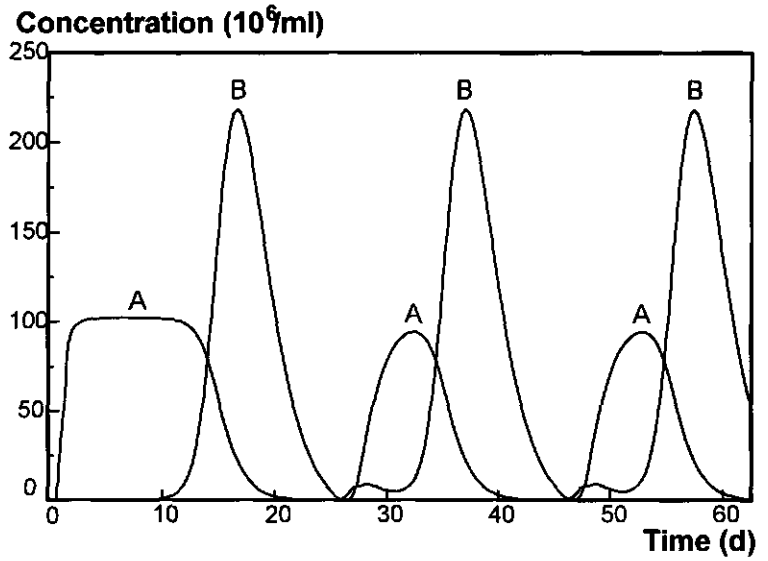


Figure 11. The von Magnus effect in a continuously-operated infection vessel as simulated by the model. The concentration of I-NOV (lines A) and D-NOV (lines B) versus time. Parameters are in table 2 under D.

if not all I-NOVs are washed out, I-NOV reproduction starts again since the chance that insect cells become correctly infected increases in that situation. This reactor behaviour could also be simulated with the model, as illustrated in figure 11. This phenomenon has been observed in our bioreactor system (Van Lier et al. 1992).

Sensitivity analysis

In order to assess the significance of each parameter a sensitivity analysis was made. Parameter values were varied along the settings as under D in table 2. The influence of parameter variations on the pseudo-steady-state infection level

Table 3. Sensitivity analysis of the parameters. *dL/dP* and $dt_{1/2}/dP$ are the effects of varying the parameter value on the pseudo-steady-state infection level (*L*) and on the time at which the infectionlevel is half the pseudo-steady-state level ($t_{1/2}$), respectively. The first and second sign denote the effect of a respectively smaller and larger parameter (*P*) value than given under D in table 2, under *ceteris paribus* conditions. + = *d/dP* is positive, o = there is hardly any influence, - = *d/dP* is negative.

PARAMETER	*dL/dP*	$dt_{1/2}/dP$
Number of virions produced at a correct infection	++	++
Number of virions produced at a defective infection	OO	-O
Cell concentration	++	OO
Number of entry sites	++	-O
Number of I-NOVs at startup	OO	OO
Number of A-NOVs at startup	OO	OO
Number of D-NOVs at startup	O-	O-
Time lapse between infection and I-NOV/A-NOV release	OO	--
Time lapse between infection and D-NOV release	OO	++
Fraction of A-NOVs produced at a correct infection	O-	O+
Fraction of D-NOVs produced at a correct infection	+O	+O
Fraction of A-NOVs produced at a defective infection	OO	O+
Fraction of D-NOVs produced at a defective infection	OO	OO

L and on the time ($t_{1/2}$) at which the infection level is only half the pseudo-steady-state level were investigated. Results are given in table 3.

The sensitivity analysis presented in table 3 shows some interesting features. The time lap between infection and D-NOV release obviously only influences $t_{1/2}$. If this time lap is smaller the D-NOVs will occur sooner, and hence $t_{1/2}$ will decrease. The influence of the time lap between infection and I-NOV/A-NOV release is much less pronounced. As the I-NOV/A-NOV ratio is not altered, the L will not be changed, and with a decreasing time lap I-NOVs and A-NOVs are produced sooner, and therefore D-NOVs have a smaller chance to infect cells. In that case the $t_{1/2}$ will occur later. The parameters that describe the composition of the virion mixture at startup are hard to determine but have no influence on $t_{1/2}$ and L. This can be explained by the large amount of virions that will be produced in a continuous culture shortly after startup (Figures 9 and 10). This also explains the greater importance of the amounts of the three virion types produced at a correct, and to a lesser extent, defective infection. These amounts control the numbers of virions produced in pseudo-steady state, and with that both L and $t_{1/2}$. A higher number of entry sites per cell will lead to more virions infecting cells. At the pseudo-steady-state level of infection the majority of the infections will consist of correct and abortive infections. The NOV mixture then consists of mainly A-NOV with little I-NOV (figures 9 and 10), and if more virions can infect cells the chance that an I-NOV infects a cell increases. With that, an increase in the fraction of correctly infected cells can be expected.

Suggested mode of operation

Figures 9 and 10 showed that a low concentration of D-NOVs is not detrimental to the infection process. This observation provides a key to a promising strategy for reactor operation, especially for semi-continuous cultures (Klöppinger et al. 1990). Figure 12 depicts a simulation of a repeated batch. Cells are grown in a fed-batch-operated growth vessel, and the infection reactor is operated batchwise. After each cycle, the volume of the infection reactor was lowered to 3%. The virions remaining in this volume will start the infection of new cells that are then pumped into the infection vessel from the cell growth vessel.

From this figure it is clear that production of I-NOVs in this system is maintained significantly longer than in a continuous culture. This can easily be explained by the repeated dilution of the D-NOVs. With that, a very low

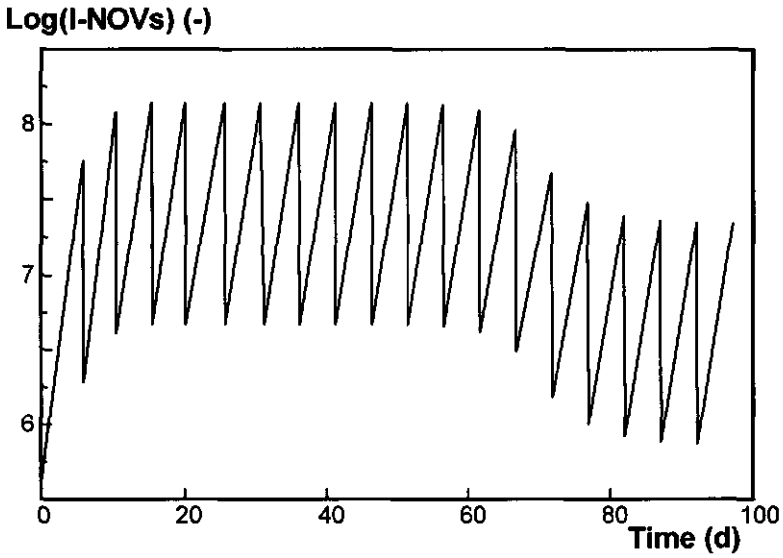


Figure 12. Simulation of a repeated batch infection process. The concentration of I-NOV versus time. Parameters as in table 2 under E.

multiplicity of infection will occur, and from that, the chance that cells are infected with both D-NOV and I-NOV will be very low. Hence, I-NOV will be produced before D-NOVs are available in significant quantities, and the infection process will continue for a longer period of time.

In a repeated fed-batch mode of operation of the infection vessel, however, insect cells are continuously grown and pumped into the infection vessel in a fed-batch mode until the reactor is completely filled. Thereafter the medium volume of the infection reactor is decreased to 3%. The remaining cells and virions start the infection process again in the next run. In a repeated fed batch the repeated dilution will be less significant, as more passages occur during the run. The run time must therefore approach a fully continuous culture, which indeed can be simulated by the model. These alternative modes of operation of reactor configurations are now experimentally tested to validate the model.

CONCLUSIONS

In the case of a continuously-operated reactor configuration, an increased number of infection reactors with the same total residence time resulted in an increased pseudo-steady-state level of infection, but a decreased run time. The observed decrease in infection level in continuously-operated series of reactors may be attributed to the passage effect. With the concept of the defective interfering virion and the non-infectious non-occluded virion, experimental data from the bioreactors can be described well with the proposed dynamic model, thereby giving an accurate description of the passage effect. Our current research is focused on the determination of the actual numbers of the three types of NOVs occurring in the continuously-operated reactor series, in order to validate the model presented here. Furthermore, an experimental confirmation

of the suggested repeated batch operation of the infection vessel in the reactor series is under way.

ACKNOWLEDGEMENTS

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NOMENCLATURE

a	production constant formed by the number of virions produced per cell, multiplied by the number of cells flowing into the reactor per unit of time	h^{-1}
B	number of entry sites on a cell	-
C	number of non-infected insect cells	-
D	dilution rate	h^{-1}
f	fraction of virions	-
F	fraction of cells with a certain infection type	-
G	number of cells that are infected	-
I	rate of virion use for infection purposes	h^{-1}
k_g	first-order cell growth constant	h^{-1}
P	propability that an entry site is infected	-
p_{max}	maximum number of passages	-
R	rate of virion release by the cells	h^{-1}
$r_{x,in}$	*ingoing flow of x*	h^{-1}

$r_{x,out}$	outgoing flow of x	h^{-1}
$r_{x,prod}$	production rate of x	h^{-1}
$r_{x,use}$	consumption rate of x	h^{-1}
t	time	h
$t_{1/2}$	time at which the infection level in a continuous culture is half the pseudo-steady-state level	h
v	amount of virions	-
x	number of cells or virions	-
Δt	time step	h
α	the number of entry sites per cell that are not occupied by any virion	-
β	the number of entry sites per cell that is occupied by A-NOVs	-
λ	the number of entry sites per cell that is occupied by D-NOVs	-
κ	the number of entry sites per cell that is occupied by I-NOVs	-

Subscripts

d	defective interfering particle (D-NOV)
i	infectious non-occluded virus (I-NOV)
m	reactor number in the series
a	NOV leading to an abortive infection (A-NOV)
o	not infected
p	passage number
tot	total

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CHAPTER 8

LONG-TERM SEMI-CONTINUOUS PRODUCTION OF RECOMBINANT BACULOVIRUS PROTEIN IN A REPEATED (FED-)BATCH TWO-STAGE REACTOR SYSTEM

SUMMARY

The baculovirus expression system is commonly used in the research and development area and in the production of diagnostics and vaccines. Because the infection of insect-cell cultures with a (recombinant) baculovirus is a lytic process the running time of an infected batch insect-cell reactor is limited. Another disadvantage of the system is the instability of the virus. In this study a two-stage reactor system was tested for its suitability for long-term semi-continuous operation. Three experimental set-ups were tested involving repeated infections in a reactor fed with cell suspension from a separate cell-growth reactor. As virus inoculum part of a previous infection was used. Best

performance with respect to long-term operation was obtained with a repeated batch system. Twelve consecutive productive runs, consisting of infections during five days, could be performed. The titers of infectious extra-cellular virus could be described well with an infection model previously developed in our laboratory.

INTRODUCTION

The baculovirus expression vector system has established itself as a powerful tool in the expression of foreign genes. The system is mainly used in research and development and for production of diagnostics and vaccines (Vlak 1990). The first steps towards clinical application have already been taken (Glaser 1993). A major disadvantage of the system, however, is the lytic character of the infection process which limits the production time of a batch process.

A continuous production process in a two-stage reactor system is possible by exploiting the typical bi-phasic infection cycle of the virus. During the first phase infected cells produce non-occluded viruses (NOVs) which are released into the medium and which are capable of infecting other insect cells. When infected with wild-type virus, the infected cells will produce virus particles packed in polyhedra during the second phase of the infection process. In a baculovirus expression vector usually the gene coding for the major late protein (polyhedrin) is replaced by a gene of choice and hence the infected cell will produce the desired protein during the second phase of virus infection (O' Reilly et al. 1992, King & Possee 1992). An alternative site for insertion is the locus of the p10 gene which has, like the polyhedrin gene, a strong promoter and which is also highly expressed during the second phase of infection (Vlak et al. 1990).

For a continuous production process insect-cell culturing and infection of cells have to be physically separated. This can be achieved by using a reactor system consisting of two continuously operated reactors in series. In the first reactor the insect cells are cultured and the effluent is used as influent for the second reactor where the cells are infected. In such a reactor system wild-type *Autographa californica* nuclear polyhedrosis virus (AcNPV) (Kompier et al. 1988) and recombinant β-galactosidase (Van Lier et al. 1992) have been successfully produced. In both studies, however, after four weeks of more or less stable production the productivity rapidly decreased. This decrease in production has been ascribed to interference of defective virus particles with intact virus (Kool et al. 1991).

To make the continuous system suitable for large-scale production the level of defective NOVs (D-NOVs) should be held low. This implies that a low multiplicity of infection (m.o.i.; number of infectious viruses available per cell) should be maintained in the infection vessel. This can not be achieved in a continuous reactor system since NOV concentration gradually builds up (about 200 NOVs are produced per cell). A batch infection has the disadvantage of long down times as a result of cleaning and sterilization of the reactor after each run. With a semi-continuous system based on repeated (fed-)batch infections the m.o.i. can be better controlled. By using a two-reactor setup the down time can be minimized (Van Lier et al. 1991, De Gooijer et al. 1992, Zhang et al. 1993).

Hink and Strauss (Hink & Strauss 1980, Hink 1982) described a production system where *Trichoplusia ni* Tn368 cells were cultured in a stirred reactor. AcNPV was propagated in a series of four spinner flasks. Every 24 hours the content from each infection vessel was pumped to the next infection vessel and the first infection vessel was filled from the cell-growth reactor and inoculated with virus. Production of polyhedra stayed constant during four runs and then

declined. This decline was probably due to lack of nutrients or accumulation of waste metabolites since medium was re-used. Klöppinger et al. (1990) reported on semi-continuous production of polyhedra in a reactor system consisting of two bioreactors. Part of the cultured cells from the first reactor was pumped into a second reactor for infection, the remaining cells were used as inoculum for a new fed-batch culture of cells. When the virus propagation reactor was harvested, part of the contents stayed in the reactor to infect the next cell batch. Klöppinger et al. (1990) observed "a constant production for several weeks". In a third study on repeated batch production, *Bombyx mori* Bm5 cells were infected with a recombinant BmNPV expressing bacterial chloramphenicol acetyltransferase (Zhang et al. 1993). In that study, four consecutive runs were performed and production of the two-stage bioreactor system was constant for 27 days.

Especially for clinical applications it is important to get insight in the stability of the baculovirus expression vector. In our laboratory a model was developed describing the infection process in insect-cell reactor configurations (De Gooijer et al. 1992). It is assumed that each insect cell has a limited number of so-called "entry sites" where virus can enter the cell. Furthermore, three types of viruses are taken into account: infectious virus (I-NOV), defective virus (D-NOVS) and abortive virus (A-NOV). Most virus belongs to this last group that uses an entry-site but is incapable of a permissive infection. With this model continuous reactor runs could be well described. This model can also be used to describe a series of repeated batch runs. De Gooijer et al. (1992) did a model simulation for a system similar to the one described by Klöppinger et al. (1990) and Zhang et al. (1993). The model predicted that after about 12 consecutive batch runs the I-NOV concentration would decrease as a result of defective interference of D-NOVs.

In the present study it was investigated how many consecutive batch infections could be done before the production declined as a result of defective virus build up. Beside the repeated batch infection experiment, two other experiments were performed where infection was conducted in a fed-batch mode. The experimental data were used to evaluate the model of De Gooijer et al. (1992) in predicting the productivity decline.

MATERIALS AND METHODS

Cells, virus, medium

The IPLB-Sf-21 cell line isolated from *Spodoptera frugiperda* by Vaughn et al. (1977) was used. The cells were grown in TNM-FH medium (Hink 1970) supplemented with 10% fetal calf serum. Cells were maintained in 25 cm^2 tissue culture flasks (Greiner). For growth in suspension methyl cellulose with a final concentration of 0.1% (w/v) was added to the medium. The recombinant baculovirus was an *Autographa californica* multiple-capsid nuclear polyhedrosis virus (AcMNPV) containing the lacZ gene of *Escherichia coli* instead of the polyhedrin gene (Summers & Smith 1987).

Bioreactor configuration

The bioreactor system consisted of two 1 dm^3 round-bottomed fermentors or two 2 dm^3 flat-bottomed fermentors (Applikon) in series. The reactors were equipped with marine impellers and maintained at 28 °C with an internal heating system connected to a waterbath with forced cooling. Air was introduced with a flow of

10 $dm^3.h^{-1}$ in the head space of the reactors through a 0.2 μm filter. Liquid was transported via silicone tubing by peristaltic pumps (Watson-Marlow). Infection of the cell-growth reactor by virus from the infection reactor was prevented with a drop former.

The first experiment consisted of a series of batch infections. The reactors were operated as shown in figure 1A. The batch time was five days. To inoculate a batch of cells, 20 cm^3 suspension of the previous run was left in the reactor.

In the second experiment (Figure 1B) the infection reactor was filled with 0.3 dm^3 cell suspension. During two days cell suspension was added continuously to the infection reactor. Additionally, upon reaching the final working volume the reactor was operated batch-wise during 1 day. To inoculate a new infection cycle 20 cm^3 of suspension was left in the reactor. The one day batch period served two purposes: it prolonged the residence time of cells in the infection reactor and it allowed for a cell suspension build-up in the cell-growth reactor needed for starting the next infection.

The third experiment was performed analogous to the second experiment except that here a three day fed-batch period was used and that the reactor volumes differed (Figure 1C).

During all operation schemes the cell-growth reactor was fed continuously with medium. During the first experiment the effluent of the cell-growth reactor was bleeded when the working volume was reached. The operation of the bioreactor systems was such that the residence time of the cells in the cell-growth reactors were similar.

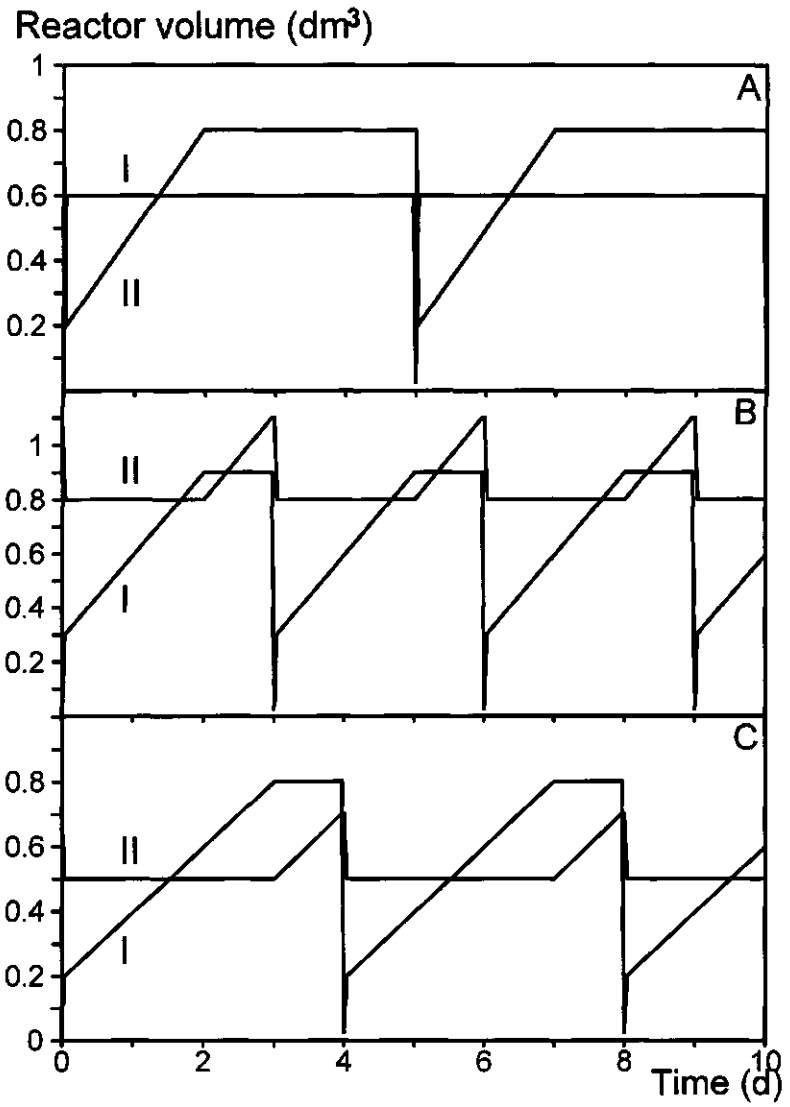

Figure 1. Reactor volumes during the infection processes. I : infection reactor, II : cell-growth reactor. A : 5-day batch infections, B : 2 day fed-batch / 1 day batch infections, C : 3 day fed-batch / 1 day batch infections.

Hence physiological differences of the cells before infection between the three experiments were eliminated as much as possible.

Bioreactor samples

Samples from the bioreactor were forced into the sample flasks by pressurizing the reactor. The sample system was cleaned by blowing sterile air through the system. Samples were taken daily. The sample volumes were about 1 cm^3 for the cell-growth reactor and about 5 cm^3 for the infection reactor. In both samples the cell number was determined.

Four cm^3 of the samples from the infection reactor were centrifuged (1500g for 5 min). The cell pellet was resuspended in 4 cm^3 phosphate buffered saline (PBS) and centrifuged again. The pellet was finally resuspended in 1 cm^3 PBS. Both the cell suspension and the supernatant from the washing step were stored at -20 °C. The supernatant from the first centrifuge step was filtered through a 0.45 μm filter and split into two aliquots of which one was stored at 4 °C for virus titer determination and the other at -20 °C for a β-galactosidase assay.

Cell counts

Cells were counted in a bright-line Neubauer haemocytometer. To distinguish between viable and non-viable cells the trypan blue exclusion method was used.

β-galactosidase assay

The cell pellet stored at -20 °C was lysed by sonification (three cycles of 30 s with 30 s intervals). β-galactosidase activity was determined in the lysed pellet and both supernatant fractions stored at -20 °C.

The activity of β-galactosidase was measured by following the hydrolysis of 2-nitrophenyl-galactopyranoside (ONPG) spectrophotometrically at 420 nm. The assay was executed at 28 °C. The activity of β-galactosidase was expressed as mol ONPG cleaved per minute. An extinction coefficient of 4.5 10^3 $M^{-1}.m^{-1}$ was used.

Virus titer

The titer of infectious virus was determined in the supernatant fraction stored at 4 °C using an end point dilution method as described by Vlak (1979). To screen for wells containing infected cells 10 mm^3 of a 0.4 $g.dm^{-3}$ solution of 5-bromo-4-chloro-3-indolyl-β-galactopyranoside (X-gal) was added to each well.

RESULTS AND DISCUSSION

Viable-cell concentration in both the cell-growth and infection reactor at the start of each infection run was relatively constant during the repeated batch infection experiment (Figure 2A). Since the residence time of the cells was similar in all three reactor configurations, similar cell densities could be expected at the beginning of each infection. However, cell densities differed from about 0.8 10^6 $cells.cm^{-3}$ (batch system) to about 1.6 10^6 $cells.cm^{-3}$ (3 day fed-batch).

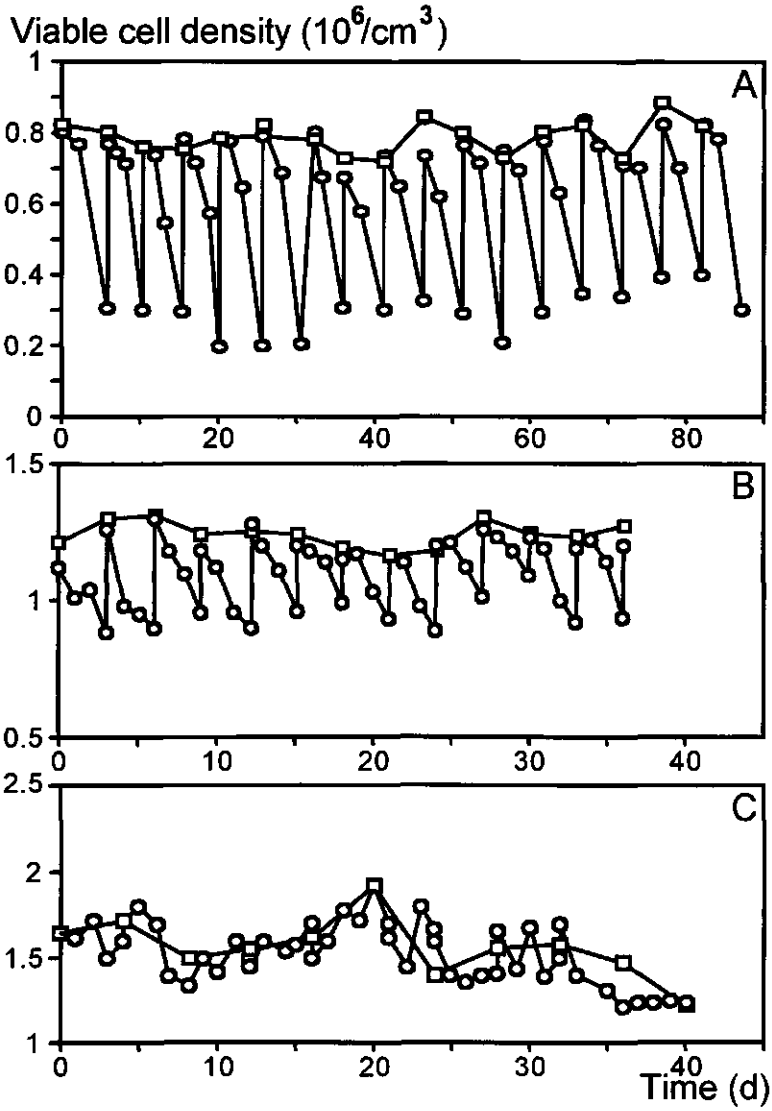

Figure 2. Viable cell density versus time. (□) : cell-growth reactor, (○) : infection reactor. A : 5-day batch infections, B : 2 day fed-batch / 1 day batch infections, C : 3 day fed-batch / 1 day batch infections.

During each infection the viable-cell concentration in the infection reactor decreased. Viable-cell concentrations during the two-day repeated fed-batch experiment showed a similar behaviour (Figure 2B). During the three-day repeated fed-batch experiment (Figure 1C), however, viable-cell density at the start of each infection run was not constant and the viable-cell density in the infection reactor did not decrease as distinctly as in the other two experiments (Figure 2C).

During twelve consecutive infections in the infection reactor of the batch operated system (Figure 1A) β-galactosidase production was reproducible and averaged 22 $U.cm^{-3}$ (Figure 3A). After the twelfth run production of β-galactosidase decreased and stayed at about 3 $U.cm^{-3}$. A total of 21 consecutive runs was performed (not all data shown). Production of β-galactosidase during the two-day fed-batch operated infection experiment (Figure 1B) declined after the fifth consecutive infection (Figure 3B). During the first five infections the β-galactosidase production was about 14 $U.cm^{-3}$, comparable to the β-galactosidase activity in the batch operated system three days after start of infection. The β-galactosidase activity in the infection reactor operated with the three-day fed-batch regime (Figure 1C) was considerably higher than in both other experiments. Here, production was on average 35 $U.cm^{-3}$ during the first seven runs with exception of the first infection (Figure 3C).

The production time of the repeated batch configuration is by far the longest. This can be expected because batch operation diminishes the build-up of D-NOVs compared to fed-batch operated systems. In a fed-batch mode of infection more virus passages can occur due to the continuous supply of non-infected cells. The fed-batch infections, however, offer a tool for model evaluation. It should be noted that long-term operation is not per se an

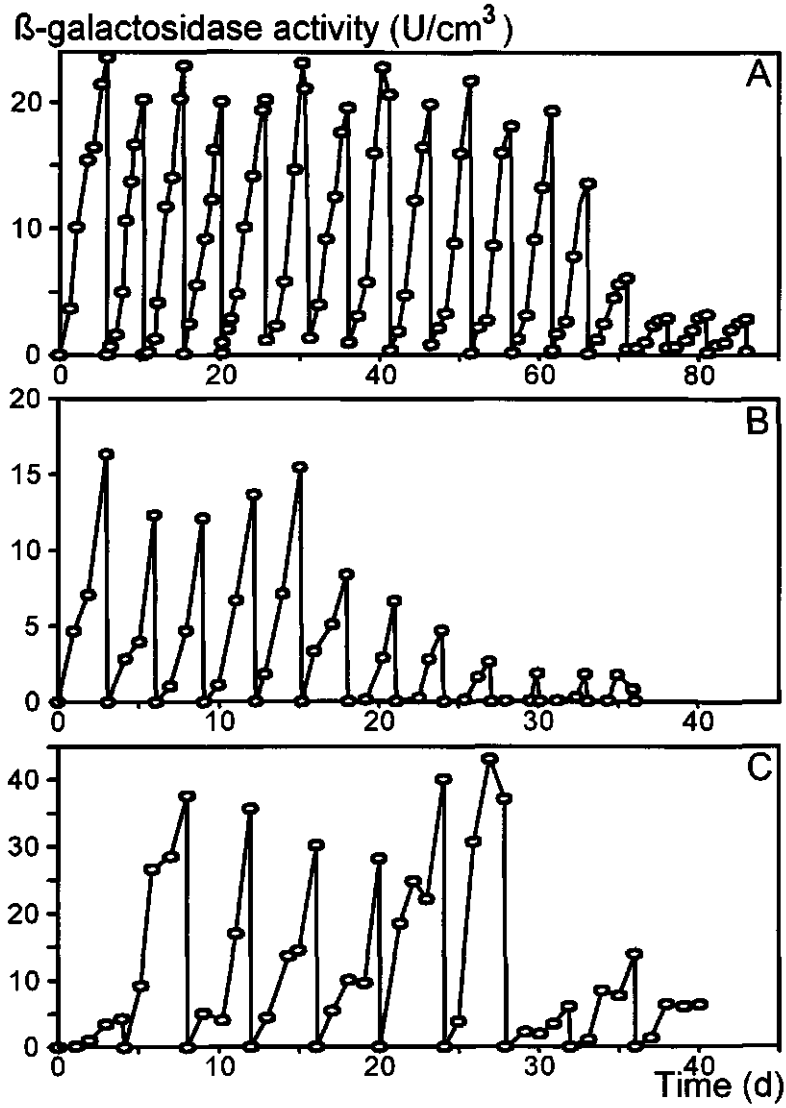

Figure 3. β-galactosidase activity versus time. A : 5-day batch infections, B : 2 day fed-batch / 1 day batch infections, C : 3 day fed-batch / 1 day batch infections.

optimization criterion. Practical reasons (maintenance, working hours) or regulatory affairs may limit the running time of a cultivation.

The lacZ gene of *E. coli* is often used as a model gene for measuring baculovirus-mediated recombinant-protein expression. Caution must be taken, however, when data obtained with lacZ-AcMNPV vectors are compared. Differences in levels of β-galactosidase expression can occur as a result of differences in cell density (resulting in different metabolic state of cell suspensions), differences in baculovirus vectors and differences in cell lines used (Wickham et al. 1992). Also differences in m.o.i. can influence β-galactosidase production (Licari & Bailey 1991, Neutra et al. 1992). Furthermore, the experimental determination of β-galactosidase activity (differences in temperature, extinction coefficient or pH) may introduce (seeming) discrepancies when different studies are compared.

Nevertheless, in table 1 production data obtained with the reactor configurations described in this paper are compared with data from the literature. These literature data are comparable with respect to the β-galactosidase activity assay conditions. In all cases the same extinction coefficient ($4.5\ 10^3\ M^{-1}.m^{-1}$) was used. The productivity of the repeated batch system is low as compared to the literature data on single batches (data are all from day 5 post infection). However, using the same virus, cells, medium and culture conditions values of about $20\ U.10^{-6}$ cells were found in our laboratory in batch cultures (Van Lier et al. 1992). Furthermore, since the bioreactors were only aerated through the head space, oxygen limitation could also be responsible for the lower yield. The productivities of the continuous system and the reactor configurations described in this study are in the same order of magnitude. The three-day fed-batch reactor system shows a somewhat higher productivity. This suggests that a fed-batch operated system can give rise to a

higher productivity. This is, however, unlikely since the average residence time of the cells in the infection reactor is less than in the batch operated infection reactor. The higher productivity can be attributed to the higher cell density.

Table 1. Comparison of several reactor systems used to produce baculovirus-expressed β-galactosidase. STR: stirred tank reactor, AR: airlift reactor. Literature: (1) Van Lier et al. 1992, (2) Neutra et al. 1992, (3) Murhammer & Goochee 1988, (4) King et al. 1992.

SYSTEM	β-GALACTOSIDASE ACTIVITY IN HARVEST ($U.cm^{-3}$)	HARVEST VOLUME PER DAY ($cm^3.d^{-1}$)	PRODUCTIVITY ($U.cm^{-3}.d^{-1}$)	TOTAL RUNNING TIME (d)
Continuous (STR) (1)	10	320	4	25
Batch (shake flask) (2)	200	8	40	5
Batch (AR) (3)	50	114	10	5
Batch (AR) (4)	64	1,560	13	5
Repeated batch	22	120	4.4	60
Repeated fed-batch / batch (2/1 d)	14	300	4.7	15
Repeated fed-batch / batch (3/1 d)	35	200	8.8	28

In all three experiments performed, the decrease in β-galactosidase production in the infection reactor coincided with a decrease in titer of infectious virus (Figure 4). This decrease is due to interference of defective rec-AcMNPV mutants which lack the ability to produce β-galactosidase (Kool et al. 1991) or compete for replication factors (Kool et al. 1993).

The kinetics of the mechanism of interference by defective AcMNPV was modeled by De Gooijer et al. (1992) for continuous systems. This model is capable of predicting the titer of NOVs and distinguishes between infectious NOVs (I-NOVs), defective NOVs (D-NOVs) and abortive NOVs (A-NOVs). For the repeated batch experiment as described in this study De Gooijer et al. (1992) calculated the virus titer profiles as shown in Figure 4A. The virus titer

profiles of both other experimental systems used in this study were calculated with the same system-independent parameters as used for the batch operated system (Table 2).

Table 2. Input parameters for the baculovirus infection model (De Gooijer et al. 1992).

VARIABLE	VALUE	UNIT
Number of D-NOVs at startup	0	-
Number of cell entry sites	33	$cell^{-1}$
Number of virions produced at a correct infection	3,340	$cell^{-1}$
of which A-NOVs	94	%
of which D-NOVs	10^{-6}	%
Number of virions produced at a defective infection	1,600	$cell^{-1}$
of which A-NOVs	71	%
of which D-NOVs	25.5	%

The calculated NOV titers are given in Figure 4B and C. In case of the two-day fed-batch infection the calculated I-NOV declined somewhat later than the measured titer. The model predicts the measured data well with respect to the repeated batch infection and the three-day fed-batch infection experiment. Especially the time when the I-NOV titer dropped is well predicted.

CONCLUSIONS

The use of repeated infections, each starting with an inoculum from the previous infection, increased the running time of a two-stage reactor system as compared to a continuously operated two-stage reactor system. There is, however, a limit to the number of consecutive infections that can be performed without significant loss in productivity. This number depends on the mode of operation of the

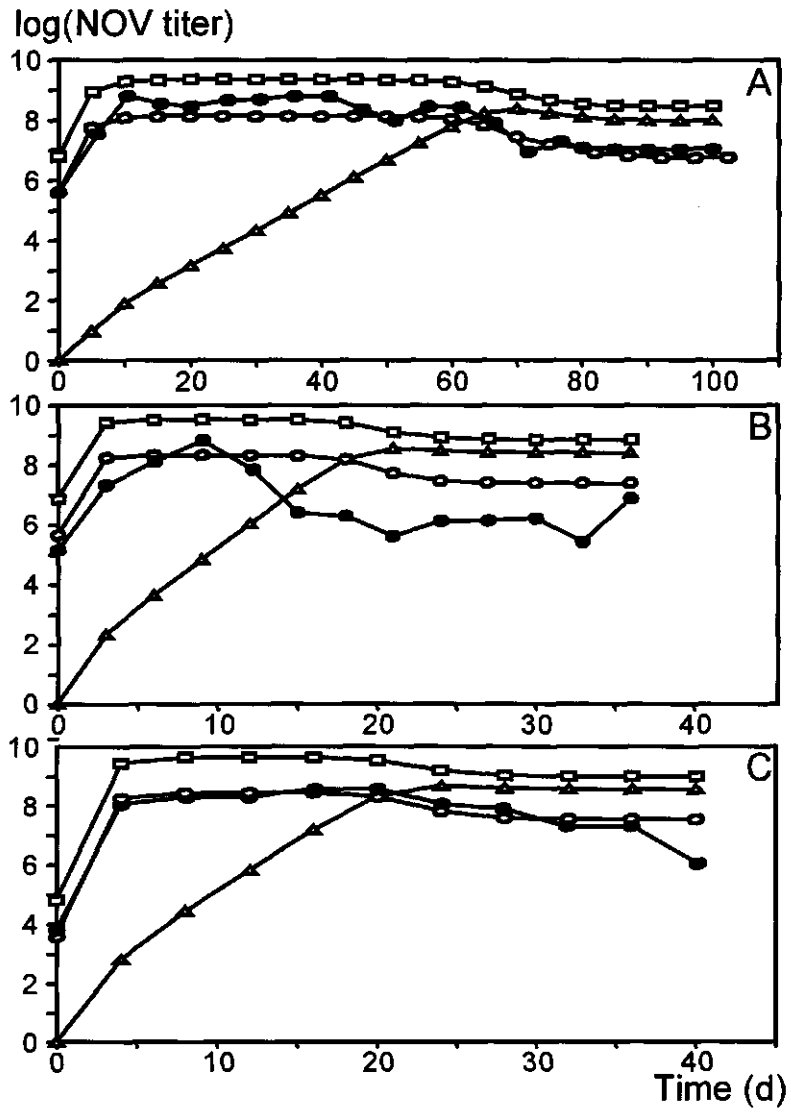

Figure 4. NOV titers. (●) : measured I-NOV titer, (○) : computed I-NOV titer, (□) : computed A-NOV titer, (Δ) : computed D-NOV titer. A : 5-day batch infections, B : 2 day fed-batch / 1 day batch infections, C : 3 day fed-batch / 1 day batch infections.

reactor system. From the three modes of operation tested, a scheme of consecutive 5 day batch infections performed best in terms of long-term operation. Repeated infections, where cells were supplied during three days to the infection reactors, followed by one day batch operation, performed best in terms of productivity.

With the model developed to describe the infection process (De Gooijer et al. 1992) the number of consecutive infections that can be performed before defective baculoviruses become predominant in the infection reactor can be predicted well.

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CHAPTER 9

SUCROSE CONVERSION BY IMMOBILIZED INVERTASE IN A MULTIPLE AIR-LIFT LOOP BIOREACTOR

ABSTRACT

A new bioreactor series within one vessel, the Multiple Air-lift Loop reactor (MAL), is introduced. In the MAL a series of air-lift loop reactors is incorporated into one vessel. From residence time distribution studies it was shown that the three-compartment MAL behaves like a series of three ideal mixers. A continuously operated MAL, containing immobilized invertase as model biocatalyst, was evaluated. The advantage of approaching plug flow by using a bioreactor cascade could be shown by comparing substrate conversion in the three-compartment MAL to that in a single vessel at the same overall dilution rate. This was done for two sets of experimental conditions, which were chosen by using a previously developed model. Intrinsic kinetic parameters of the

immobilized enzyme, needed for the model calculations, were determined experimentally. Model calculations gave good approximations of the results. The model incorporates external mass-transfer resistance and diffusion and reaction in the biocatalyst beads.

INTRODUCTION

Cascades of continuously operated stirred-tank reactors can be flexible tools for the optimization of bioprocesses (Pirt, 1975, Luyben & Tramper, 1982, Shimizu & Matsubara, 1987, Hill & Robinson, 1989, Malcata & Cameron, 1992). The Multiple Air-lift Loop reactor (MAL) is a new type of bioreactor, consisting of a series of air-lift loop reactors (ALRs) within one vessel (De Gooijer, 1989). With a series of ideal mixers the behavior of a plug-flow reactor can be approximated (Levenspiel, 1972). On laboratory scale (V = 0.01 m^3), and with a regular aspect ratio (H/D = 13), ALRs behave like nearly ideally mixed vessels (Chisti, 1989, Van 't Riet & Tramper, 1991). Therefore, as a whole, the MAL is an approximation of an aerated plug-flow bioreactor when sufficient ALRs (N > 20) are placed in series. Thus the advantages of both reactor types are combined in this reactor series: the possibility of improved substrate conversion in the plug-flow approximation, and suitable conditions for measurement and control in the nearly ideal mixers. The MAL can be used to study applications of reactor series in biotechnology.

In 1982, Luyben & Tramper derived an analytical expression for the optimal design of a cascade of continuous stirred-tank reactors with the minimal overall reactor volume required for a specific substrate conversion as criterion. They limited their design to biocatalytic reactions with suspended enzymes following

Michaelis-Menten kinetics. This expression was extended to immobilized enzymes that obey intrinsic Michaelis-Menten kinetics, and validated experimentally by De Gooijer et al. (1989) for reactors in series. Further studies on reactor series containing suspended enzymes were made by Malcata (1988, Malcata & Cameron, 1992) for different kinetics, taking minimal capital investment as optimization criterion. The above authors (Luyben & Tramper, 1982, Malcata, 1988, De Gooijer et al., 1989) observed that the decrease in required overall reactor volume for a desired conversion is the largest when going from one to two reactors in series. Only slight improvement is found when going from 2 to 3, and even more reactors in the cascade is economically not feasible (Malcata, 1988). From these observations it was chosen to use a MAL with three reactors in series incorporated in it for this study.

Sucrose conversion by invertase was selected as a convenient and cheap biological model system to compare the performance of the MAL to that of a single vessel of the same volume. Obviously there can be all kinds of other arguments, like economical motives (not taken into account here), to select a different model system or to prefer the use of a single vessel.

In the present contribution, a MAL was operated continuously with invertase immobilized in gel beads as a biological model system. The gel beads were moving freely and kept fluidized and well mixed in each MAL compartment. Substrate conversion in a three-compartment MAL was compared to that in a single vessel at the same overall dilution rate. This experimental comparison was not made by De Gooijer et al. (1989). Statistically significant improvement (based on the difference in steady-state concentrations with no overlay of their 95% confidence intervals) of substrate conversion in the new bioreactor series over a single, nearly ideally mixed, vessel could be demonstrated. The theoretical development and the calculation procedures of De Gooijer et al.

(1989) were used as a tool to choose the experimental conditions, using intrinsic kinetic parameters that were determined experimentally. With this model the simultaneous diffusion and consumption of substrate in the biocatalyst bead is described, resulting in an estimation of the substrate concentration profile in the bead. The trend of the model estimations agreed with reality.

The assumption that the MAL behaves like a cascade of three nearly ideal mixers was validated by residence time distribution (RTD) measurements. The RTD of a tracer was determined in the effluent for various influent and gas flow rates. In all cases the MAL could be described best as three mixers in series.

The Multiple Air-lift Loop reactor

The central MAL-compartment is a conventional internal-loop ALR with aeration in the annulus (Figure 1). Subsequent compartments in the MAL are concentric. The annular-shaped compartments have a circular baffle which splits them in a riser and downcomer section.

Fresh medium is supplied to the central ALR; from there it flows into the downcomer of the next compartment. There it is mixed with the down-flowing stream. In this way medium travels through the cascade of ALRs. The MAL can be constructed in many configurations for various applications (De Gooijer, 1989). By supplying different gasses to the subsequent compartments for example, aerobic and anaerobic processes can be carried out in series within one reactor vessel.

Advantages of the MAL compared to single ALRs, or bubble columns, in series are that no extra pumps or hoses are needed. Also the reactor series can be sterilized as one reactor, and old reactor vessels can be reused and upgraded to a MAL. Disadvantages of the MAL in this comparison are that, on a

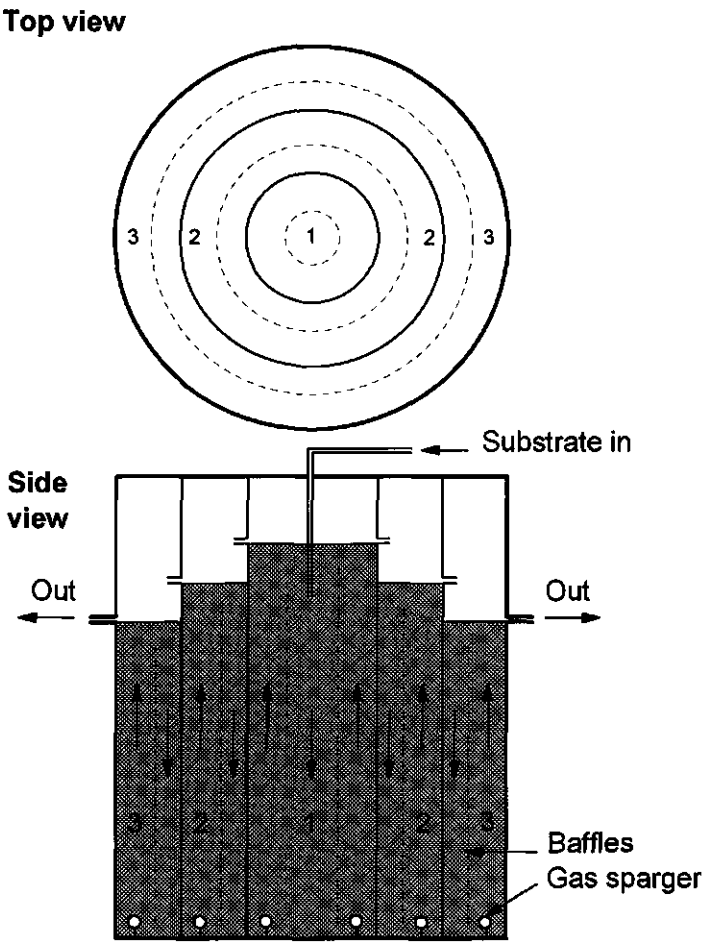

Figure 1. Three-compartment multiple air-lift loop reactor cross-sectional side and top view. Geometric data are given in table I.

lab-scale, the compartments are narrow which makes cleaning difficult. Further, the gas distributor is relatively complex.

Hydrodynamics and mixing are greatly influenced by reactor geometry, these subjects were investigated previously (Bakker et al. 1993) in a MAL of a

Table I. Geometric data of the multiple air-lift loop reactor (MAL).
[a] The working volume is the liquid volume corrected for gas holdup and foam formation during the experiments. [b] Outer diameters are given. Perspex wall thickness was 5 mm, except for the two tubes marked with (4), where it was 4 mm. [c] The distance between the baffles and the bottom (1.5 cm) is included in the baffle height.

	MAL COMPARTMENT 1	MAL COMPARTMENT 2	MAL COMPARTMENT 3
Liquid volume (10^{-3} m^3)	8.07	7.29	6.89
Working volume[a] (10^{-3} m^3)	7.71	6.97	6.57
Liquid height (cm)	29.7	23.7	16.5
Diameter[b] (cm)	19.9 (4)	30	40 (4)
Baffle diameter[b] (cm)	11	24	34
Baffle height[c] (cm)	29.1	23.1	15.5

larger scale (0.034 m^3 per compartment) than the MAL used in the present study. In that previous study, the second compartment of a MAL was used as a model for the new internal-loop reactor geometry. Liquid velocities, gas holdup and mixing were comparable with those of conventional ALRs with an internal loop. Complete mixing was established within four liquid circulations (τ_m < 54 s) for all gas flow rates applied. This implied that, also on this larger scale, the subsequent MAL compartments could be regarded as nearly ideally mixed.

MATERIALS AND METHODS

MAL

The vessel used in this study was a 0.022 m^3 three-compartment MAL made of transparent perspex (Figure 1). Geometric data are given in table 1. The three compartments were arbitrarily chosen to be of nearly equal volumes. The circular sparger rings were constructed of porous Accurel polypropylene membrane tube

(type V8/2, maximum pore size: 0.51 µm, a kind gift of AKZO, Obernburg, Germany). The spargers were positioned at the riser entrance to prevent entrainment of gas bubbles in the downcomer. Gas was distributed over the circular risers by the membrane spargers at a flowrate of 9.7 10^{-6} $m^3.s^{-1}$ for each MAL-compartment. The gas flow rate was kept low to prevent excessive foam formation.

Enzyme immobilization

Microgranular anion exchanger (DE32-cellulose), sodium alginate, and other chemicals were described previously (De Gooijer et al. 1989). The invertase (Maxinvert P) was a kind gift of Gist-brocades, Delft, The Netherlands. Invertase was coupled to DE32-cellulose before immobilization in alginate (De Gooijer et al. 1989). DE32-cellulose (50 $kg.m^{-3}$) was equilibrated for 48 h in sodium-phosphate buffer (10 $mol.m^{-3}$, pH 7.0). Invertase was added (5 $kg.m^{-3}$) and stirred with the DE-32 particles for 3 h at room temperature. Non-adsorbed invertase was then removed by washing three times with the equilibrating sodium-phosphate buffer and finally once with a sodium-acetate buffer (10 $mol.m^{-3}$, pH 4.6). The latter wash liquid did not show enzymatic activity anymore. The DE32-cellulose invertase then was added to a 2% (w/w) sodium-alginate solution in acetate buffer. In this way a 35 $kg.m^{-3}$ gel load, based on dry-complex weight, was obtained in the beads resulting from immobilization. This immobilization was performed with a resonance nozzle (Hulst et al. 1985) at 35 °C. Alginate drops were collected in 200 $mol.m^{-3}$ $CaCl_2$ at 5 °C. To obtain perfect spheres, decane was layered on the $CaCl_2$ solution (Wijffels et al. 1991). The decane-$CaCl_2$ was kept at 5 °C in order to initiate the gelation. After

solidification for 2 h, the beads were kept in a 50 mol.m^{-3} $CaCl_2$ acetate buffer at 4 °C. The Sauter mean bead diameter d_{32} was 1.74 mm.

For the determination of intrinsic kinetic parameters, beads were prepared, in the same way as described above, with five different gel loads (9, 33, 69, 83 and 100 kg of dry enzyme complex per cubic meter gel beads (kg.m^{-3})). To obtain those gel loads the amount of DE32-cellulose invertase added to the sodium-alginate solution in acetate buffer was changed. The beads were used directly for activity assays to determine apparent kinetic constants (i.e. including transport limitation).

Analyses

Glucose and sucrose concentrations were determined with a D-glucose kit and a sucrose kit, respectively (Boehringer, Germany). Sucrose concentrations were measured in the influent stream. Sucrose was found to be converted stoichiometrically into glucose and fructose. Reactor sucrose concentrations thus could be calculated from glucose analysis. Samples (1.5 cm^3) were heated for 5 minutes at 100 °C to inactivate the freely suspended enzyme-DE32-complex used for the determination of the kinetic parameters. Consequently this procedure was used for all samples to warrant uniform sample treatment. After heating, the samples were frozen at -20 °C until analysis to prevent microbial degradation of the sugars.

Activity assays

For all determinations of kinetic parameters (intrinsic and for freely suspended ezyme complex), substrate conversion was measured as a function of time until sucrose was completely converted. The initial sucrose concentration was 600 mol.m^{-3} and the batch-wise experiments were done in acetate buffer at 30 °C in a stirred vessel. For the determination of a loss in enzyme activity with time, beads (gel load: 100 kg.m^{-3}) were stored at 30 °C in acetate buffer between the determinations to obtain the same conditions as during the MAL-experiment. Parameters were estimated by fitting the integrated Michaelis-Menten equation to each set of experimental data with non-linear regression (Van 't Riet & Tramper 1991). The 95% confidence intervals of the kinetic parameters were calculated with the use of the Student t test value from one set of experimental data (i.e. substrate versus time) for the different measurements (Zwietering et al. 1990).

MAL experiments

The MAL was operated continuously twice at 30 °C for several days. The substrate was 630 mol.m^{-3} sucrose in acetate buffer. In the two MAL experiments the reactor gel holdup, 6.3% and 9.1% (v/v), respectively, was equal for each of the three MAL-compartments. Beads were kept in each compartment by a stainless steel sieve (hole diameter = 0.5 mm) at the overflows to the next compartment. After a change in substrate feed rate, steady-state conditions were established after four hydraulic residence times through the cascade of airlift reactors. Subsequently, during three MAL-volume changes, samples were taken regularly from all three MAL-compartments, and

thus the attainment of a steady-state was verified. Enzyme decay was assumed to be negligible during the short period (compared to the duration of the complete experiment) of a steady-state measurement. For the two MAL experiments at 6.3% and 9.1% gel holdup substrate was supplied at a constant rate of 2.65 10^{-6} and 2.4 10^{-6} $m^3.s^{-1}$, respectively.

The central compartment of the MAL was used as a single-vessel reference. To this end, it was operated at a different substrate feed rate (0.94 10^{-6} and 0.85 10^{-6} $m^3.s^{-1}$ for the two experiments at 6.3% and 9.1% gel holdup, respectively). Consequently, the overall residence time (2.2 and 2.5 h, respectively) in the comparison between the three-compartment MAL and the single compartment was equal. Here residence times were arbitrarily based on the liquid volume plus beads, that is without gas holdup and foam formation (Table I).

Mixing in the MAL

Macroscopic mixing in the MAL was investigated by measurement of the residence-time distribution (RTD). For that, the MAL was operated continuously under various conditions as explained in the Results and Discussion section. The effluent response of an inlet salt pulse (30 cm^3 4000 $mol.m^{-3}$ NaCl) was measured with a conductivity electrode.

The RTD-curves were characterized by the mean ($\bar{\theta}$) and variance (σ^2) of the distribution (Levenspiel, 1972). The distributions were normalized with respect to time (θ = time (s) / one hydraulic residence time (s)) and concentration (C = concentration ($kg.m^{-3}$) / initial concentration ($kg.m^{-3}$)) to make them comparable at different hydraulic residence times. The distribution curve was known at a number of discrete time values i from which the mean $\bar{\theta}$ and the

variance σ_θ^2 were calculated as described by Levenspiel (1972). From this the theorethical number of equal-size ideal mixers N in the series could be derived from $N = 1 / \sigma_\theta^2$. Finally, the area under the RTD-curve represented the fraction of tracer recovered.

The conditions during the MAL experiments with the immobilized enzyme as described above, were within the applied range with respect to the gas flow-rate, and lower for the liquid flow-rate.

Model parameter values

Parameter values used for the model calculations were: maximum substrate consumption rate V_m (at t = 0) = 0.10 mol.kg^{-1}.s^{-1}, Michaelis-constant K_m = 310 mol.m^{-3}, gel load = 35 kg.m^{-3}, effective diffusion coefficient (De Gooijer et al. 1989) D_e = 3.85 10^{-10} m^2.s^{-1}, Sauter mean bead diameter d_{32} = 1.74 mm, inlet substrate concentration = 630 mol.m^{-3}, substrate feed rate = 2.4 10^{-6} m^3.s^{-1} (MAL experiment) and 0.85 10^{-6} m^3.s^{-1} (single-vessel experiment), reactor gel holdup = 9.1%, liquid/solid mass-transfer coefficient $k_{l,s}$ = 1 10^{-5} m.s^{-1}, zero-order decay rate k_d = 4.0 10^{-8} mol.kg^{-1}.s^{-2}. The first steady-state measurement was done after running the MAL continuously for 3.5 days (t = 3.5 d), therefore this time was used to make a correction for the ezyme decay in the model calculations.

RESULTS AND DISCUSSION

Kinetic parameters

Alginate beads with five different gel loads of enzyme complex (DE32-cellulose-invertase) immobilized therein were used to determine intrinsic kinetic parameters of the Michaelis-Menten equation:

$$r(s) = \frac{V'_m S}{K'_m + S} = \eta \frac{V_m S}{K_m + S} \qquad [1]$$

where $r(s)$ is the specific reaction rate (mol.kg^{-1}.s^{-1}), S the substrate concentration (mol.m^{-3}), V'_m and V_m the apparent and intrinsic maximum

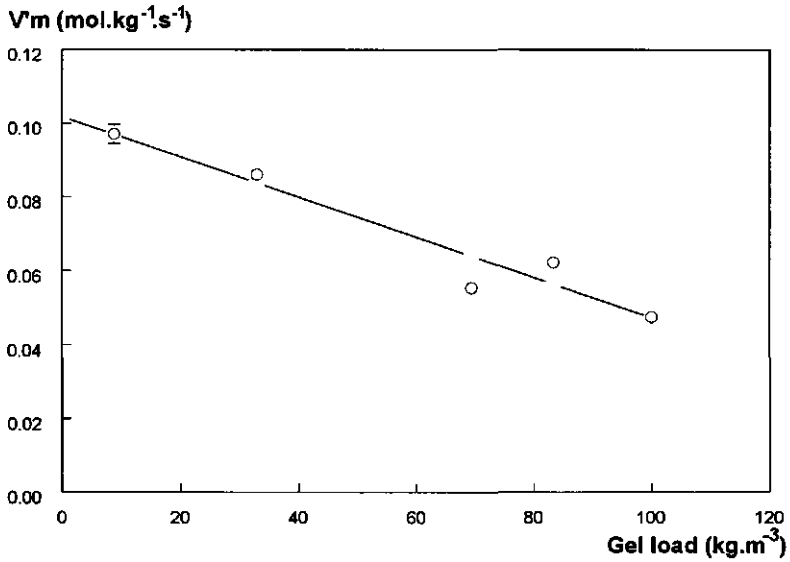

Figure 2. The apparent maximal substrate consumption rate V'_m as a function of the gel load. Bars give 95% confidence intervals, when invisible overlayed by the datum points. Solid line: linear regression line for extrapolation to the intrinsic V_m at zero gel load.

substrate consumption rate (mol.kg^{-1}.s^{-1}), respectively, K'_m and K_m the apparent and intrinsic Michaelis-constant (mol.m^{-3}), respectively, and η the efficiency factor (-).

Extrapolation to zero gel load gives the diffusion free, intrinsic, kinetic constants V_m and K_m (Van Ginkel et al. 1983, Van 't Riet & Tramper 1991). Here a linear relationship between the apparent kinetic constants V'_m and K'_m and gel load is assumed. The validity of this assumption was statistically assessed by using the Student *t* test (Table 2).

Table 2. Statistical evaluation of the linear regression analysis of the kinetic parameters determined from immobilized enzyme complex with five different gel loads, and enzyme inactivation in time. Also in this table: intrinsic kinetic data obtained from the immobilized enzyme complex and from the freely suspended enzyme complex.
[a] Boldface data indicate acceptance of the linear model with the t test. [b] Means and confidence intervals for the freely suspended enzyme complex were calculated from six independent experiments. [c] Min and max are 95% confidence limits for the parameter values.

LINEAR MODEL STATISTICS	TEST ON THE SLOPE COEFFICIENT	FIGURE 2	FIGURE 3	FIGURE 4
	t-statistic[a]	**-7.43**	1.95	**-8.72**
	t-table	-3.18	3.18	-4.3
	parameter	V_m	K_m	k_d
	unit	mol.kg^{-1}.s^{-1}	mol.m^{-3}	mol.kg^{-1}.s^{-2}
Enzyme complex immobilized in gel beads	value	0.1		4.0 10^{-6}
	min[c]	0.08		2.1 10^{-6}
	max[c]	0.12		6.0 10^{-6}
Freely suspended enzyme complex[b]	value	0.1	310	
	min[c]	0.09	205	
	max[c]	0.11	415	

For the intrinsic V_m (Figure 2) the slope coefficient was sufficiently different from zero to warrant a significant correlation (Table 2). The intrinsic V_m, i.e. the intercept of the linear regression line in figure 2, was 0.10 $mol.kg^{-1}.s^{-1}$ (Table 2). No statistically reliable intrinsic K_m could be determined in this way because the slope coefficient (Figure 3) was found not to differ significantly from zero (Table 2). Furthermore, the kinetic parameters V_m and K_m were also determined by using freely suspended DE32-cellulose-invertase complex, assuming negligible diffusion limitation. The average V_m obtained from six independent determinations was 0.10 $mol.kg^{-1}.s^{-1}$, and the K_m 310 $mol.m^{-3}$ (Table 2). This average V_m agrees very well with the above intrinsic parameter obtained from the beads with different gel loads (Table 2). Therefore no effect due to immobilization in alginate, like for example a change in conformation of the ezyme, on this kinetic parameter of the enzyme complex could be shown. The accuracy of the apparent constants K'm was limited as illustrated by the large 95% confidence intervals (Figure 3), and the rejection of the linear model (Table 2). This limitation of accuracy is more often encountered in the determination of immobilized enzyme-kinetics (Hooijmans et al. 1992). It was assumed that K_m was also left unaffected by the immobilization, and thus the average of 310 $mol.m^{-3}$ for the freely suspended enzyme complex represented the intrinsic K_m. This K_m was in the same order of magnitude as the earlier reported 198 $mol.m^{-3}$ for a different batch of enzymes (De Gooijer et al. 1989), and other literature data. Typical values of K_m, for different binding methods and different invertase preparations, are in the range 50 - 270 $mol.m^{-3}$ (Johansen & Flink 1986, Mansfeld & Schellenberger 1987, Mansfeld et al. 1991).

For all determinations of kinetic parameters, the initial substrate concentration was in the same range (600 $mol.m^{-3}$) as applied during the MAL experiments (630 $mol.m^{-3}$). For those concentrations possible effects of

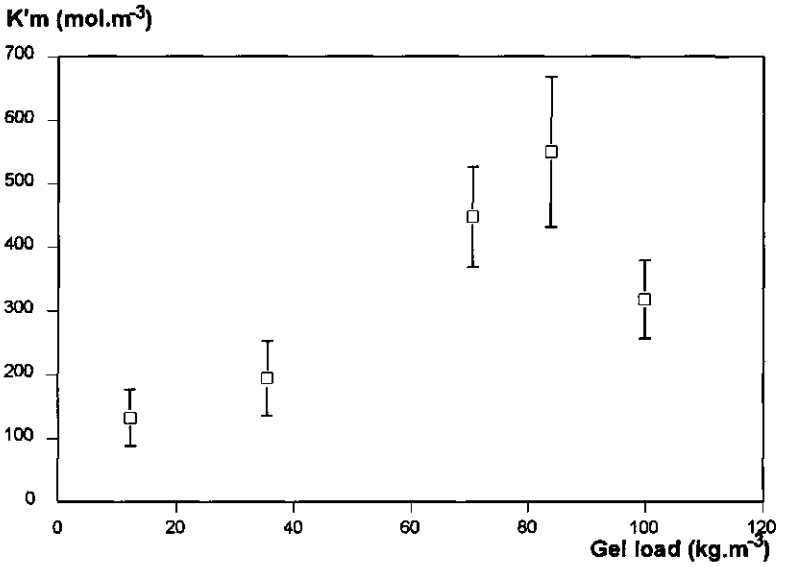

Figure 3. The apparent Michaelis-constant for the rate limiting substrate K'_m as a function of the gel load. Bars give 95% confidence intervals.

substrate or product inhibition on the reaction rate (Combes & Monsan 1983, Mansfeld & Schellenberger 1987, Mansfeld et al. 1991) were found to be negligible because all experimental results could be described well with the Michaelis-Menten equation.

To make a correction for the loss of enzyme activity in time, the inactivation rate was measured by determining V'_m from the same beads for four days (gel load : 100 kg.m^{-3}). Figure 4 shows a linear loss of enzyme activity in four days. The zero-order decay rate k_d was 4.0 10^{-8} mol.kg^{-1}.s^{-2}. This slope coefficient k_d was statistically significant different from zero (Table 2). The decay rate k_d was comparable to the 29% activity loss reported for invertase coupled to DE-cellulose after 4 days at 30 °C (Suzuki et al. 1966).

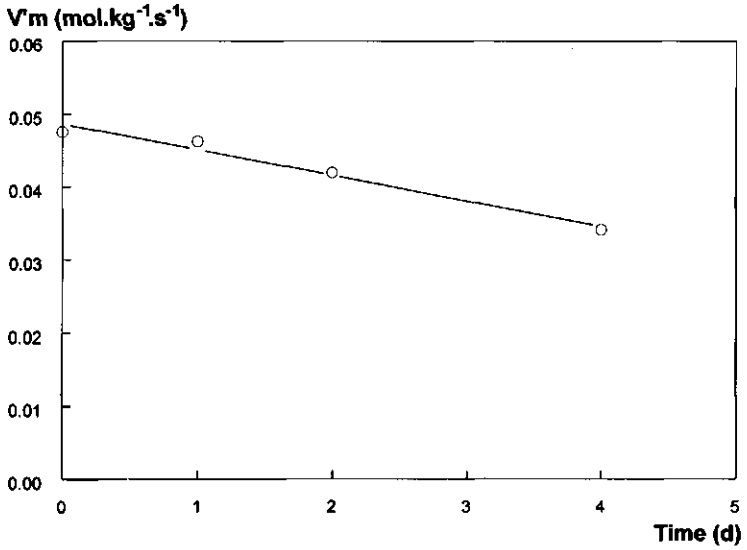

Figure 4. The apparent maximal substrate consumption rate V'_m as a function of time. Bars for the 95% confidence intervals are invisible because they are overlayed by the datum points. Solid line: linear regression line giving the inactivation rate.

Mixing in the MAL

A model assumption for the prediction of substrate conversion was that the continuously operated MAL behaved like a series of three ideal mixers. This was experimentally validated by RTD-measurements as illustrated in figure 5.

The theoretical number of equal-size ideal mixers *N* was derived from the RTD-curves, and is given in table 3. Even under the extreme experimental conditions applied (low gas flow rates, and for biological systems relatively short residence times), the mixing in the MAL was like that in a series of three ideal mixers (N = 3, see table 3). To illustrate this, model calculations by mass-balance equations (at short time intervals of 0.1 s) for two, three and four

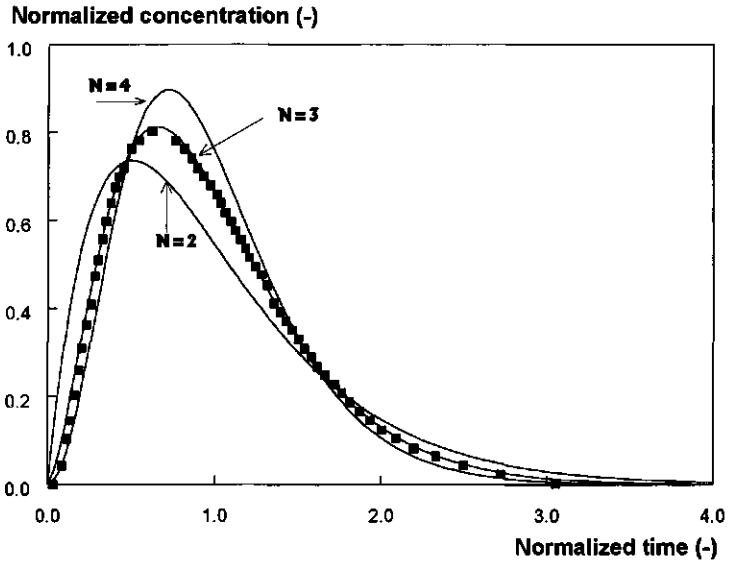

Figure 5. Normalized salt concentration *C* as a function of the normalized time θ, resulting in a typical residence time distribution (RTD) curve (experiment number 1 in table 3). Solid lines: model calculations for two, three and four ideal mixers in series.

Table 3. Results of the mixing studies by residence time distribution measurements.

NR.	OVERALL LIQUID RESIDENCE TIME (s)	GAS FLOWRATE PER MAL COMPARTMENT (10^{-6} $m^3.s^{-1}$)	MEAN (-)	VAR. (-)	$N = 1 / \sigma_\theta^2$ (-)	NaCl RECOVERY (%)
1	851	14	0.93	0.34	2.98	96
2	851	42	0.96	0.33	3	97
3	552	8.3	0.97	0.31	3.24	96
4	556	14	0.97	0.32	3.09	97
5	554	42	0.98	0.32	3.11	99

ideal mixers in series in the MAL are given in figure 5. The model calculations for three ideal mixers in series were, as illustrated in figure 5, also found to be in good agreement with the RTD-curves obtained from the other experiments.

From these results it was concluded that the mixing in the MAL during the sucrose conversion experiments indeed could be described as that in a series of three ideal mixers. Residence times applied there were an order of magnitude larger than those for the mixing experiments, thus allowing sufficient time for complete mixing in the MAL-compartments.

Mixing per MAL-compartment was also investigated during the MAL experiments with immobilized invertase. Samples were drawn at the same time at different places over the circle of each compartment. The low spread in the concentrations (Figure 6) indicated good mixing within the compartments. The aeration of the compartments resulted in such a hydrodynamic behavior that all alginate beads were kept fluidized and were circulating through the riser and the downcomer (visually observed) in the three well-mixed MAL-compartments.

Sucrose conversion in the MAL

Steady-state sucrose concentrations for the MAL and for a single vessel, both at 9.1% gel holdup, together with model calculations are given in figure 6. For practical convenience the same beads were used for several days while substrate conversion in the MAL was compared to that in a single vessel. Figure 6 clearly shows the advantage of using a MAL reactor series over a single vessel. Substrate conversion in the MAL improved to 83% compared to 73% in the single vessel. The difference in substrate concentrations was statistically shown to be significant (no overlay of the 95% confidence intervals). The single vessel experiment was conducted one day before and one day after the MAL experiment. Both steady-state sucrose concentrations for the single vessel were averaged to account for enzyme inactivation.

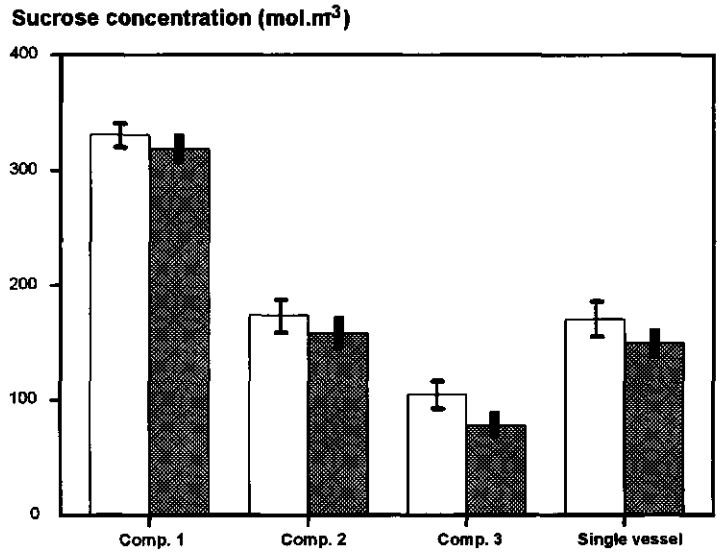

Figure 6. Results of the multiple air-lift loop reactor experiment per compartment, comparison with single vessel (white bars), line bars give 95% confidence intervals. Model estimates (grey bars), here the solid black bars give the range of model estimates using no film theory ($k_{l,s} = \infty$) and $k_{l,s} = 5\ 10^{-6}$ m.s^{-1}.

For the model calculations shown in figure 6 the working volumes of the MAL-compartments given in table 1 were used. A correction of the maximal substrate consumption rate V_m was made using the experimentally determined inactivation rate, taking into account the number of days between startup and steady-state measurement. The calculation procedures used were based on the work of De Gooijer et al. (1989) and incorporated internal diffusion and reaction in the beads and external mass transfer resistance. Parameter values used for the calculations are given in the Materials and Methods section.

The contribution of the liquid/solid mass-transfer coefficient $k_{l,s}$ to the total mass-transfer resistance can be estimated from the Biot number, which is defined as the ratio of the mass transfer resistance in the stagnant layer around

the bead and in the bead (De Gooijer et al. 1989, Wijffels et al. 1991). The $k_{l,s}$ was very roughly estimated from the relation of Brian & Hales (1969), which was selected because the Reynolds number for the beads was estimated to be 20 (based on the particles moving at the rate of free fall). The resulting Biot number (Bi = 26) indicated that external mass-transfer resistance was nearly negligible compared to internal mass-transfer resistance. Therefore, $k_{l,s}$ was chosen to be infinite (i.e. no stagnant layer present) and half the estimated value of $k_{l,s}$ = 1 10^{-5} m.s^{-1} to give a range of model estimates (Figure 6). This range is in agreement with estimations made for $k_{l,s}$ using recent correlations for ALRs proposed by Mao et al. (1992): $k_{l,s}$ = 2 10^{-5} m.s^{-1}, and Kushalkar & Pangarkar (1994): $k_{l,s}$ = 4 10^{-6} m.s^{-1}. All other model parameters were determined experimentally and the calculations agreed well with experimental results (Figure 6).

Model calculations

Model estimates of the sucrose conversion under various conditions were made to choose the experimental conditions, and thus the amount of experiments needed could be reduced. The residence time and the gel holdup were selected as variables that can easily be adapted. The inlet sucrose concentration was chosen such that large absolute differences in the steady-state concentrations between both reactor configurations could be expected. From the model, those differences were found to increase with increasing inlet sucrose concentration.

On the other hand, the influent concentration was chosen not too extreme, such that substrate and product inhibition were negligible. The parameters gel load and bead diameter were chosen arbitrarily. In figure 7 model estimates are

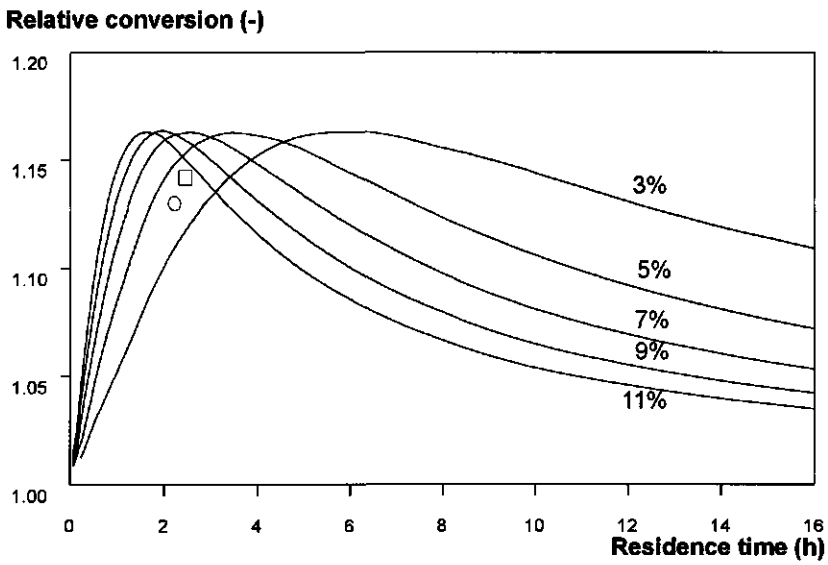

Figure 7. Model estimates for the relative conversion of sucrose in the MAL compared to a single vessel as a function of gel holdup (percentages shown in the graph) and overall residence time. Experimental results for 6.3% gel holdup (○) and 9.1% gel holdup (□).

given for the relative conversion as a function of the overall residence time and gel holdup. Other parameter values were as mentioned before.

The same relative conversion optimum can be reached under different conditions. For example, lowering the gel holdup requires increasing residence times (Figure 7). In this example the sucrose conversion in both reactors is low. This means that the absolute differences between the steady-state concentrations will be small, and difficult to show experimentally. Figure 7 also shows the experimentally determined relative conversion at 6.3% and at 9.1% gel holdup. Both experimental results are in rather good agreement with the model estimates (Figure 7).

CONCLUSIONS

The novel multiple air-lift loop reactor was evaluated with immobilized invertase as a biological model system. The conversion of sucrose in the MAL reactor series was higher than that in a single vessel with the same overall residence time. The difference was statistically significant. From RTD-measurements it was found that the three-compartment MAL could be described as three ideal mixers in series. Thus the MAL proved to be a suitable tool for the experimental evaluation of reactor series in biotechnology.

No effect of the immobilization in alginate on the kinetics of the DE32-cellulose-invertase complex could be shown. Immobilized enzyme complex was observed to inactivate as a function of time with a decay rate that was not negligible with respect to the duration of the experiment. Therefore enzyme decay was incorporated in the model calculations.

Model estimations for the sucrose conversion based on the work of De Gooijer et al. (1989), incorporating internal diffusion and reaction in the beads and external mass transfer resistance, were in good agreement with the experimental results.

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NOMENCLATURE

Bi	Biot number	-
C	normalized salt concentration	-
d_{32}	Sauter mean bead diameter	m
D	diameter	m
D_e	effective diffusion coefficient	$m^2.s^{-1}$
H	height	m
k_d	zero-order decay rate	$mol.kg^{-1}.s^{-2}$
$k_{l,s}$	liquid/solid mass-transfer coefficient	$m.s^{-1}$
K_m	Michaelis-constant for the rate limiting substrate	$mol.m^{-3}$
K'_m	apparent Michaelis-constant for the rate limiting substrate	$mol.m^{-3}$
N	theorethical number of equal-size ideal mixers	-
S	substrate concentration	$mol.m^{-3}$
V	volume	m^3
V_m	maximal substrate consumption rate	$mol.kg^{-1}.s^{-1}$
V'_m	apparent maximal substrate consumption rate	$mol.kg^{-1}.s^{-1}$

Greek symbols:

θ	normalized time	-
$\bar{\theta}$	mean normalized time	-
σ_θ^2	variance of the normalized distribution curve	-
η	effectiveness factor	-

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CHAPTER 10

GENERAL DISCUSSION

The perspective of this thesis is best described by the paradigm of Herbert (1964) : "If one fermenter gives good results, two fermenters give better results and three fermenters better still. This is sometimes true, but often false."

In chapter 1, a classification of bioprocesses in series of bioreactors is presented : a division is made between processes with constant and variable overall stoichiometry. For the first group, processes with constant overall stoichiometry, a further classification is made into enzymatic and autocatalytic bioprocesses.

ENZYMATIC BIOPROCESSES

For enzymatic processes chapter 1 shows that it can be worthwhile to use optimally designed series of CSTR's in almost all cases. However, if the cost of

capital investment is considered, it is unlikely that the use more than three bioreactors in series is economically feasible. Since a series of CSTR's approaches a plug-flow reactor, it is not surprising that many enzymatic processes in industry are reported to be executed in columns, usually of the packed-bed type (Tanaka et al. 1993).

In chapter 2 the procedure is described to do the design of a series of bioreactors containing immobilized enzymes. Here, the effectiveness factor η was incorporated in the dimensionless residence time :

$$\tau_i = \frac{\eta_i \theta_i V_m X \varepsilon}{S_0} \qquad [1]$$

Subsequently, the design of the series was done as if the enzyme was dissolved (no diffusion limitation).

Mathematically, the effectiveness factor is a function of the substrate concentration, and hence it would have been a more correct approach to incorporate this factor in the general equations for the optimum design :

$$\frac{\partial\left[\sum_{j=1}^{N} \tau_j\right]}{\partial \alpha_i} = 0 \qquad i=1, 2, \ldots (N-1) \qquad [2]$$

and

$$\tau_i = \frac{(\alpha_{i-1} - \alpha_i)(\kappa + \alpha_i)}{\eta_i \alpha_i} \qquad [3]$$

If subsequently a relation is fitted to the data points relating the effectiveness factor to the generalized Thiele modulus, the optimum design can be carried out. Although this remains to be worked out in detail, the case of only two bioreactors in series is easier to handle mathematically. Figure 1 was obtained by rather

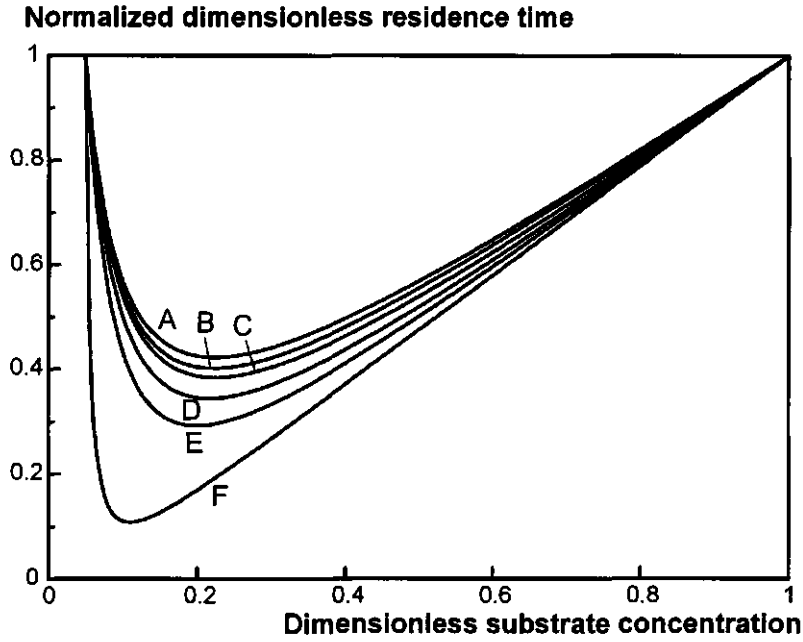

Figure 1 : The dimensionless total residence time of two CSTR's in series divided by the volume of a single vessel with the same substrate concentration at the exit versus the dimensionless substrate concentration (S/S_0) in the first vessel. Parameter is the diffusion coefficient ($m^2.s^{-1}$): A=10^{-4}, B=10^{-6}, C=10^{-10} , D=8 10^{-12} , E=5 10^{-12} , F=3.7 10^{-12}

straightforwardly applying mass balances for substrate to both vessels, and calculating the volumes of both vessels with varying substrate concentrations in the first vessel. In this way, a minimum total residence time can be found. Thereby, the substrate concentration in the second vessel was kept constant, and the same values for all parameters were used as in chapter 2, except for the diffusion coefficient, which was varied. As shown in figure 1, the design procedure described in chapter 2 is correct for common ranges of the diffusion coefficient : the minimum of curves B and C in figure 1 is attained at the same dimensionless substrate concentration. Higher diffusion coefficients than

CONCLUSIONS

This thesis presents a classification of bioprocesses carried out in continuously operated serial reactor configurations. Moreover, the theory of optimal design is worked out for a wide variety of processes. A logical continuation of this thesis is the physical characterization of the bioreactor device presented in chapter 9, after which, as has been shown in this thesis, the application thereof should be feasible.

NOMENCLATURE

α	Dimensionless concentration (S / S_0)	-
ε	Gel holdup in reactor	-
η	Overall effectiveness factor	-
θ	Residence time	s
κ	Dimensionless Michaelis-Menten constant (K_m/S_0)	-
τ	Dimensionless residence time ($\theta V_m \eta_s \varepsilon X / S_0$)	-
K_m	Michaelis-Menten constant	$mol.m^{-3}$
N	Number of reactors in series	-
S_0	Substrate concentration at the inlet of the first reactor	$mol.m^{-3}$
V_m	Maximum reaction rate	$mol.kg^{-1}.s^{-1}$
X	Gel load	$kg.m^{-3}$

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SUMMARY

In this thesis a common phenomenon in *bioprocess engineering*[1] is described : the execution of a certain bioprocess in more than one bioreactor. Chapter 1, a review, classifies bioprocesses by means of a number of characteristics :

i) processes with a variable *stoichiometry*,

ii) processes with a constant stoichiometry using *biocatalysts*,

iii) processes with a constant stoichiometry that are *autocatalytic*.

This chapter also offers a method to decide in which cases it can be worthwhile to use more than one bioreactor. The possible advantage is gained by a possible reduction in the total *residence time* needed to accomplish a certain degree of conversion. The shorter that residence time, the smaller the bioreactor(s) can be, and with that the capital investment reduces. The minimal residence time is attained if the bioreactors all have a different volume. In general the volume of each bioreactor decreases along the series. Moreover, the total volume of the series decreases if an increasing number of bioreactors are used in the series, although that decrease becomes increasingly less. The largest decrease in total residence time occurs by using two bioreactors in series instead of one single bioreactor, whereas the use of more than three bioreactors

[1] Words printed in italics are explained in the list at the end of the summary.

in series usually offers little advantage since the extra costs for pumps and similar additional parts is getting too high.

Chapter 2 describes the optimum design of a series of bioreactors for the case that the biocatalysts are *immobilized*, and chapter 3 describes this for immobilized autocatalytic systems. In chapter 3 some rather straightforward assumptions are made for the behaviour of immobilized growing cells, which may not be true in reality. Chapters 4 and 5 show the dynamic behaviour of the cells, including an experimental evaluation of such a system. As a model system *Nitrobacter agilis* cells were used. These cells perform the conversion of nitrite in nitrate, which is of importance for waste-water treatment, more precisely the removal of ammonia. Hereby ammonia is first converted to nitrite, nitrite to nitrate, and nitrate finally is converted to nitrogen gas. The model that is derived in chapter 4 however, has a more general applicability.

Chapters 6, 7 and 8 describe a system with a variable stoichiometry. In a first bioreactor *insect cells* are produced, which are infected with a *baculovirus* in one or two subsequent bioreactors. The infected cells then will produce *polyhedra*, which have a use as bioinsecticide. According to the current knowledge, insects cannot develop resistency against baculoviruses. Moreover, baculoviruses are extremely specific for an insect species, which means that useful insects are not affected. In these chapters it is shown that the production in continuously operated series of bioreactors is not unlimited in time, and that that is caused by the so-called passage effect : if the viruses have infected a cell a number of times, their infectivity decreases and the reaction stops. The model described in chapter 7 can predict what should be the optimal reactor configuration, and in chapter 8 this is experimentally shown : the cells must be grown in a bioreactor with a feed of medium, and if that bioreactor is filled part of its contents are pumped to a second bioreactor in which infection with

baculovirus occurs. During the time that the bioreactor in which the cells are grown is filled, the infected cells in the infection bioreactors produce polyhedra. After that the infection bioreactor is largely emptied, so that some virus remains in the reactor, and new cells are added, after which the infection proceeds, and so on. In this manner the time that the production process runs can prolongate fourfold as compared to a fully continuous process.

The number of applications of series of bioreactors is limited. An important cause for this is that, in practice, the for most bioprocesses required sterility is not easily maintained if the process is executed in more than one bioreactor. Chapter 9 shows a possible solution to that problem : in the presented Multiple *Air-Lift Loopreactor* up to three air-lift loopreactors in series are incorporated into one bioreactor.

List of explained words.

Air-Lift Loopreactor	A bioreactor without stirrer that consists of two compartments : a riser and a downcomer. Mixing and oxygen transfer are accomplished by sparging air at the bottom of the riser.
Autocatalytic	A reaction where the biocatalyst itself is produced.
Baculovirus	A rod-shaped virus occuring in insects.
Biocatalysts	Compounds, usually enzymes, that accelerate a reaction but do not take part in the reaction.
Bioprocess Engineering	The application-oriented science of the integration of one or more biological disciplines and process engineering. Chapter 7 is a good example of the integration of virology and process engineering.

Immobilized	In this thesis this means the inclusion of cells in a carrier, to retain them in a bioreactor.
Insect cells	Cells of an insect (in this case *Spodoptera frugiperda*), capable of growth in suspension.
Polyhedra	The form of baculoviruses occurring in nature. To protect the virions against environmental influences they are packed in protein matrices, the polyhedra. Once arrived in the gastro-intestinental tract, the polyhedra are dissolved and the virus particles are released, after which the insect is infected.
Stoichiometry	The ratio, on a molar basis, between the substrate offered and the product formed.
Residence time	The average time spent in a bioreactor.

SAMENVATTING

In dit proefschrift wordt een algemeen verschijnsel in de *bioprocestechnologie*[1] beschreven : het uitvoeren van een bepaald proces in meer dan één bioreactor. Hoofdstuk 1, een overzichtsartikel, deelt bioprocessen in aan de hand van een aantal karakteristieken :

i) processen met een variabele *stoichiometrie,*

ii) processen met een constante stoichiometrie die werken met *biokatalysatoren,*

iii) processen met een constante stoichiometrie die *autokatalytisch* zijn.

Dit hoofdstuk biedt tevens een leidraad om te kunnen beslissen in welke gevallen het voordelig kan zijn om met meer dan één bioreactor te werken. Dat voordeel schuilt in de totale *verblijftijd* die nodig is om een bepaalde *omzettingsgraad* te bewerkstelligen. Hoe korter die verblijftijd, hoe kleiner de bioreactor(en) kan of kunnen zijn, hoe lager de te plegen investering wordt. De minimale verblijftijd wordt bereikt in het geval dat de bioreactoren allemaal een verschillend volume hebben, en niet als meerdere bioreactoren met hetzelfde volume worden gebruikt. In het algemeen daalt het bioreactorvolume

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[1] Woorden die cursief zijn gedrukt worden verklaard in de woordenlijst aan het eind van de samenvatting.

verdergaand in de serie. Tevens daalt het totale volume van de serie naarmate er meer bioreactoren in de serie worden geplaatst, al wordt die daling steeds minder naarmate er meer bioreactoren in de srie worden geplaatst. De grootste daling treedt op door in plaats van één bioreactor, twee bioreactoren te gebruiken; meer dan drie bioreactoren heeft meestal weinig zin meer, doordat de extra kosten voor pompen en dergelijke dan te hoog worden.

Deze indeling is terug te vinden in de overige hoofdstukken van het proefschrift. Hoofdstuk 2 beschrijft hoe een serie optimaal kan worden ontworpen als de biokatalysatoren *geïmmobiliseerd* zijn, en hoofdstuk 3 beschrijft dit voor geïmmobiliseerde autokatalytische systemen. In hoofdstuk 3 zijn een aantal ruwe aannames gedaan voor het gedrag van geïmmobiliseerde groeiende cellen, die in werkelijkheid kunnen afwijken. De hoofdstukken 4 en 5 laten zien hoe het dynamische gedrag is van die geïmmobiliseerde groeiende cellen, inclusief een experimentele toetsing van het voor zo'n systeem opgestelde model. Als modelsysteem is daarbij gebruik gemaakt van *Nitrobacter agilis* cellen, die een omzetting uitvoeren die van belang is voor de afvalwaterreiniging : nitriet wordt omgezet in nitraat. Dit is een stap in het proces om ammonia te verwijderen, waarbij ammonia eerst wordt omgezet in nitriet, nitriet in nitraat, en nitraat in het onschuldige stikstofgas. Het in hoofdstuk 4 ontwikkelde model heeft echter een meer algemene geldigheid.

De hoofdstukken 6 tot en met 8 gaan over een systeem met variabele stoichiometrie. Hierbij worden in een eerste bioreactor *insectecellen* geproduceerd, die in een of twee volgende vaten worden geïnfecteerd met een *baculovirus*. De geïnfecteerde cellen gaan vervolgens *polyeders* produceren, die kunnen worden gebruikt als gewasbeschermingsmiddel. Insecten kunnen, voorzover bekend, geen resistentie tegen baculovirussen ontwikkelen. Tevens zijn baculovirussen extreem soortspecifiek, zodat de nuttige insecten intact

blijven. In deze hoofdstukken wordt aangetoond dat de produktie in continu bedreven bioreactoren niet ongelimiteerd is, en dat de oorzaak daarvan moet worden gezocht in het passage-effect : als de virussen een aantal malen een cel hebben geïnfecteerd, neemt hun infectiviteit af en stopt de reactie. Met het in hoofdstuk 7 ontwikkelde model kan worden voorspeld wat de optimale reactorconfiguratie zou moeten zijn, en in hoofdstuk 8 wordt dit experimenteel aangetoond. De cellen moeten worden gekweekt in een reactor met een toeloop van medium, en als de bioreactor vol zit wordt een gedeelte eruit gepompt naar een tweede bioreactor, waarin infectie met baculovirus optreedt. In de tijd dat de bioreactor waarin de insectecellen groeien volloopt, produceren de geïnfecteerde cellen in de infectiereactor polyeders. Daarna wordt de infectiereactor leeggepompt, er blijft een beetje virus achter, en er worden weer nieuwe cellen toegevoerd, waarna de infectie weer op gang komt, enzovoorts. Op deze manier is een vier maal zo lange produktie mogelijk als met een continu bedreven systeem.

Het aantal toepassingen van series van reactoren is beperkt. Een belangrijke reden hiervoor is dat het in de praktijk niet eenvoudig is om de voor bioprocessen noodzakelijke steriliteit te handhaven als het proces zich afspeelt in meerdere bioreactoren. Hoofdstuk 9 laat daarvoor een mogelijke oplossing zien : in de gepresenteerde Meervoudige *Air-Lift Loopreactor* staan tot drie air-lift loopreactoren in serie, in een enkele bioreactor.

Verklarende woordenlijst.

Air-Lift Loopreactor Een bioreactor die geen roerder heeft, en uit twee compartimenten bestaat : een stijgbuis en een daalbuis. Menging en zuurstofoverdracht treden op door lucht onderin de stijgbuis te blazen.

Autokatalytisch	Een reactie waarbij de biokatalysator wordt gevormd.
Baculovirus	Een staafvormig virus dat voorkomt bij insecten.
Biokatalysatoren	Stoffen, veelal enzymen, die een reactie versnellen maar er zelf niet aan deelnemen.
Bioprocestechnologie	Het wetenschapsgebied dat zich bezighoudt met de integratie van één of meerdere biologische basisdisciplines en de proceskunde, gericht op een bepaalde toepassing. Hoofdstuk 7 is een mooi voorbeeld van de integratie van de virologie en de proceskunde.
Geïmmobiliseerd	In dit proefschrift is dat het insluiten van cellen in een dragermateriaal, zodat ze in de bioreactor blijven.
Insectecellen	Cellen van een insect (in dit geval *Spodoptera frugiperda)* die in suspensie kunnen groeien.
Omzettingsgraad	De behaalde omzetting.
Polyeders	De in de natuur voorkomende vorm van baculovirussen. Om de virusdeeltjes zelf te beschermen tegen de invloeden van het milieu zijn ze ingepakt in een eiwitmantel, de polyeder. Eenmaal in het spijsverteringskanaal van een insect beland lost de polyeder op en komen de virusdeeltjes vrij, waarna ze het insect infecteren.
Stoichiometrie	De verhouding, op molbasis, tussen de aangeboden grondstof en het produkt.
Verblijftijd	De gemiddelde tijd die in een bioreactor wordt doorgebracht.

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CURRICULUM VITAE

Kees de Gooijer werd op 11 mei 1960 geboren in Ede. In 1978 werd het diploma VWO-B behaald aan het Christelijk Lyceum Veenendaal.

De in datzelfde jaar aangevangen studie Levensmiddelentechnologie aan de Landbouwuniversiteit Wageningen werd in maart 1985 afgesloten (met lof). Afstudeervakken waren Proceskunde, Informatica en Bedrijfskunde.

Vanaf 1984 werkte hij bij het softwarebedrijf Fytoconsult in Wageningen, een latere dochteronderneming van Cebeco Handelsraad te Rotterdam.

In november 1987 werd hij universitair docent bij de sectie Proceskunde van de Landbouwuniversiteit te Wageningen.