Multi-pahse fermentation
Meer, Abele Broer van der

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
1993

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):
Meer, A. B. V. D. (1993). Multi-pahse fermentation: reaction engineering models for the productio of optically active 1,2-epoxyoctane from 1-octene by free and immobilized pseudomonas oleovorans cells. s.n.

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In biotechnological processes living organisms, cells or parts of these are applied to produce or modify products. Traditionally, the conversion of substrates into products takes place in an aqueous phase. Various microorganisms, however, are able to convert organic substrates which are poorly soluble in the aqueous phase. In this case a second organic liquid phase is present in the fermentation system leading to a four-phase reaction system. Such complex microbial four-phase reaction systems are rapidly gaining interest. Applications are particularly found where organic substrates are converted into new products, or products which are difficult to produce economically by chemical reaction (e.g. chiral compounds).

The modeling and optimization of microbial four-phase reaction systems is still in its infancy. An aim of the present research project is to develop engineering models predicting the performances of microbial reaction systems under practical multi-phase fermentation conditions. A second aim is to investigate the opportunities of immobilizing the living cells on inert carriers to increase both the production capacity and production selectivity. For this thesis the production of optically active 1,2-epoxyoctane from 1-octene by Pseudomonas oleovorans is chosen as a model system.

In this reaction system four phases can be present: an aqueous phase, a suspended biomass phase, a dispersed gas phase (oxygen supply) and a dispersed organic phase. Both substrates, 1-octene and oxygen, have to be transferred, from the organic liquid phase and the gas phase, respectively, via the aqueous phase, to the cells. Depending on the operation conditions both the mass transfer of oxygen and 1-octene can be the rate controlling resistance for cell growth and 1,2-epoxyoctane production. Cell damage as a result of direct contact of the cells with the organic liquid phase may reduce the cell growth and epoxide production rate. An additional complication is the release of lipopolysaccharides from the outer membrane of the cells. These emulsifiers may influence the mass transfer of both substrates. Moreover, the presence of small organic droplets in the aqueous phase may enhance the mass transfer of oxygen to the aqueous phase. Finally, the product 1,2-epoxyoctane is toxic to the cells. To gain an understanding of the simultaneous processes taking place in the fermentation system, cell growth, cell damage, product inhibition, epoxide formation, gas/liquid mass transfer and liquid/liquid mass transfer (including the effect of the in situ generated emulsifiers) were each investigated in separate experimental systems.

n-Octane is catabolized via the same metabolic route as 1-octene. By applying gaseous n-octane instead of liquid 1-octene as the organic substrate all complications mentioned above are prevented and uninhibited cell growth on molecular dissolved n-octane can be observed. In Chapter 2 kinetic rate equations for both true cell growth and substrate consumption, and a reactor model predicting cell growth on gaseous n-octane in both fed-batch and continuous stirred tank reactors are presented. The cell growth rate constant
and the cell death rate constant appear to differ for the fed-batch and the continuous culture system. At low biomass concentrations the growth kinetics were found to be rate limiting, whereas at relatively high cell concentrations cell growth is controlled by the gas/liquid mass transfer of n-octane. Unbalanced growth was observed in the intermediate regime.

By adding the product 1,2-epoxyoctane to the oxygen and n-octane containing gas phase of the three-phase reaction system the cell growth rate constant can be obtained as a function of product concentration. The product inhibition model parameters thus obtained are presented in Chapter 3. It turns out that also these parameters are different for the fed-batch and the continuous bioreactor. From dynamic experiments it could be concluded that inhibition of cell growth is a relatively fast process, compared to the process of growth rate recovery following a decrease in product concentration.

In an other three-phase reaction system, using gaseous l-octene as the organic substrate, epoxide formation was studied without cell damage and emulsifier production. This work is described in Chapter 4. Oscillations in biomass and epoxide concentration were observed in the continuous stirred tank reactor. These could be explained by a model based on epoxide production coupled to cell growth with time delayed product inhibition. This model also predicts the performance of the corresponding fed-batch reactor system.

Chapter 5 deals with the growth of Pseudomonas oleovorans on liquid n-octane in a four-phase fed-batch stirred tank reactor. From a two-substrate (oxygen and n-octane) model sub-models are derived for three rate controlling regimes: fermentation controlled by cell growth kinetics, by mass transfer of oxygen, and by mass transfer of n-octane, respectively. These sub-models are used to determine the relevant kinetic and physical model parameters, and to predict the rate controlling resistance for cell growth. To maximize the cell growth rate it appears to be necessary to increase the mass transfer of n-octane. Within experimental accuracy the true cell growth kinetic rate constant appears not to be significantly affected by stirrer speed, organic phase hold-up and partial pressure of oxygen. The rate constant for endogenous metabolism seems to increase by changing from air to oxygen and with increasing stirrer speed. Much stronger, however, is the effect of the presence of the liquid organic phase. Compared to the three-phase fermentation system with gaseous n-octane the rate constant for endogenous metabolism turns out to be increased by a factor of 10 to 40. Therefore, it is attractive to develop new reactor concepts where direct contact between cells and organic phase can be avoided.

The effects of hydrodynamics, organic phase hold-up and surfactant concentration on both the gas/liquid and the liquid/liquid mass transfer in the four-phase bioreactor are presented in Chapter 6. In the absence of the organic phase, small amounts of biosurfactant lower the gas/liquid volumetric mass transfer coefficient of oxygen and relatively large amounts of the emulsifier increase this coefficient. The Sauter mean diameter of the n-octane droplets is strongly influenced by the presence of
biosurfactant. For surfactant concentrations typically found in the four-phase fermentation systems approximately 70 % of the droplets have diameters below \(0.5 \times 10^{-6}\) m. These small droplets appear to be responsible for a significant enhancement of gas/liquid oxygen mass transfer up to a factor of 1.6. As theoretically expected, the enhancement factor turned out to increase with the concentration of small n-octane droplets. As far as the authors know, such a mass transfer enhancement effect so far has been observed in stirred cells with a flat gas/liquid interface only, where mass transfer coefficients usually are an order of magnitude lower relative to the stirred tanks used in the present investigation. The results indicate that surfactants can be advantageously used to increase the relatively low fermentation rates.

A reaction engineering model describing actual epoxide production in the four-phase fermentation system using liquid 1-octene as the organic substrate is presented and experimentally verified in Chapter 7. At low biomass concentrations the true reaction kinetics were found to be rate limiting. At relatively high cell densities either mass transfer or product inhibition becomes rate controlling. Both for the overall rate controlled by kinetics and for the transition regime the model predictions are in good agreement with the experimental results. Oscillations in biomass, oxygen and epoxide concentration could be predicted with reasonable accuracy. At high cell densities the discrepancies between the actual performance of the reactor and the predictions from our mathematical model become more significant. Here, increased accuracy can only be obtained by inclusion of both the effect of the in situ generated emulsifiers on the gas/liquid and liquid/liquid mass transfer parameters, and of the effect of the reaction conditions on the biomass/liquid mass transfer parameters. The net cell growth rate constant turned out to decrease with increasing organic phase hold-up. The observed specific epoxide production rates in the four-phase system are up to a factor of 7 higher than in the three-phase system with gaseous 1-octene as the organic substrate. This positive result appears to be caused by the presence of the liquid organic phase which acts as a sink for the toxic product. The selectivity for epoxide production equals the selectivity in the three-phase system: 0.3 kg epoxide formed per kg 1-octene consumed.

Relative to free cells, well known advantages of immobilized cells are easy handling of the biocatalyst, easy separation of the cells from the product, no wash-out of cells, reduction of infection risks and a reduced reactor volume due to an increased cell concentration. A possible additional advantage of applying immobilized *Pseudomonas oleovorans* cells in the production of 1,2-epoxyoctane from 1-octene is minimizing cell damage by preventing direct contact of the cells with liquid 1-octene. As a result the specific epoxide production rate may be increased and the release of emulsifiers, which leads to foam formation, may be reduced.
Chapter 8 deals with epoxide production by immobilized *Pseudomonas oleovorans* cells both in a packed bed recycle reactor and in a fluidized bed recycle reactor. In the packed reactor relatively large alumina beads (particle diameter = 4 mm) were used as biomass carriers whereas relatively small calcium alginate beads were applied in the fluidized bed (0.5 ≤ particle diameter ≤ 2.0 mm). The latter particles were stabilized with polyethyleneimine and glutardialdehyde. In the packed bed the epoxide production rate turned out to be controlled by both external and internal mass transfer of both oxygen and 1-octene. By applying the organic substrate as the continuous phase, external mass transfer limitation of 1-octene can be eliminated. For the macro porous alumina beads, however, this resulted in medium limitation due to medium wash-out. Medium leakage from the beads could be successfully prevented by using the stabilized alginate beads in the fluidized bed recycle reactor. In this reaction system intra particle diffusion and external mass transfer limitation turned out to be negligible for bead diameters smaller than 0.55 mm. Relative to free cells, higher selectivities (0.4-0.9 versus 0.3) and specific epoxide production rates (0.30-0.50 versus 0.02-0.15 kg m⁻³ hr⁻¹) could be realized with the immobilized cell systems. This possibly is a result of preventing cell damaging contact between cells and organic phase by immobilization. No foam formation occurred in the immobilized cell systems. The epoxide production rate and the total production per batch probably can be further increased by increasing the medium concentration in the beads, by co-immobilization of an additional pH buffer and by optimizing the partial pressure of oxygen. These are prospective areas for further research.