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Conclusions and summary

This thesis describes the biotechnological application of the alkane-hydroxylase system of *Pseudomonas oleovorans* to produce fine chemicals in two-liquid phase systems. As outlined in chapter 1, using this enzyme system several interesting products can be made. Especially the production of 1,2-epoxyoctane, adipic acid and dodecanedioic acid could be promising. Despite the optimization efforts by a number of different groups [56,127,129,140,141], laboratory scale alkane-oxidation rates observed thus far have not exceeded 3 U/g cells. These rates must be raised at least one order of magnitude to approach an economically favourable production process [164].

In this thesis several approaches improving the production rate for oxidized aliphatics by *P. oleovorans* are described. These complementary approaches include reactor engineering to develop suitable bioreactors for two-liquid phase biocatalysis and to optimize physiological conditions for production in these reactors, and cell engineering to construct strains specifically suited for production processes in two-liquid phase systems. A number of investigations concerning these subjects are described in this thesis.

First, the alkane-hydroxylase system of *P. oleovorans* was studied in more detail: induction and expression were measured with several techniques. By using antibodies raised against the purified enzyme, expression in different hosts could be measured as well as its cellular localization in these hosts (chapter 2 and 3).
Second, the development of suitable bioreactors and optimal conditions for alkane oxidation are described in chapter 4 and 5. The production of octanol and epoxyoctane by wild-type and engineered strains of \textit{P. oleovorans} in these bioreactors was optimized (chapter 6).

Finally, biopolymer production by \textit{P. oleovorans} was also studied. This resulted in the optimization of the polymer formation process and the characterization of a whole range of different biopolymers (chapter 7).

The results obtained in each of these three areas are briefly summarized and discussed below.

**Alkane-hydroxylase**

The process of alkane induction was studied in several ways: by looking at changes in growth rate and changes in protein synthesis and protein composition upon addition of octane. Irrespective of the method used, the total process of induction takes about 1 hour under conditions of no repression. Looking at induction during growth on other carbon sources, it appeared that the enzymes for alkane oxidation were induced only when cells grow faster on octane than on the other substrate. Obviously, \textit{P. oleovorans} looks for the carbon source in which it grows best.

Looking in more detail at the changes at the protein level during induction by octane, the expression of a 41 kDa band in the membrane fraction was especially striking. This band was shown to be alkanehydroxylase. Induction of this protein in the membrane fraction appeared behind its appearance in total lysates. In the latter case the maximum amount of alkane-hydroxylase was already reached in 25 minutes, during which time the enzyme increased from about 30 to about 35,000 protein copies per cell under fully induced conditions. Therefore either the \textit{alkB}-promotor must be a very strong but strictly regulated promotor, or the mRNA transcript must be very stable. For better understanding of this phenomenon genetic studies have to be carried out in which the promotor is isolated and studied and the half-life time of the \textit{alkB}-mRNA is determined.

Looking at the cellular localization of alkane-hydroxylase by immuno electron microscopy we showed that this enzyme is not simply a membrane localized enzyme as has been assumed. Instead, it turns out to be an inner membrane protein. However, detailed studies followed by immuno-EM showed that the enzyme is synthesized in the cytoplasm, while large invaginations were observed where the enzyme seemed to be localized. These invaginations were also observed in the \textit{alkB}-mRNA.
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 results. However, detailed studies in which the induction process was 
 followed by immuno-EM showed that the enzyme was mainly localized in the 
 cytoplasm, while large membrane invaginations were observed with which 
 the enzyme seemed to be associated. It was clearly demonstrated that 
 these invaginations were exclusively due to the induction of the alkB A/R 
genodes.

To explain such a localization two models were presented. First, the 
enzyme is synthesized in the cytoplasm, associates with lipids and these 
structures are fused with the cytoplasmic membrane. The second model 
assumes that the enzyme is synthesized near the cytoplasmic membrane, 
which is saturated with protein and starts to invaginate. This latter 
explanation seems at this moment more favourable, since during the 
induction process the amount of alkane-hydroxylase increases more rapid-
ly in the membrane fraction than in the cytoplasm. Nevertheless, more 
research will be needed to choose between these two models. Whatever the 
correct explanation may be, a consequence is that the limit of alkane-
hydroxylase expression is not likely to be dictated by the available 
membrane surface. The use of multicopy plasmids containing alkB must 
deduct or even higher expression levels can be attained. On the other 
hand, it remains to be seen whether the other two components of the 
system, rubredoxin and rubredoxin-reductase [91,93,99,156], are truly 
cytoplasmic or are also associated in the same manner with the intra-
cellular vesicle structures. It is also questionable whether all cyto-
plasmic alkane-hydroxylase is really necessary for growth on alkanes.

Growth and production in two-liquid phase systems

In order to test and optimize production of oxidized aliphatics by 
Pseudomonas strains a computer controlled bioreactor especially suited 
for two-liquid phase biocatalysis was developed. Using the wild-type 
strain, several growth parameters were optimized in these bioreactors.

As a consequence of the use of hydrophobic substrates combined with a 
biocatalyst in the water phase a two-liquid phase system is obtained, in 
which mass transfer limitations of the substrate towards the biocatalyst 
may occur as predicted by a number of theoretical studies 
 [8,27,114,160]. To estimate mass transfer rates we studied exponential
growth in different water/alkane mixtures from which actual octane- and octene transfer rates could be calculated. These experiments revealed that at least 300 - 500 μmoles alkane or alkene/min/liter reactor volume can be transferred, which is considerably higher than predicted by various mass transfer models. Higher transfer rates may well be possible, since the mentioned rates were already found at amounts of 20% (v/v) organic phase and relatively low stirrer speeds. Furthermore, the production of emulsifiers by the cells should also increase the mass transfer rate of the substrate towards the cells.

Conditions optimized for alkane utilization were also favourable for 1-octanol and epoxyoctane production, as expected. Using these conditions in the bioreactors epoxyoctane production occurred at initial rates of 50 U/g in the exponential growth phase. These rates decreased in the stationary phase. During growth on octene, a rate of 7 U/g could be maintained for at least 100 hours. During growth on octane, P. oleovorans produced octanol at a rate of 8 U/g when entering the stationary phase, but this production decreased rapidly in time. This was probably due to product inhibition. Application of a second organic phase which withdraws the product from the water phase more effectively might give longer production times and higher conversion rates.

The combination of host strains resistant to bulk apolar phases and the genetic information needed to express the alkane-hydroxylase system, leads to especially suited production strains. Results obtained with such engineered strains look very promising: during the early exponential phase initial conversion rates for octane to octanol were found to exceed 50 U/g, which is close to the maximum attainable conversion rate calculated from growth experiments in chapter 5. Epoxidation rates were however very low. Probably the host strains used are more sensitive to organic phases or epoxyde. Therefore, further attempts should be made to select strains resistant against bulk apolar phases, and better alkanol-dehydrogenase negative mutants should also be selected.

In conclusion, the conversion rates needed to approach an economically favourable epoxidation or hydroxylation process can indeed be reached by cell- and reactor engineering. Although high conversion rates were only found during several hours, further attempts should be made to find more stable biocatalysts. The necessary regeneration of cofactor (NADH) inside the cell must also be taken into account.
which actual octane- and e experiments revealed in/liter reactor volume higher than predicted by rates may well be. For example, the actual reactor volume found at amounts of 20% speeds. Furthermore, the also increase the mass were also favourable for two speeds. Using these conditions, occurred at initial rates rates decreased in the rate of 7 U/g could be growth on octane. P. oleovorans when entering the rapidly in time. This of a second organic phase more effectively conversion rates.

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Biopolymers

The formation of a PHB-like material by P. oleovorans during growth on octane as originally described by de Smet [42] was also investigated.

First the chemical structure of the methanolized polyester-like material was compared to chemically synthesized chiral standards. It was found that the biopolymer produced on octane consisted of a mixture of (R)-3-hydroxyoctanoate and (R)-3-hydroxyhexanoate, of which the former is the predominant species.

In order to optimize biopolymer production an assay was developed to determine the amount of biopolymer in culture samples. Using this assay the production of the polyester was followed during growth and in the stationary phase. During growth only small amounts of the material were found, but when the cells entered the stationary phase due to nutrient limitations polymer synthesis was started. A maximum of about 20% of the cell dry weight was reached at about 22 hours stationary phase, after which a decline in the amount of biopolymer was observed. Comparison of different nutrient limitations revealed that nitrogen and phosphate limitations yielded the highest amounts of polymer. In contrast to PHB, which consists for more than 95% of the same monomers, the biopolymer formed on alkanes can differ considerably in composition and therefore probably also in polymeric characteristics. Using different alkanes as the carbon source the composition of the monomers was strongly influenced, varying from a copolymer consisting of 3-hydroxy-dodecanoates, -decanoates, -octanoates and -hexanoates on dodecane, to a homopolymer of 3-hydroxyhexanoates on hexane. Thus, simply varying the carbon source resulted in different monomeric compositions of the polymer and thus to different polymers.

An even more special polymer was found when octene is used as the carbon source. About 50% of the monomers contained a terminal C=C bond. This polymer is especially suited for subsequent chemical modifications. In comparison to PHB, where routinely more than 80% of the cell dry weight can consist of polyester, the amount of polymer produced is still low. In order to raise this amount, further optimization of the physiological conditions is needed. Moreover, the breakdown of the polymer
observed after prolonged cultivation must be avoided, probably by
deletion of the genes involved in polymer degradation. A more efficient
isolation procedure must also be developed to yield high amounts of
polymer from bacterial cultures, after which the polymer characteristics
should be determined and compared to other polyesters including PHB.
Their biodegradability and varying monomeric composition should make
this set of biopolymers suited for a large number of applications.

Samenvatting

Biotechnologie is eigentijds wetenschap. Het bedrijf maakt men wijn,
moderner produkt als pen. Er zijn onderwerpen die onder de heugenis
Echter, zo'n 10 tot 15 jaar, belangrijke ontdekkingen mogelijk is binnen de bio
en de microbiologie, stofcellen of celonderdelen daarmee iets te produceren
als biotechnologie. Een belangrijke ontdekking is, dat de mens
levende dingen gaan doen

Eén klein onderwerp heeft geschift behandeld, namelijk:

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