Escherichia coli as a potential hydrocarbon conversion microorganism. Oxidation of aliphatic and aromatic compounds by recombinant E. coli in two-liquid phase (aqueous-organic) systems

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The increased interest in the study of hydrocarbon utilizing microorganisms in recent years has been stimulated by the possibility of using their monooxygenases in the selective oxidation of aliphatic and aromatic compounds. As an example, long chain ($> C_{16}$) n-alkanes are converted to dicarboxylic acids by yeast. This type of bioconversion is useful because the regioselective and stereospecific introduction of oxygen into unactivated organic substrates by classical synthetic chemistry remains very difficult.

Medium chain length (C_6-C_{12}) fatty acids, ω-hydroxyfatty acids and dicarboxylic acids are also potentially interesting industrial synthons. They can in principle be prepared from the corresponding alkanes with the alkane oxidation system of *Pseudomonas oleovorans*. However, since *P. oleovorans* efficiently utilizes monocarboxylic acids as a source of energy and carbon, the desired product accumulation must compete against a very efficient β-oxidation pathway. In effect, the bioconversion of hydrocarbons should be uncoupled from cell growth; cells should therefore grow not on alkanes but on a second carbon source, such as a water-soluble sugar. This might be attained with β-oxidation mutants of *P. oleovorans* but thus far we have not been able to produce such mutants.

We have turned therefore to *Escherichia coli* as a possible candidate for alkane bioconversions. Its β-oxidation system is normally repressed, and becomes active only after induction by C_{12} or longer fatty acids. Thus, recombinant *E. coli* strains containing the *alk* genes of *P. oleovorans* might be expected to accumulate medium-chain length fatty acids from the corresponding n-alkanes. However, one of the potential drawbacks of such a two liquid phase fermentation system is that *E. coli* is more sensitive than *P. oleovorans* to bulk apolar phases. Indeed, organic solvents are generally not conducive to microbial growth and often they are toxic.

Growth characteristics of *E. coli* W3110 in the presence of different apolar solvents.

The growth and the morphology of *Escherichia coli* were studied in different two-liquid phase (aqueous-organic) systems in a 1 liter stirrer tank reactor (Chapter 2). The growth on sugars of *E.coli* W3110, a wild type K-12 strain, is not affected by the presence of large amounts of hydrocarbons with a solvent polarity equal to or higher than that of n-heptane or cyclooctane. Solvents with a polarity lower than or equal to that of n-octene (hexane, cyclohexane) completely prevent growth of *E. coli* W3110 in two-phase media. Freeze fracture electron microscopic studies of *E. coli* grown on water-n-octane media showed no cell damage during the exponential phase whereas during the stationary phase the cytoplasmic membrane is heavily damaged. This membrane damage was accompanied by a loss of cell viability of 98 - 99% in the stationary phase. As long as the cells grew exponentially, however, they remained fully viable, which suggests that *E. coli* W3110 is able to survive in the presence of apolar solvents because of energy requiring active processes.

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Production of octanoic acid from n-octane by *E. coli* W311047, a strain containing *alk* genes from *P. oleovorans*.

The *alk* genes from the catabolic plasmid OCT of *Pseudomonas oleovorans*, which encode the enzymes involved in the oxidation of n-alkanes to carboxylic acids, were introduced in *E. coli*. The resulting recombinants convert n-octane in a two-liquid phase medium into the corresponding alkanoate and excreted this compound into the aqueous phase (Chapter 2). We have develop an improved fermentation arrangement in which the octanoate yield was maximized. The rate of octanoic acid production in such improved conditions, 45 µmoles/ min. g of cells, is better than the maximum alkane oxidation rate of *P. oleovorans*. recorded thus far [1,2] . However, the biooxidation of n-octane to octanoic acid by *E. coli* W311047 was limited to about 25 mM of octanoic acid in the aqueous phase. During such fermentations we noted that there was a decrease of cell viability, loss of the tetracyclin marker, and a negative effect of *alk* induction on cell growth (Chapter 3). Based on these observations we concluded that the biooxidation of n-octane by *E. coli* W311047 was limited not by the altered cell physiology which results from the expression of the *alk* genes in *E. coli* W3110.

Improvement of the host-vector system.

There is little information in the literature about the influence of the host on the expression of recombinant proteins. It therefore appeared interesting to us to see if the expression of the *alk* genes has the same dramatic effects in different *E. coli* hosts that it has in *E. coli* W3110.

Accordingly, the plasmid pGEc47 containing the *alkB-L/ST* loci was introduced into different *Escherichia coli* hosts. These were grown in the same mineral medium in the presence of 20% (v/ v) n-octane in a 1 liter tank reactor and the cell growth, the glucose concentration, and the amount of octanoate produced were monitored and compared to that seen for*E. coli* W311047 grown under the same conditions. *E. coli* HB101 appeared to be a better host than *E. coli* W3110. The recombinant *E. coli* HB10147 containing the plasmid pGEc47 seemed not to be bothered by the alk proteins and was able to reach higher cell dry weight levels than *E. coli* W311047 (Chapter 4).

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[1] **Witholt, B., de Smet, M. J., Kingma, J., Van Beilen, J., Kok, M., Lageveen, R. G., and Eggink, G.** 1990. Bioconversions of aliphatic compounds by *Pseudomonas oleovorans* in multiphase bioreactors: backround and economic potential. TIBTECH. **7**: 46-52.

[2] **de Smet, M.-J., Kingma, J., Wijnberg, H., an Witholt, B.** 1983. *Pseudomonas oleovoran*s as a tool in bioconversions of hydrocarbons: growth, morphology, and conversion characteristics in different two-phase systems. Enzyme Microb. Technol. **5:** 352-360.

Physiology and octanoate formation of a recombinant *Escherichia coli* in two-liquid phase continuous cultures

Since cell growth is apparently important for maintenance of cell viability and enzyme activity in the presence of a bulk apolar phase, we have studied the growth of a wild type *E. coli* and the formation of octanoic acid by a recombinant strain in two-liquid phase continuous cultures using n-octane as the organic phase.

The morphology and the physiology of the wild type *E. coli* W3110 was studied in a two-liquid phase chemostat at different dilution rates (D) in the presence of n-octane. Experiments indicated that *E. coli* was able to withstand the presence of a bulk apolar phase for a period as long as one week. The growth and the production of octanoate from n-octane by the recombinant *E. coli* HB10147 was studied in two-liquid phase chemostats. Studying the growth of recombinant organisms in a chemostat can be complicated by two factors, First, plasmid stability is not always maintained. In the absence of selective pressure, cells can lose their plasmids and grow faster than the cells which contain the plasmid. Eventually the chemostat becomes populated with only plasmid-free cells. In some cases the problem can be reduced by using a medium which exerts a selective pressure for cells that contain plasmids.

A more difficult problem to avoid in the continuous culture of recombinant microorganims is the occurrence of spontaneous mutations leading to "structural instability". In this situation, some cells undergo mutation events leading to a decrease in or elimination of their ability to produce the recombinant protein of interest, although they still retain the recombinant plasmid. These mutated cells are able to metabolize the limiting substrate more efficiently than cells producing the recombinant protein. Consequently, the nonproducers gradually replace the cells that synthesize the recombinant protein.

An examination of different recombinant strains (host-vector systems) revealed that *E. coli* HB10147 was particularly suited to two-liquid phase continuous culturing: stable cultures could be maintained for at least 200 hours. The volumetric productivity was determined as a function of growth rate and showed a maximum at a dilution rate of 0.32 h^{-1}, reaching 0.5 g octanoate/ liter. hour.

The production of oxidized hydrocarbons by recombinant *Escherichia coli* in high cell density two-liquid phase cultures.

Thus far, relatively low concentrations of octanoate were obtained in two-liquid phase systems because of low biocatalyst concentrations. Therefore, recombinants *E. coli* must be cultured at high cell densities in two-liquid phase media while retaining a sufficient oxidation activity. We developed a fed-batch system specially designed for two-liquid phase culture requirements. In such two-liquid phase fed-batch cultures, we obtained 50 g cell dry weight/ l of *E. coli* HB101 growing on glucose as the sole carbon source and in the presence of 10% (v/ v) n-octane. The recombinant *E. coli* HB10147 was also cultured to a high biomass density, and exposed to a bulk n-octane phase. The recombinant strain was able to convert the alkane to the

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corresponding alkanoate resulting in the accumulation of 46 mM octanoate in the water phase, which was the highest concentration obtained so far.

Genetic studies in the past few years have enabled the transfer of catabolic genes to *Escherichia coli*, thereby expanding the range of organisms which can be used in bioconversion applications. We introduced the *xylMA* genes from the TOL plasmid of *P. putida* mt-2 which codes for the enzymes catalizing the oxidation of various aromatic compounds such as toluene, p- and m-xylene, into *E. coli*. We used a similar fermentation arrangement to grow the resulting recombinant, *E. coli* HB10163 which contains the *xyl* genes, to high biomass and studied the bioconversion of styrene to chiral styrene epoxide in a two-liquid phase system. In such high cell density cultures, styrene was efficiently transformed into styrene epoxide, with a 94% enantiomeric excess, resulting in the accumulation of 91 mM S-styrene epoxide of the product in the bulk apolar phase.

Conclusion.

The use of microbial transformations instead of traditional chemical methods has many potential advantages in the fine and specialty chemical industries. One of the potential applications of microbial transformation is in the production of fine chemicals such as oxidized aromatic and aliphatic compounds.

Genetic studies in the past few years have enabled the transfer of catabolic genes to *E. coli*, thereby expanding the range of organisms which can be used in bioconversion applications. Reasons for using *E. coli* in two-liquid phase biotransformations have been demonstrated in this thesis and include:

1. The ease with which *E. coli* can be modified genetically, thus facilitating the development of pathways to produce specific compounds.
2. The absence of catabolite repression of the expression of the *alk* genes.
3. The well established plasmid technology.
4. The fact that there is considerable experience with fermentations of recombinant *E. coli* strains.

These reasons contribute to facilitate the development of a desired bioconversion. For both bioconversions studied in this thesis, n-octane to octanoic acid and styrene to (S) styrene epoxide, higher bioconversion rates and product concentrations were obtained with *E. coli* than have previously been reported for *Pseudomonas* strains. These are two illustrations of the possible uses of *E. coli* in two-liquid phase bioconversions.

Other applications can be found. One example is the microbial synthesis of indigo by recombinant *E. coli* . Given the popularity of blue jeans, there is a worldwide market estimated to be in excess of $100 million for indigo. In fact, genes from *P. putida* which code for the enzymes responsible for naphthalene oxidation have been cloned and expressed in *E. coli* to create a

construct capable of indigo synthesis [3]. Thus, a suitable strain is available. However, suitable fermantation technology remains to be developed. Thus far, high concentrations of the dye in the water phase have prevented proper cell growth. One can imagine a two-liquid phase system such as described in this thesis might help solve this problem, to provide the basis for a practical fermentation system for one industrial synthesis of indigo.

To conclude, we shall say that two-liquid phase fermentations of *E. coli* cells might bring new opportunities for the production of chemicals by microorganisms, leading to processes which may be less traditional in their characteristics and demands, and might even represent radical departures from the usual, requiring considerable inventiveness in their biological, chemical and engineering aspects. Finally, despite all of the futuristic discussion about the displacement of traditional chemical processes by new biotechnological methods, each new process must surmount one last and overwhelming barrier: the demand that, in real terms, these new processes must be commercially viable. Such is the real world.

[3] **Ensley, BD., Ratzkin, B., Osslund, T.D., Simon, M.J., Wackett, L.P., Gibson, D.T.** 1983. Expression of naphtalene oxidation genes in *Escherichia coli* results in the biosynthesis of indigo. Science **222:** 167-169.

Synthese van geoxide

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