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CONTINUOUS COENZYME DEPENDENT STEREOSELECTIVE SYNTHESIS OF SULCATOL BY ALCOHOL DEHYDROGENASE

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SUMMARY
6-methyl-5-hepten-2-one was reduced to sulcatol ((+)-6-methyl-5-hepten-2-ol) by using alcohol dehydrogenase from *Thermoanaerobium brockii* in a continuous process. The co-factor NADP(H) was retained by a charged UF-membrane and regenerated by oxidation of isopropanol to acetone. Use of native NADP in a charged UF-membrane reactor proved to be superior to use of PEG-coupled NADP in a uncharged UF-membrane reactor.

INTRODUCTION
In the past few years interest in enantiomerically pure compounds has substantially increased, especially in the chemical and pharmaceutical industries. As biotransformations with enzymes or microorganisms are capable of performing stereoselective reactions, much attention has been placed upon these types of catalysts for chemical synthesis. It is well known that different enantiomers of one substance may have different physiological effects. One example is the pheromone sulcatol (6-methyl-5-hepten-2-ol) which in its S-(+)-form attracts the bark beetle *Gnathotrichus retusus*, while the R-(-) alcohol is inactive (Stokes and Oehlschlager, 1987).

It is our goal to develop continuous enzymatic processes for the production of hydrophobic fine chemicals, for which sulcatol was chosen as a model product. Since alcohol dehydrogenase from *Thermoanaerobium brockii* (TBADH) can tolerate high concentrations of organic solvents and accept a broad range of hydrophobic compounds as substrates, it was suggested to use TBADH for the stereoselective reduction of 6-methyl-5-hepten-2-one (sulcatone) (Belan, 1987). The expensive cofactor NADP(H) can be regenerated using the coupled substrate approach by oxidation of isopropanol to acetone (fig.1).

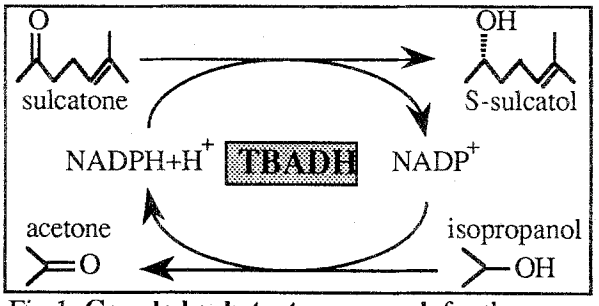

Fig.1: **Coupled substrate approach** for the production of S-sulcatol

MATERIALS AND METHODS
TBADH was purchased from Sigma, NADP(H) (research grade) from Serva, 6-methyl-5-hepten-2-one and (±)-6-methyl-5-hepten-2-ol from Aldrich and purified by vacuum distillation. NADP-PEG was synthesized according to Bückmann (1980). All other sub-

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stances were research grade. Membranes (NTR 7410 and 7430) were a kind gift from NITTO Denko, Japan.
Sulcatone and sulcatol were detected by GC equipped with a fused silica Anabond wax (Polyethylenglycol) column from Analyt. Enantiomeric purity was determined after derivatization with isopropyl-isocyanate on a chiral GC column (XE-60-S-Val-S-α-PEA , Chrompack), (König et al., 1982)[1]. Enzyme activity was measured by following the change of NADPH at 340 nm on a Hitachi UV/VIS photometer in 0.1 mol/l Tris/HCl buffer, 150mM isopropanol at 30°C (Lamed and Zeikus, 1981).

RESULTS AND DISCUSSION

TBADH reduces sulcatone at about 6 % of the rate it obtained with isopropanol as substrate. Thus NADPH is effectively regenerated in the coupled reaction and will not be reaction rate limiting. Even at very low sulcatone concentrations (0.01% v/v) high reaction rates were achieved and enantiomeric excesses always ranged above 97%. The K_m for NADP is 20 µmole/l, but very low concentrations of NADP (10 µmole/l) are sufficient for full catalytic activity. This may result from the fact that the cofactor does not need to dissociate from the enzyme in the coupled substrate system.
Since the solubility of sulcatone and sulcatol in water is about 20 mmole/l and the NADP concentration was chosen to be around 50 µmole/l, the theoretical maximum NADP turnover number which can be achieved is 400. This turnover number would not be sufficient to develop an economical process, thus, either the cofactor needs to be retained or the sulcatone concentration has to be increased. The latter would also reduce subsequent product recovery costs.

BATCH EXPERIMENTS

Since TBADH is a relatively expensive enzyme it would be expected that major production costs would result from enzyme purchase. Thus, it was tried to stabilize the enzyme with several additives. It was found that adenosine-5-mono-phosphate (AMP) both stabilizes and activates the enzyme. With AMP, storage half life times of 1200h were measured under operating conditions. This high stability of the enzyme also makes it more likely that enzymatic transformation can compete with biotransformation processes which utilize microrganisms.
In order to achieve higher solubilities of sulcatone some hydrophilic organic solvents were tested with respect to enzyme activity and stability. It was found though that dioxane, ethanol and high concentrations of isopropanol inhibit or destabilize the enzyme. Thus, an isopropanol concentration of < 5 % v/v was found to be optimal. This concentration still allowed complete reduction of sulcatone (>97 % conversion) up to concentrations of 0.1% v/v.
Reaction rates observed in aqueous/organic two-phase systems with n-hexane or benzene as an organic solvent were considerably lower than in the aqueous system and are not reported further here.

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[1]We thank Mrs. I.Schaible and Dr. H.Hasenfratz-Schreier for determination of enantiomeric excesses.

CONTINUOUS EXPERIMENTS

Continuous experiments in the aqueous system were conducted in 0.05 mol/l Tris buffer, pH 7.5. In this case it is aimed to retain and regenerate the coenzyme as much as possible. For coenzyme dependent transformations a charged UF-membrane enzyme reactor proved to be useful for retention of the cofactor (Howaldt, 1988; Kulbe et al., 1989). Another method for retaining the coenzyme is also based on ultrafiltration, but in this case the coenzyme has to be artificially enlarged with e.g. polyethylenglycol (PEG) to be rejected by uncharged UF-membranes (Wichmann et al., 1981). Fortunately, TBADH accepts this enlarged coenzyme PEG-NADP and so both methods for retaining the cofactor were compared. However,TBADH shows a reduced V_{max} (84% of that with $NADP^+$) and an increased K_m (33µmole/l) with PEG-NADP. In one experiment native NADP was used and retained by a highly charged UF-membrane with a small molecular weight cutoff (NTR 7430, NaCl rejection ≈ 30%), in the other PEG-NADP and a less charged UF-membrane with a higher cutoff (NTR 7410, NaCl rejection ≈ 10%) was used. This membrane rejects NADP-PEG quantitatively and allows for higher fluxes. With the charged membrane, the cofactor, which is mainly present in its reduced form, is rejected at 99.9% since both the cofactor and the membrane carry a net negative charge. Both methods were tested for sulcatone reduction, the cofactor being recycled by oxidation of isopropanol with TBADH in a continuous stirred-tank reactor (CSTR, see fig.2).

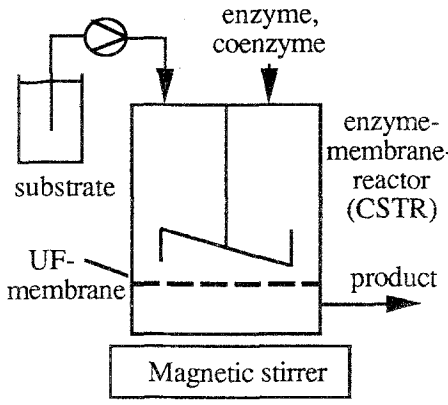

Fig. 2 : Laboratory setup for CSTR experiments

From fig.3 it can be seen that the process with native NADP resulted in higher conversion. This could be due to the changed kinetic parameters using PEG-NADP or decreased adsorption of enzyme on the highly charged UF-membrane. The latter was expected since the enzyme carries a negative net charge at pH 7.5 and should also be rejected due to electrostatic repulsion. This fact was proved since the reactor equipped with the highly charged UF-membrane showed higher specific enzyme activities in samples which were withdrawn from the reactor than the one with the less charged UF-membrane.

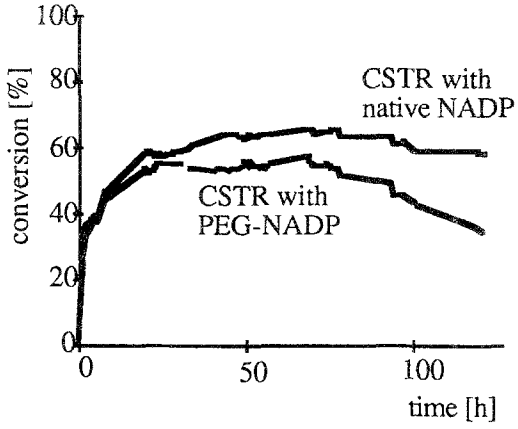

Fig.3: Comparison of native NADP to PEG-NADP in a CSTR equipped with an integrated UF-membrane (see fig.2).
Initial NADP(-PEG) conc.: 100 µmol/l,
feed stream: sulcatone conc. 13 mmole/l
isopropanol conc. 0.65 mole/l,
residence time ≈ 5h, Reactor volume: 15ml,
Enzyme conc.: 0.5 U/ml

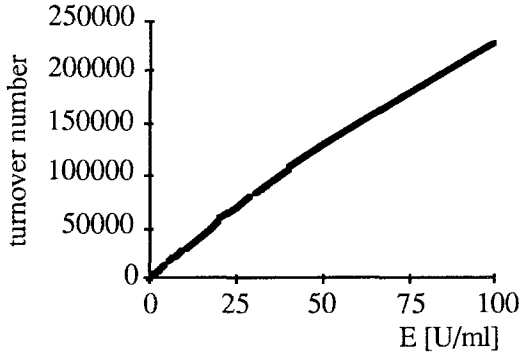

Fig.4: Achievable turnover numbers for NADP as a function of enzyme concentration. Parameters: 10mmole/l sulcatone, 3% isopropanol, 50 µmole/l NADP, retention coefficient $_{NADP(H)}$=99.9% , t $_{1/2}$ for NADP(H) ≈ 110h.

Theoretically, turnover numbers up to 230,000 may be achieved with the setup shown in fig.2 depending on the enzyme concentration used (fig. 4). High coenzyme recycling efficiency can be attained when high amounts of substrate are converted with a given amount of coenzyme. Thus, higher enzyme concentrations will yield higher turnover numbers. In the aqueous system the turnover number is mainly limited to the stability of NADPH. The turnover numbers which were experimentally achieved in the aqueous system ranged around 4500 since only very low enzyme concentrations were used. The tunover numbers for enzyme (µmole product/ Unit enzyme) were around 4.400.

CONCLUSIONS

Continuous production of sulcatol with TBADH was succesfully carried out in an aqueous system where the expensive cofactor NADP(H) is retained in a charged UF-membrane enzyme reactor.

It should be kept in mind, however, that downstream processing costs usually are of major contribution to overall production costs. Thus, it may be more feasible to use a two-phase system in order to achieve both higher sulcatone concentrations and retention of enzyme and cofactor. A reverse micellar system seems especially promising for this application. It has the advantage over conventional two-phase systems in that it exhibits less enzyme deactivation and also reduces diffusion limitation. The reverse micellar system with various surfactants and organic solvents is currently under study.

ACKNOWLEDGEMENT

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