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A novel reactor concept for the enzymatic reduction of poorly soluble ketones

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Abstract

Reductions of poorly soluble ketones often suffer from low total turnover numbers conferring to the coenzyme and large volumes which are needed for the conversion. The novel emulsion membrane reactor overcomes these limitations. From an emulsion consisting of an organic substrate and an aqueous buffer phase, the aqueous phase is separated selectively by using a hydrophilic ultrafiltration membrane and fed to a subsequent enzyme membrane reactor. The product outflow is recirculated to the emulsion stirred vessel and, due to the partition coefficients, the aqueous phase is recharged with substrate while the product is extracted. This new reactor concept will be compared to the classical enzyme membrane reactor. The latter was operated under the same conditions over a period of 4 months at a space–time yield of 21.2 g l^{-1} day^{-1}. As a model system the enantioselective reduction of 2-octanone to (*S*)-2-octanol (ee > 99.5%) is used, carried out by a carbonyl reductase from *Candida parapsilosis*. NADH is regenerated by formate dehydrogenase from *Candida boidinii*. In comparison to the classical enzyme membrane reactor the total turnover number could be increased by a factor 9 using the novel emulsion membrane reactor. © 1998 Elsevier Science B.V.

Keywords: Oxidoreductase; Enantioselective reduction; Emulsion membrane reactor; Enzyme membrane reactor

1. Introduction

Optically active secondary alcohols are used as synthons for the preparation of chiral natural products and related compounds such as many pharmaceuticals [1–3]. (*S*)-2-Octanol is also a versatile starting material for the synthesis of ferroelectric liquid crystals [4].

In the literature three main biocatalytic ways are known to produce (*S*)-2-octanol by biotransformations: lipase catalyzed hydrolysis and transesterification [3,5–8], oxidoreductase catalyzed reductions [9,10] and whole cell catalyzed reductions [11].

Alcohol dehydrogenases show two main advantages compared to lipases for the enantioselective production of alcohols: (i) direct enantioselective reduction; (ii) 100% conversion (opposed to 50% conversion for the racemic resolution). These advantages are only applicable

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without restrictions if the ketone of interest is watersoluble. If hydrophobic substrates are converted oxidoreductases are less favored. In the first place only a low total turnover number (ttn) conferring the cofactor is reached. The ttn serves as a measure of the efficiency of the cofactor regeneration and is defined as mole product formed per mole cofactor consumed [12,13]. Secondly, large volumes of aqueous phase are needed to solubilize the substrate which leads to complicated downstream processing. In contrast to lipases, alcohol dehydrogenases show only a very low stability in the presence of organic interfaces making the direct use of emulsions not always possible. Up to now only a limited number of reactor concepts are known which overcome these limitations [14–20].

Using the enantioselective reduction of 2-octanone to (*S*)-2-octanol (Fig. 1), which is catalyzed by the carbonyl reductase from *Candida parapsilosis* (*CP*CR), (EC 1.1.1.*, the natural substrate is not known) [21,22] as an example, we will show that by combination of two membrane processes the production of hydrophobic alcohols is possible at lab scale. Since the oxidoreductase needs nicotinamide adenine dinucleotide (NADH/NAD^+) as cofactor [23,24], the cofactor regeneration is carried out by formate dehydrogenase (FDH) from *Candida boidinii* (EC 1.2.1.2) [25,26]. Due to the practical irreversible oxidation of formic acid to CO_2 catalyzed by FDH, the equilibrium of the product-forming reaction is shifted in the direction of complete conversion [27].

The newly developed reactor will be compared to the classical enzyme membrane reactor [28–33], working in the solubility range of the substrate. In the latter one the long term stability was tested.

2. Materials and methods

2.1. Enzymes, chemicals

Carbonyl reductase from *Candida parapsilosis* and formate dehydrogenase from *Candida boidinii* were supplied by the Institute of Enzyme Technology (Jülich, Germany). β-Nicotinamide adenine dinucleotide (NAD^+) was obtained from Biomol Feinchemikalien (Hamburg, Germany). All other chemicals were purchased from Fluka (Buchs, Switzerland).

2.2. Enzyme activity assay

All activity tests were carried out at standard conditions ($T = 25°C$; triethanolamine (TEA)-buffer, 50 mM, pH 7, 1 mM NADH). The kinetic parameters were determined by initial rate measurements followed by fitting of the model to the measured velocity–concentration pairs by nonlinear regression [34,35]. Scientist

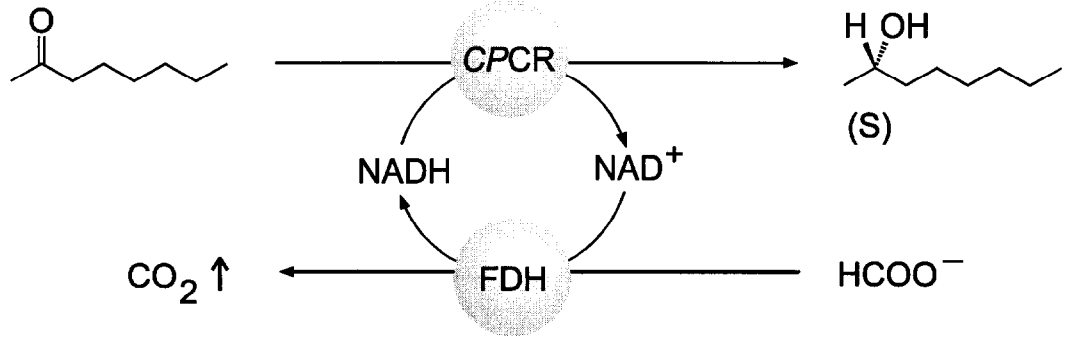

Fig. 1. Reaction scheme of the enantioselective reduction of 2-octanone to (*S*)-2-octanol catalyzed by the carbonyl reductase from *Candida parapsilosis* (*CP*CR). The cofactor NADH is regenerated by formate dehydrogenase (FDH) from *Candida boidinii* by oxidizing formate to carbon dioxide.

for Windows V. 2.0 (Micromath, Salt Lake City, UT, USA) was used for parameter estimation.

2.3. Determination of conversion and enantiomeric excess

The conversion was determined by means of GC on a permethylated cyclodextrin column (FS-cyclodextrin, β I/P 50 m × 0.32 mm ID, Macherey and Nagel, Düren, Germany), using H_2 as carrier gas in an isothermal mode at 100°C, FID. Typical retention times were: 2-octanone; 4.5 min, (*S*)-2-octanol; 6 min. For determination of the enantiomeric excess the alcohol had to be derivatized with acetanhydride beforehand. Samples (1 ml) were extracted with dichloromethane (1:1) and dried over anhydrous sodium sulfate. After addition of 200 μl pyridine and 200 μl acetanhydride, the sample was heated to 100°C for 30 min.

2.4. Classical enzyme membrane reactor

The substrate solution was pumped through a continuously operated stirred tank reactor containing a hydrophilic ultrafiltration membrane (UF-PA-20H, cut-off 20 000 Da, Hoechst, Frankfurt/Main, Germany) made out of polyaramid. The reactor volume was 10 ml. No sterile filter was used since it was unstable against the ketone enriched aqueous phase. Prior to use the whole reactor was sterilized with 0.01% peracetic acid. To prevent adsorption of the oxidoreductases on the membrane, the membrane was precoated with 1 mg of bovine serum albumin per ml reactor volume.

2.5. Emulsion membrane reactor

The reactor as shown in Fig. 2 consisted of a stirred emulsion vessel (1) in which the aqueous phase was separated from the organic phase (= pure substrate) by a hydrophilic ultrafiltration membrane (UF-PA-20H, Hoechst) [36]. The substrate saturated aqueous phase entered the enzyme membrane reactor (2) where 2-octanone was reduced enantioselectively to (*S*)-2-octanol by the *CP*CR. The enzyme membrane reactor consisted of a loop containing a circulation pump (3) (pump 504U, Watson–Marlow, Rotterdam, The Netherlands) which was operated at a pump rate of 18 l h^{-1} and an ultrafiltration module (Filtron Minisette with a membrane stack, cut-off 10 000 Da, Filtron, Karlstein, Germany) which was thermostated at 25°C. The

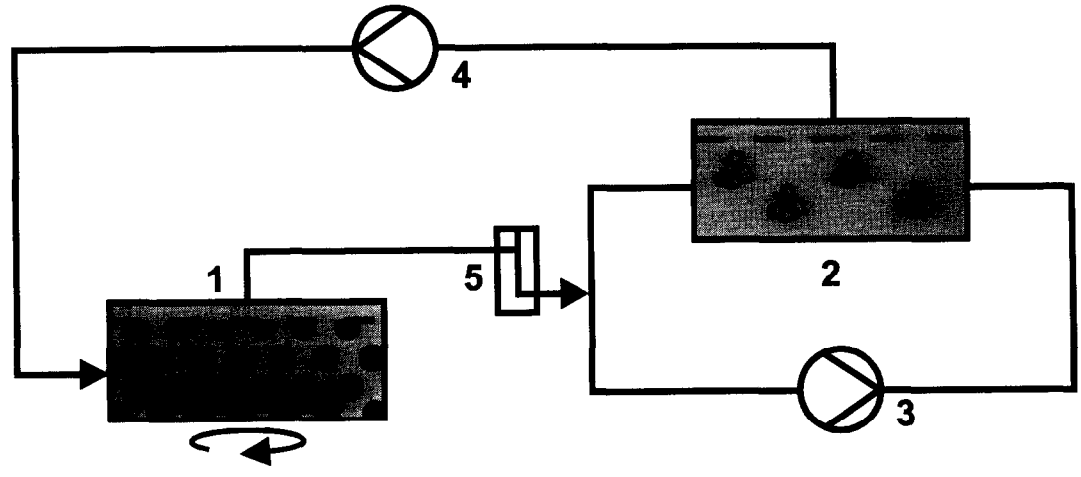

Fig. 2. Flow scheme of the emulsion membrane reactor: 1; stirred emulsion vessel with hydrophilic ultrafiltration membrane, 2; enzyme membrane reactor loop with ultrafiltration module, 3; circulation pump for enzyme membrane reactor, 4; circulation pump for total reactor, 5; bubble trap.

product outflow was recirculated to the stirred emulsion reactor by using a Pharmacia P 500 pump (4) (Pharmacia, Freiburg, Germany) at a pump rate of 150 ml h^{-1}, where, due to the partition coefficients the product was extracted by the organic phase and the aqueous phase was recharged with substrate. The total volume of the reactor was 100 ml (volume ratio of the organic phase (= substrate) is 1% v/v). Prior to use the whole reactor was sterilized with 0.01% peracetic acid. The ultrafiltration membrane in the enzyme membrane reactor was precoated with 300 mg of bovine serum albumin to prevent adsorption of the oxidoreductases.

3. Results and discussion

3.1. Reaction conditions and kinetic parameters

The stability and activity of the enzymes under operating conditions are important parameters which determine the feasibility of a process. Therefore comprehensive studies of the reaction conditions and the kinetics were carried out. It is known that the carbonyl reductase from *Candida parapsilosis* can be stabilized by addition of 1 mM dithiothreitol [37]. The substrate 2-octanone and the product of the reduction (*S*)-2-octanol do not have any destabilizing effects on the *CP*CR in the range of the solubilities. Besides the chemicals, the activity and stability is mainly influenced by the two parameters temperature and pH (Fig. 3). With rising temperature the activity of the *CP*CR is also increased in contrast to the half-life of the enzyme, which is decreased. As a working temperature 25°C is chosen as a compromise (half-life $\tau_{1/2} = 31.1$ days). Using the Arrhenius–Trautz-correlation an activation energy of 61 kJ mol^{-1} is determined for the reduction of 2-octanone. In the case of the pH the activity and the stability have also to be compared. As optimal value pH 7 is chosen exhibiting the highest stability and activity of the *CP*CR under the conditions described. The operating pH-value and temperature are determined by the *CP*CR to guarantee an optimal usage of the carbonyl reductase.

Since the emulsion membrane reactor shows the characteristics of a batch reactor, it is important to know the kinetics of the enzyme system. Any reaction exhibiting substrate surplus inhibition should not be carried out in such a batch reactor setup resulting in longer reaction times. The reason is the high substrate concentration at startup that is lowering the reaction velocity. In such a case a continuously operated stirred tank reactor is preferred that is operated at a high conversion in the steady state resulting in a low

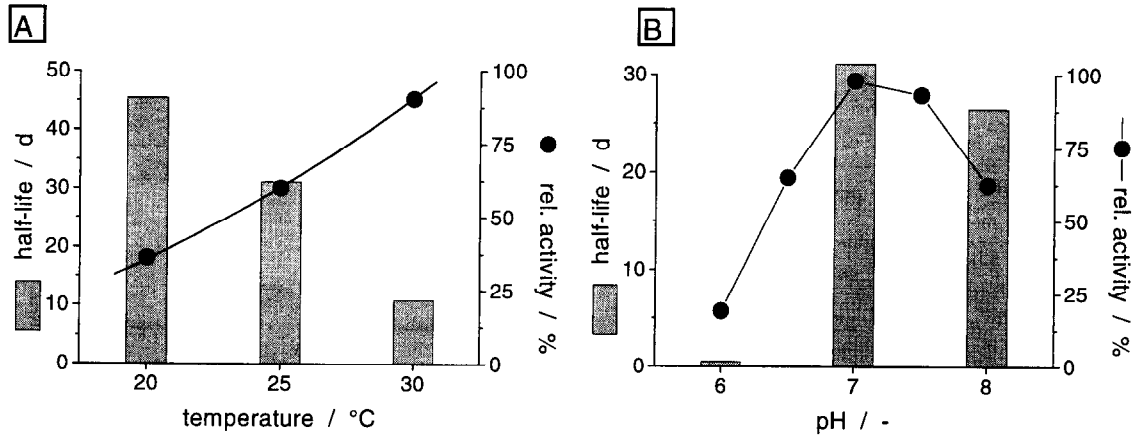

Fig. 3. Reaction conditions of the *CP*CR: half life and activity as function of temperature (A) and pH (B) (6 mM 2-octanone, 1 mM NADH, 1 mM DTT, 50 mM TEA buffer, pH 7, 25°C).

Table 1
Kinetic parameters for the *CP*CR-catalyzed enantioselective reduction of 2-octanone and oxidation of (*S*)-2-octanol

Reduction	Oxidation
V_{max} (U mg^{-1}) = 7.26 ± 0.31	V_{max} (U mg^{-1}) = 24.7 ± 5.23
$K_{m,ket}$ (mM) = 0.55 ± 0.088	$K_{m,alc}$ (mM) = 0.46 ± 0.08
$K_{m,NADH}$ (mM) = 0.12 ± 0.023	K_{m,NAD^+} (mM) = 0.58 ± 0.02
$K_{i,alc}$ (mM) = 1.46 ± 0.33	K_{i,NAD^+} (mM) = 2.00 ± 0.75
K_{i,NAD^+} (mM) = 0.29 ± 0.073	$K_{i,ket}$ (mM) = 0.07 ± 0.02
	$K_{i,NADH}$ (mM) = 0.01 ± 0.002
$V_0 = V_{max} \cdot \frac{[ket]}{K_{m,ket} \cdot (1 + [alc]/K_{i,alc}) + [ket]} \cdot \frac{[NADH]}{K_{m,NADH} \cdot (1 + [NAD^+]/K_{i,NAD^+}) + [NADH]}$	$V_0 = V_{max} \cdot \frac{[alc]}{K_{m,alc} \cdot (1 + [ket]/K_{i,ket}) + [alc]} \cdot \frac{[NAD^+]}{K_{m,NAD^+} \cdot (1 + [NADH]/K_{i,NADH}) + ([NAD^+]^2/K_{i,NAD^+}) + [NAD^+]}$

substrate concentration. The development of a reactor design in case of strongly product inhibited reactions was demonstrated in a previous publication [31]. As an oxidoreductase, the *CP*CR follows an ordered bi–bi mechanism [37] meaning that there is a defined order of binding/dissociation of the cofactor and substrate to/from the active site of the enzyme [34,38]. By incorporation of the formate dehydrogenase catalyzed cofactor regeneration, converting formate to carbon dioxide, the reversible *CP*CR catalyzed enantioselective reduction of 2-octanone becomes irreversible. This allows the use of a formal kinetic model based on the Michaelis–Menten kinetics, which does not take into account any equilibria. The kinetic parameters of the double substrate Michaelis–Menten model were fitted to the data measured under initial reaction condition by means of non-linear regression. In Table 1 the kinetic parameters and the kinetic equations are listed. The strong product inhibition by NAD^+ observed for the reduction of 2-octanone is not of relevance since under production conditions the NAD^+ concentration is almost zero due to the cofactor regeneration. The slight product inhibition of 2-octanol appears tolerable. The oxidation of (*S*)-2-octanol (reverse reaction) is strongly inhibited by the substrates of the reduction reaction (forward reaction). The kinetics of the formate dehydrogenase from *Candida boidinii* under production conditions were published previously [39].

Both membrane reactor experiments were started with NAD^+ instead of NADH due to the lower costs of the oxidized cofactor.

3.2. Emulsion membrane reactor

The emulsion membrane reactor could be viewed as a fed batch process with continuous extraction. The aqueous phase is repeatedly charged with substrate in range of the maximal solubility due the partition coefficients, whereby the product is continuously extracted.

There are three main advantages of such a reactor design. In the first place the product is concentrated in the organic phase of the emulsion. No additional extraction steps are needed. Secondly, the enzyme will not be in contact with an organic solvent interface. And last but not least, the ttn will be increased significantly. Due to the high charge density of NAD^+/NADH the cofactor is partitioned to the aqueous phase and is recirculated. The partitioning of 2-octanone and 2-octanol in an 1:1 emulsion (organic:aqueous) is shown in Fig. 4. In the upper graph the ratio of 2-octanol/2-octanone in the organic phase and in the lower graph the maximal solubility of both components in the aqueous phase as function of the mole fraction are shown. If the organic phase consists of 100% 2-octanone, 7 mM of ketone is found in the aqueous phase, which equals the maximal solubility of 2-octanone in 50 mM TEA buffer at pH 7 and 25°C. By increasing the mole fraction of 2-octanol in the organic phase from 0 to 0.1, meaning substitution of ketone with alcohol in the organic phase, the solubility of 2-octanone in the aqueous phase is also increased. This is due to the solubility enhancement by the alcohol.

The conversion–time course for the reduction of 2-octanone by *CP*CR in the emulsion membrane reactor is shown in Fig. 5. In the case of a standard batch reaction a hyperbolic curve is

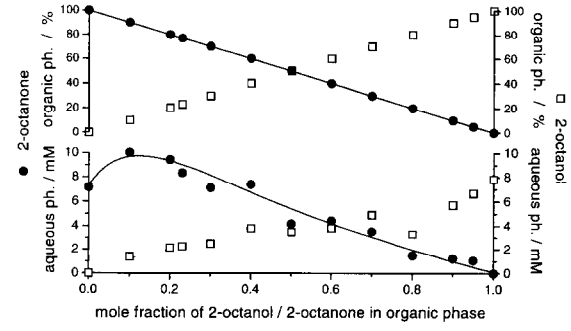

Fig. 4. Partition of 2-octanol/2-octanone in an emulsion (1:1, organic:aqueous). In the upper graph the ratio of 2-octanol/2-octanone in the organic phase and in the lower graph the maximal solubility of both components in the aqueous phase as function of the mole fraction are shown.

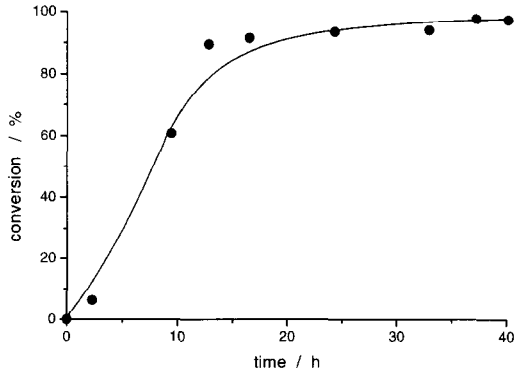

Fig. 5. Conversion as function of time for the enantioselective reduction of 2-octanone in the emulsion membrane reactor (68 mM 2-octanone, 0.5 mM NAD^+, 225 mM formate, 1 mM DTT, 0.48 U ml^{-1} *CP*CR, 1.44 U ml^{-1} FDH). At 91% conversion a space–time yield of 11 g l^{-1} day^{-1} and a ttn of 124 is reached.

expected. The slight increase in the reaction velocity which is observed might be a result of leaking some organic phase by the emulsion separating membrane.

In this emulsion membrane reactor a ttn of 124 and a space–time yield of 11 g l^{-1} day^{-1} at a conversion of 91% was reached. The enzyme consumption of the *CP*CR was determined to 22 380 U kg^{-1} (*S*)-2-octanol.

The applied amount of substrate is, due to the repeated charging of the aqueous phase, no longer determined by its maximal solubility in water but instead by the volume of the organic phase. Since the appropriate cofactor concentration is still determined by the K_m value of the enzyme, an increase in the total turnover number is the result. In this emulsion membrane reactor there is still a potential for an increase of the ttn by a factor of 10 if the volume of the organic phase (= substrate) is increased from 1% to 10% v/v.

In the course of the reaction an increase of the transmembrane pressure (1 bar) of the hydrophilic membrane (UF-PA-20H) in the stirred emulsion vessel was observed. This might be caused by swelling of the membrane due to the organic ketone phase.

3.3. Enzyme membrane reactor

The enzyme membrane reactor works under product outflow conditions, meaning that the concentrations in every volume element are the same as those at the outlet of the reactor. If the

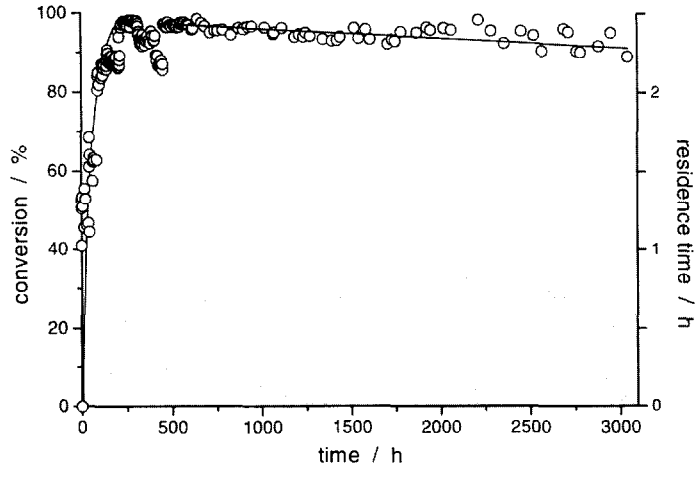

Fig. 6. Conversion as function of time for the continuous enantioselective reduction of 2-octanone in the enzyme membrane reactor (7 mM 2-octanone, 0.5 mM NAD^+, 12 mM (first 200 h) 50 mM (> 200 h) formate, 1 mM DTT, 1.5 U ml^{-1} *CP*CR, 3.2 U ml^{-1} FDH). At 97% conversion a space–time yield of 21.2 g l^{-1} day^{-1} and a ttn of 13.6 is reached.

steady state is reached, the concentrations are independent of time and place. The conversion is controlled by the enzyme concentration and the residence time. The ttn is determined by the kinetic parameters of the reaction system and the maximal solubility of the substrate under the specific reaction conditions.

In Fig. 6 the conversion–time course for the continuous production of (*S*)-2-octanol over a period of > 4 months is shown. During the first 200 h a stable conversion of 87% (residence time 1 h) was reached. Simulation of the conversion–time course for the continuous stirred tank reactor using the above determined kinetic parameters showed, that the formate concentration of 12 mM was the limiting factor. By increasing the formate concentration to 50 mM a stable conversion of 97% (residence time: 1 h) was reached. This equals a space–time yield of 21.2 g l^{-1} day^{-1}. Lowering the residence time by a factor of 2 the space–time yield increased to 40.7 g l^{-1} day^{-1} and the conversion dropped to 93%. The maximal conversion obtainable under the reaction conditions is determined by the product of the enzyme concentration and the residence time. Therefore a reduction of the residence time at high enzyme concentration will have a high effect on the space–time yield, but only a small effect on the conversion. From 450 h onwards the reactor was run at a residence time of 1 h. The process was stopped after 4 month when the conversion decreased below 90% due to enzyme deactivation. The operational half-life of the enzymes in the continuous process was in case of the *CP*CR: $\tau_{1/2} = 67$ d and in case of the FDH: $\tau_{1/2} = 88$ d. The stability of the carbonyl reductase was increased by a factor > 2 in comparison to the above determined half-life (*CP*CR: $\tau_{1/2} = 31.1$ d). A reason might be the antibacterial effect of 2-octanol. Using these values the enzyme consumption of the *CP*CR is determined to 720 U kg^{-1} (*S*)-2-octanol. For the cofactor a ttn of 13.6 was reached under these conditions.

In the case of the classical enzyme membrane reactor, the enzyme consumption is very low compared to the novel emulsion membrane reactor. One has to take into account that the first reactor is a continuously and the latter one a discontinuously operated reactor. Additionally, it is also possible that the emulsion separating membrane did leak some organic phase resulting in an enzyme deactivation. With regard to the total turnover number, the emulsion membrane reactor shows a much better performance. The ttn is increased by a factor of 9. The economic optimum depends on the relative costs of enzyme versus NAD^+.

4. Conclusion

The enantioselective reduction of 2-octanone to (*S*)-2-octanol (ee > 99.5%) is carried out by the carbonyl reductase from *Candida parapsilosis*. As a continuous reactor, the enzyme membrane reactor could be operated over a period of > 4 months at a space–time yield of 21.2 g l^{-1} day^{-1} (97% conversion, ttn = 13.6). In the newly designed emulsion membrane reactor even poorly soluble ketones can be reduced with higher total turnover numbers. In the case of the 2-octanone system the ttn could be increased by factor 9 to 124, which is due to the bigger applied mole of 2-octanone. Since the product is concentrated in the organic phase the downstream processing is simplified.

5. Abbreviations

*CP*CR	*Candida parapsilosis* carbonyl reductase
ee	enantiomeric excess
FDH	formate dehydrogenase
FID	flame ionization detector
K_m	Michaelis–Menten constant
K_i	inhibitor constant
NAD^+	nicotinamide dinucleotide (oxidized form)
NADH	nicotinamide dinucleotide (reduced form)

$\tau_{1/2}$	half-life
TEA	triethanolamine
ttn	total turnover number
V_{max}	maximal reaction velocity

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