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Coupling of permeabilized cells of *Gluconobacter oxydans* and *Ralstonia eutropha* for asymmetric ketone reduction using H_2 as reductant

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ABSTRACT

A combined two-cell reaction system containing *Gluconobacter oxydans* and *Ralstonia eutropha* was evaluated with regard to asymmetric ketone reduction using H_2 as the reductant. Whole cells permeabilized by EDTA/toluene were used, and synthesis was performed in a biphasic aqueous/organic reaction medium. The two-cell system was compared with a system in which *G. oxydans* alone was used for both ketone reduction and cofactor regeneration, using an alcohol as co-substrate. The two-cell system exhibited almost twice the initial reaction rate of the single-cell system, a higher yield (75% vs. 48%) but slightly lower enantiomeric purity (93% vs. 98%) of the product (S)-2-octanol. The permeabilized *R. eutropha* cells are worth evaluating for byproduct-free NADH regeneration in combination with other whole cell catalysts.

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1. Introduction

The use of oxidoreductases for industrial applications has attracted increased interest over recent decades, mainly due to their high potential for asymmetric synthesis. There is a huge market for chiral molecules, which is expected to increase in the future (Xu, 2005). Asymmetric reduction has been widely studied; the reduction of ketones being a typical example. These reductions are performed by NAD(P)H-dependent dehydrogenases/reductases. Alcohol dehydrogenases from horse liver (HLADH) and baker's yeast are among the most studied, but in recent decades several other dehydrogenases/reductases have been introduced, making the asymmetric reduction of a large variety of ketones possible (Kroutil et al., 2004; Nakamura et al., 2003). An efficient biocatalyst for production of chiral alcohols by stereospecific reduction of ketones is the acetic acid bacterium *Gluconobacter oxydans*, which has been used both as a whole-cell catalyst and as isolated 2-keto reductase (Adlercreutz, 1991a,b; Nanduri et al., 2000). The main use of *Gluconobacter* bacteria in biotechnology is in the selective oxidation of carbohydrates and alcohols to aldehydes, ketones and organic acids (Deppenmeier et al., 2002). Complete sequencing of the genome of one *G. oxydans* strain revealed the presence of 75 open reading frames encoding putative dehydrogenases/oxidoreductases of unknown function (Prust et al., 2005). This means that one should be aware that the transformation of a certain substrate can be catalyzed by more than one enzyme.

In general isolated enzymes often have the advantages of high volumetric productivity and few side reactions, while the drawbacks are the cost of purifying the enzyme and also possibly lower enzyme stability (Goldberg et al., 2007a,b). An alternative to using an active whole cell as a catalyst is to use a resting cell with no active metabolism, but with the desired enzyme in an active form. In this context, cell permeabilization is an important technique in facilitating the transfer of substrates and products into and out of the cells (Felix, 1982). Hydrophilic substrates can be converted efficiently in aqueous solutions, while it is often beneficial to use addition of organic solvents for conversion of hydrophobic substrates. Both homogeneous mixtures of water and water miscible solvents and organic/aqueous two-phase system have been successfully used (Butler, 1979; Adlercreutz and Mattiasson, 1987). Often organic solvents have a permeabilizing effect on the cells, which can be beneficial for the biotransformation.

The oxidoreductases used for bioreduction are NAD(P)H dependent, and an efficient cofactor regeneration system is necessary for the process to be economically feasible (Hummel, 1999). The regeneration of cofactors can be achieved by chemical, electrochemical or enzymatic methods; the enzymatic method being considered to be the most promising (Wichmann and Vasic-Racki, 2005). Many of the enzymatic regeneration methods can be applied not only in aqueous systems but also in the presence of organic solvents (Adlercreutz, 1996). Enzymatic cofactor regeneration can be divided into substrate-coupled and enzyme-coupled approaches. The dominating technology today is an enzyme-coupled system

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employing formate dehydrogenase and formate (Eckstein et al., 2004). However, a serious limitation of this method is that the regeneration reaction causes a change in pH that is difficult to control unless the reaction takes place in a large aqueous phase (Maurer et al., 2005).

One regeneration system that produces no side products is to use hydrogenases that utilize the cheapest reducing agent, H_2. There are few publications describing the practical applications of hydrogenases/H_2 (Andersson et al., 1998; Mertens et al., 2003). Many hydrogenases are reported in the literature to be quite unstable (Cammack et al., 1986). One hydrogenase that has been shown to be relatively stable is the soluble hydrogenase in the aerobic H_2-oxidizing bacterium *Ralstonia eutropha*. This hydrogenase has been demonstrated to be even more stable when it is retained in its natural host, and used as a permeabilized, resting whole-cell catalyst for NADH regeneration (Andersson et al., 1998). We have used *Ralstonia eutropha* as a whole-cell catalyst, together with a free enzyme (HLAD), for bioreduction in a previous study (Andersson et al., 1998).

In this study a broader area of application of the hydrogenase catalyst was investigated by combining it with another whole-cell catalyst, namely *Gluconobacter oxydans*. If both biocatalysts can be used in the form of permeabilized cells, laborious enzyme purification can be omitted and possibly enzyme stability can be improved. However, for this concept to work, coenzyme transport in and out of both cell types must be efficient. The concept of combining permeabilized microorganisms was first introduced by Zhang et al. in 2006, who combined a reductase-containing microorganism with a glucose-dehydrogenase-containing cell for combined ketone reduction and cofactor regeneration (Zhang et al., 2006, 2009). Reaction systems with combined permeabilized cells can be considered very flexible, as a large variety of oxidoreductase-containing native or recombinant microorganisms can be combined with a few very effective cofactor-regenerating organisms, resulting in effective reaction systems for bioreduction.

In this study, the possibility of combining a hydrogenase-containing microorganism with a keto-reductase-containing microorganism was evaluated. A model reaction in which 2-octanone was reduced to (S)-2-octanol was studied, and hydrogenase/H_2 cofactor regeneration was compared with a more conventional substrate-coupled method.

2. Materials and methods

2.1. Chemicals

2-Octanone and (S)-(+)-2-octanol were purchased from Sigma–Aldrich (St. Louis, USA) and (R)-(+)-1-phenylethyl isocyanate was supplied by Fluka Chemie (Buchs, Switzerland). All other chemicals were of analytical grade.

2.2. Cultivation of Gluconobacter oxydans

G. oxydans (DSM 50049) was cultivated on a glycerol medium (Holst et al., 1982). Cultivation was performed in batches of 100 ml in 1 l baffled Erlenmeyer flasks on a rotary shaker (150 rpm) at 28 °C. Cells were harvested by centrifugation (15,000 × *g*, 4 °C, 15 min), and washed once with 0.2 M Tris–HCl buffer (pH 7.5).

2.3. Cultivation of Ralstonia eutropha

R. eutropha (DSM 428) was cultivated on a fructose and glycerol medium (Andersson et al., 1998). Cultivation was performed in batches of 250 ml in 1 l baffled Erlenmeyer flasks on a rotary shaker (180 rpm) at 30 °C. Cells were harvested by centrifugation (15,000 × *g*, 4 °C, 15 min) and washed twice with 50 mM Tris–HCl buffer (pH 8). The hydrogenase activity was determined by the reduction of NAD (ε = 6.22 mM^{-1} cm^{-1} for NADH at 340 nm), using hydrogen gas as the substrate (Friedrich et al., 1981), and found to be 0.02 U/mg dry weight.

2.4. Preparation of permeabilized cells

A cell suspension of ~25 mg/ml (based on dry weight) was prepared in 0.1 M Tris–HCl (pH 8.0) containing 5 mM EDTA, and toluene was added to a concentration of 2% (v/v). The mixture was incubated with magnetic stirring, for 30 min at room temperature and for 30 min at +4 °C. It was then centrifuged at 13,400 rpm for 15 min, the supernatant was discarded, and the cell pellet was used immediately or stored at −20 °C until further use.

2.5. Standard procedure for the reduction of 2-octanone by G. oxydans using 2-butanol for cofactor regeneration

The reactions were performed in a biphasic aqueous/organic system. The aqueous phase contained permeabilized cells of *G. oxydans* (9 mg/ml) suspended in 1 ml 0.2 M Tris–buffer (pH 7.5) containing 10 mM $MgCl_2$, 10 mM $CaCl_2$ and 1 mM NAD^+. The organic phase consisted of dodecane containing 200 mM 2-octanone and 2 M 2-butanol. The total reaction volume was 1.25 ml, and the aqueous/organic phase volume ratio was 4:1. Each reaction was performed in a 4 ml septum-capped vial using an HTMR-131 thermo-mixer (HLC, Bovenden, Germany) at 28 °C and 600 rpm. Samples (10 μl) were taken from the organic phase at regular intervals and analysed by double injection on a gas chromatograph. The initial reaction rate was calculated for the first 6 h of reaction, and after 5 days of reaction the product was derivatized with optically pure phenylethyl isocyanate to determine the enantiomeric purity.

2.6. Standard procedure for the reduction of 2-octanone by G. oxydans using R. eutropha and H_2 for cofactor regeneration

These reactions were also performed in a biphasic aqueous/organic system. The aqueous phase contained permeabilized cells of *R. eutropha* and *G. oxydans* suspended in 0.2 M Tris–HCl buffer (pH 7.5), containing 10 mM $MgCl_2$, 10 mM $CaCl_2$ and 2 mM NAD^+. The organic phase consisted of dodecane and 200 mM 2-octanone. The total reaction volume was 1.25 ml with an aqueous/organic phase volume ratio of 4:1. The mixture was contained in 4 ml septum-capped vials and was saturated with H_2. The vials were incubated in the HTMR-131 thermo-mixer as described above. Samples were taken at regular intervals from the organic phase and analysed using gas chromatography. The headspace of the reaction vials was sparged with H_2 after each sample was removed. The initial reaction rate and optical purity were determined as described above.

2.7. Analytical methods

The reaction samples were analysed on a Varian 430 gas chromatograph (Varian, Middelburg, The Netherlands), equipped with a split/splitless injector, a flame ionisation detector and an autosampler. The separation of 2-octanone and 2-octanol was performed on a FactorFour™ VF-1ms column (15 m in length, 0.25 mm i.d. and 0.25 μm d.f.) from Varian (Varian, Middelburg, The Netherlands), using a programmed temperature of between 50 and 240 °C. The injector temperature was 200 °C and the detector temperature 240 °C. Helium at a pressure of 10 psi was used for isobaric analysis, and the retention times were 3.3 min for 2-octanone and 3.5 min for 2-octanol.

The enantiomeric excess of (S)-2-octanol was determined by derivatization of the 2-octanol produced with

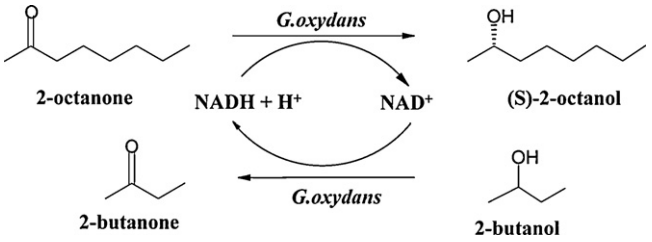

Fig. 1. Reaction scheme for the reduction of 2-octanone using 2-butanol for cofactor regeneration.

(R)-(+)-1-phenylethyl isocyanate. Generally, the derivatization reagent was added at 100% excess, compared with the amount of 2-octanol produced, together with toluene, and the mixture was incubated either for 1–2 h at 60 °C, or at room temperature overnight. The derivatization reaction was stopped by adding 10 μl ethanol and incubation either at 60 °C for 15 min, or at room temperature for 30 min. The solvent was evaporated and the sample was redissolved in toluene before the GC analysis. The same temperature program and column were used as for the determination of the total 2-octanol concentration. The retention times were 8.6 min for the derivative of (S)-2-octanol, and 8.7 min for the derivative of (R)-2-octanol.

2.8. Measurement of the partitioning of 2-butanol between water and dodecane

1 ml of water was mixed with 0.25 ml dodecane containing 0.4–3 M 2-butanol. The mixtures were incubated on a thermomixer at 28 °C and 600 rpm overnight. Samples were taken from the organic phase and the 2-butanol concentration was determined by a slightly modified version of the GC method described above. The partition coefficient for 2-butanol between water and dodecane was determined as the ratio between analysed concentration in the organic phase and calculated concentration in the aqueous phase.

3. Results and discussion

3.1. Reduction of 2-octanone with G. oxydans using 2-butanol for cofactor regeneration

Cells of *G. oxydans* permeabilized with EDTA/toluene were used for the asymmetric reduction of 2-octanone. The enzymes within the cells were activated by adding Ca^{2+} and Mg^{2+} to the reaction medium, as described previously (Adlercreutz, 1991b). The method used to regenerate NADH was the addition of 2-butanol as a second substrate, which was oxidized during the reaction (Fig. 1). Initially, comparisons were made between the pure aqueous and the aqueous/organic reaction systems. n-Dodecane was chosen as the organic phase because it was expected to be a suitable solvent regarding enzyme stability and product yield, as previously described for similar systems (Li et al., 2007). The results showed that higher product concentrations were obtained using the biphasic system than with the pure aqueous medium (data not shown). An organic phase can be favourable for both the reaction equilibrium position and in limiting substrate/product enzyme inhibition (Halling, 1994).

The effect of the 2-butanol concentration was studied in the biphasic system with n-dodecane as the organic phase (Fig. 2). Full conversion cannot be expected since there will be an equilibrium position between the two substrates and the corresponding products. In this study, a yield close to 50% was obtained for a total 2-butanol concentration of 600 mM. A total 2-butanol

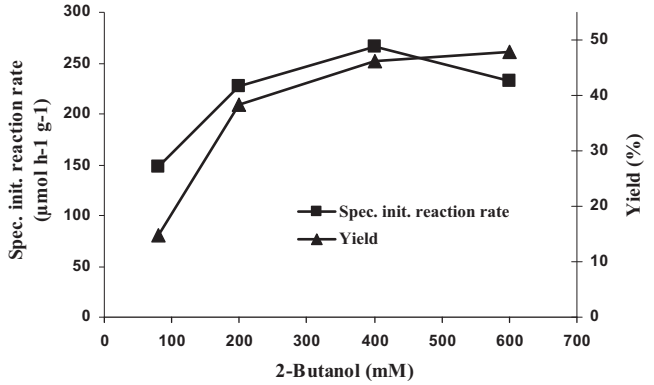

Fig. 2. Effect of the concentration of 2-butanol as the second substrate on the reduction of 2-octanone. The reaction mixture contained 9 mg/ml *G. oxydans*, 200 mM 2-octanone and 1 mM NAD^+.

concentration of 400 mM gave the highest initial reaction rate of 266 μmol h^{-1} g^{-1} cells. Concentrations of 2-butanol above 100 mM in a pure aqueous reaction system have previously been reported to be inhibitory to *G. oxydans* (Adlercreutz, 1991b). Partitioning of 2-butanol to an organic phase might decrease its concentration in the aqueous phase. To investigate this potential effect, the partition constant for 2-butanol was measured in the biphasic system used for the conversions. The partition constant was 0.08, which shows that the presence of the dodecane phase did not decrease the aqueous phase concentration of 2-butanol significantly. Thus other effects than partitioning of 2-butanol must have caused better tolerance of the *G. oxydans* cells towards 2-butanol in the present system compared to previous studies. The main function of the dodecane phase was to help in solubilising the hydrophobic substrate 2-octanone.

3.2. Reduction of 2-octanone with G. oxydans using R. eutropha and H_2 for cofactor regeneration

Cells of *G. oxydans* and *R. eutropha* were permeabilized with EDTA/toluene and used for asymmetric reduction of 2-octanone. 2-octanone was chosen as a model substrate but *G. oxydans* has a quite broad substrate specificity (Adlercreutz, 1991b). The reaction medium was presaturated with hydrogen gas, and the hydrogenase-containing *R. eutropha* cells regenerated NADH without the formation of any side products (Fig. 3). The same aqueous/organic reaction medium with n-dodecane was used. The organic phase is also favourable in this case regarding the increase in the overall amount of dissolved hydrogen in the reaction mixture. The solubility of hydrogen gas at the reaction temperature is 0.8 mM in pure water, while it is 3.5 mM in decane (Brunner, 1985; Pray et al., 1952). The K_m value of the hydrogenase can be assumed to be rather low since the K_m value of the soluble hydrogenase of the related strain *R. eutropha* H16 is 12 μM (Keefe et al., 1995). The rate of the enzymatic reaction is determined by the aqueous phase concentrations of the substrates, while the organic phase

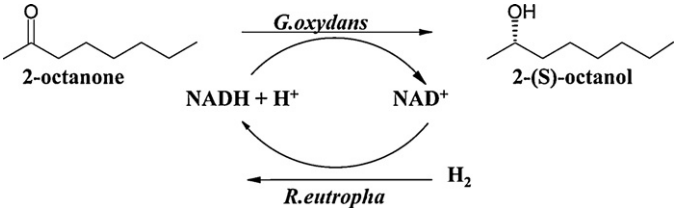

Fig. 3. Reaction scheme for the reduction of 2-octanone using *R. eutropha* for cofactor regeneration.

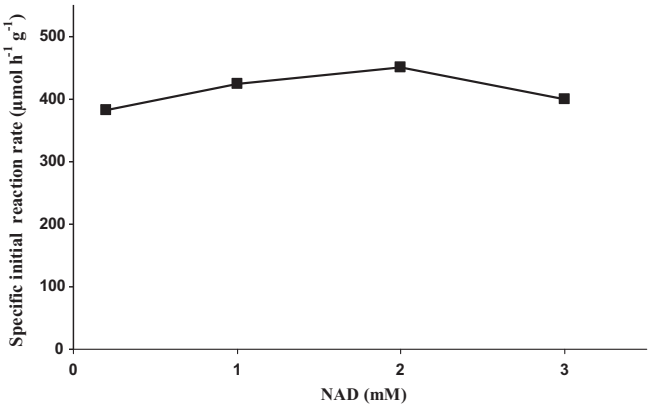

Fig. 4. Effect of the concentration of NAD^+ on the reduction of 2-octanone when *R. eutropha* is used for cofactor regeneration. The reaction mixture contained 5 mg/ml *G. oxydans*, 5 mg/ml *R. eutropha*, 2 mM NAD^+ and 200 mM 2-octanone. The specific initial reaction rate is based on the amount of *G. oxydans*.

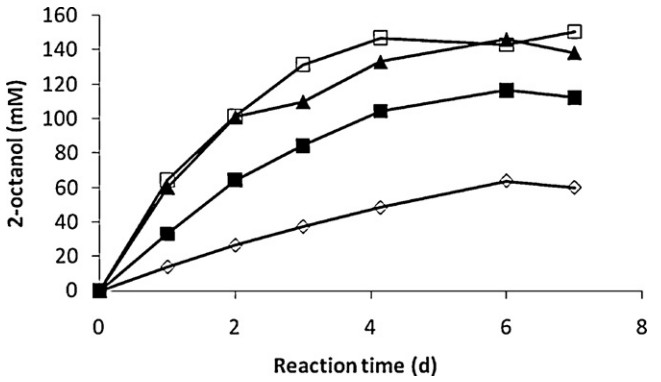

Fig. 5. The effect of the concentration of *G. oxydans* cells on the reduction of 2-octanone when *R. eutropha* cells were used for cofactor regeneration. The reaction mixture contained 2 mM NAD, 200 mM 2-octanone, 3 mg/ml (◊), 5 mg/ml (■), 9 mg/ml (▲), 12.4 mg/ml (□) *G. oxydans* cells. All reaction mixtures contained 5 mg/ml *R. eutropha*.

Table 1
Comparison between the highest values obtained for the 2 different reaction systems.

Cofactor regeneration method	Initial reaction rate ($\mu mol\ h^{-1}\ g^{-1}$)	Yield (%)	Enantiomeric excess (%)
Two-substrate	266	48	98
Two-cell	450	75	93

acts as a reservoir for 2-octanone and hydrogen. Aqueous hydrogen concentrations above 1 mM were shown to inhibit the *R. eutropha* H16 hydrogenase (Keefe et al., 1995). The reaction mixture should be properly mixed to assure efficient mass transfer between the phases.

In the approach using two types of permeabilized cells, it is necessary that the coenzyme can be effectively transported in and out of both cell types, which might require higher overall coenzyme concentrations than in other regeneration methods. It was therefore important to evaluate the influence of the coenzyme concentration on the overall conversion rate. Experiments were performed with NAD^+ concentrations in the interval between 0.2 and 3 mM. All the concentrations investigated provided higher initial specific reaction rates than the reaction system with 2-butanol (Fig. 4), which shows that the transport and regeneration of the coenzyme worked very well. Coenzyme concentrations in this range are quite often used for biocatalytic redox conversions. It should be kept in mind that when using 2-butanol as the co-substrate, the *G. oxydans* cells are used both for the reduction of 2-octanone and the oxidation of 2-butanol. In case these two reactions are catalyzed by the same enzyme there will be a competition for that enzyme which means that not all of the enzyme can be used for the ketone reduction. This effect could decrease the specific activity of the *G. oxydans* cells when measured in the reduction reaction compared to the case when these cells are only used for the reduction. The initial reaction rates obtained in the two-cell method were higher than those reported for an aqueous system for 2-octane reduction by *G. oxydans* and formate dehydrogenase (FDH) (Adlercreutz, 1991a).

The reaction time course for reactions containing different amounts of *G. oxydans* cells is presented in Fig. 5. The initial reaction rate and the maximum yield increased with increasing concentration of *G. oxydans* cells up to 9 mg/ml, and the best final yield reached about 75%. No further conversion was observed after about 6 days reaction, possibly due to enzyme inactivation. The enantiomeric excess for the products were in the interval 90–95%. The fact that lower enantiomeric excess was obtained with this regeneration method compared to the two-substrate approach, can be due to multiple enzymes catalyzing the reduction reaction with different stereoselectivity. The enantiomeric excess of the product obtained will differ if the reaction conditions used for the two regeneration methods influence the activities of the competing enzymes differently. Alternatively, the occurrence of the back-reaction (in this case oxidation of 2-octanol) might lower the enantiomeric excess. However, this explanation is less likely since this type of effect is more likely to occur in the two-substrate approach (Yang et al., 1997).

The two reaction systems studied are compared in Table 1. The potential of the two-cell system is high regarding the initial reaction rates, and high reaction yield. An advantage of the two-substrate system is the higher enantiomeric purity of the product. A big advantage of the two-cell system is that the reaction mixture contains no side product from the co-substrate and this will enhance the product purification. The method of cofactor regeneration should be chosen depending on which criteria are most important.

4. Conclusions

In this study it has been demonstrated that whole cells of *R. eutropha* can be used as a cofactor regeneration system in a combined two-cell reaction system, for asymmetric ketone reduction by *G. oxydans*. This approach, using H_2 as reductant, gave higher initial reaction rates both compared to two-substrate cofactor regeneration and FDH/formate (Adlercreutz, 1991a).

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