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

Fabian Rundbäck, Milica Fidanoska, Patrick Adlercreutz*

Department of Biotechnology, Lund University, P.O. Box 124, SE-221 00 Lund, Sweden

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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.

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

* Corresponding author. Tel.: +46 46 2224842; fax: +46 46 2224713.
E-mail address: Patrick.Adlercreutz@biotek.lu.se (P. Adlercreutz).

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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