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Synthesis of ethyl (*R*)-mandelate using recombinant *Carboxydothermus hydrogenoformans* alcohol dehydrogenase produced by two yeast species

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ABSTRACT

Yeast cell catalysts carrying a recombinant *Carboxydothermus hydrogenoformans* alcohol dehydrogenase (ChADH) gene were used to synthesise ethyl (*R*)-mandelate. Transgenic *Arxula adeninivorans* and *Hansenula polymorpha* strains were constructed to produce recombinant ChADH at high concentrations. Biochemical parameters such as pH and temperature optima, thermostability and substrate specificity were determined for the enzyme synthesized in *Arxula adeninivorans*. The recombinant enzyme combined with a substrate-coupled cofactor regeneration system and permeabilized *Arxula adeninivorans* and *Hansenula polymorpha* cell catalysts co-expressing *ChADH* and *Bacillus megaterium* glucose dehydrogenase (*BmGDH*) for enzyme-coupled cofactor regeneration, were used to synthesize ethyl (R)-mandelate. Comparison of purified recombinant ChADH/BmGDH and both of the yeast cell based catalysts ability to synthesize ethyl (*R*)-mandelate demonstrated that *Hansenula polymorpha* cell catalysts were able to produce the highest yield (*Hansenula polymorpha* 6.07 mmol l^{-1} h^{-1}) while *Arxula adeninivorans* produced approximately half this amount (3.07 mmol l^{-1} h^{-1}). The maximum conversion achieved was 98% with a high enantiomeric excess (>98%).

1. Introduction

Chiral alcohols are key building blocks for the fine chemicals industry [1]. Alcohol dehydrogenases (EC 1.1.1.1; ADHs) can be used for the synthesis of these compounds under mild ambient temperature and atmospheric pressure conditions. This avoids the problems of isomerization, racemization, epimerization or rearrangement of compounds associated with the harsh conditions used in conventional chemical processing. While chemical synthesis produces an equal amount of each enantiomer, i.e. the maximum conversion for one enantiomer is only 50%, enzymatic reactions can be conducted with remarkable stereoselectivity which permits the production almost exclusively of one enantiomer. As well as acting as chiral catalysts with their natural substrates, many enzymes are also able to catalyze reactions with a range of other substrates [2].

The *ChADH* gene (*chy1186*) was sourced from the genome of the thermophilic bacterium, *Carboxydothermus hydrogenoformans* Z-2901 [3] which was isolated from a hot spring and has an optimal growth rate at 78 °C. In the cell, ChADH participates in fatty acid and polyunsaturated fatty acid biosynthesis and is known as a 3-ketoacyl-ACP (acyl-carrier-protein) reductase.

In a previous study [4], the *ChADH* gene was overexpressed in *E. coli* and the resulting enzyme was purified and characterized. ChADH has been annotated as an SDR (short-chain dehydrogenases/reductases) with the molecular mass of about 30 kDa [3]. The highest enzymatic activity for the reduction reaction occurred when an asymmetric reduction of ethyl benzoylformate and the synthesis reaction of the reduction product were performed [4].

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Synthesis of ethyl (R)-mandelate by ChADH with ethyl benzoylformate as substrate was also conducted with *Saccharomyces cerevisiae* cells entrapped in alginate fibres [5]. The superiority of yeast cells over bacterial cells is robustness in organic solvents due to the cell wall construction [6]. The organic phase allows solubilisation of substrates which are not soluble in water and thus allows the synthesis reaction to occur.

Ethyl benzoylformate is an important chiral building block for organic synthesis [7]. It is often used as substrate in microbial reductions because of its similarity to pyruvate [8,9]. Ethyl (R)-mandelate, the product of ethyl benzoylformate reduction, is an intermediate in the synthesis of cyclandelate, hydrobenzole or pemoline [10] and it has been used in the study of stereochemistry [11].

This study describes the use of the yeast *Arxula adeninivorans* and *Hansenula polymorpha* as hosts for the synthesis of recombinant ChADH using purified enzymes and permeabilized yeast cells as catalysts for the enzymatic conversion of ethyl benzoylformate to ethyl (R)-mandelate. Expression of the enzyme encoding gene in two different yeast species was explored because both are known as effective producers of recombinant proteins [12,13] and it is possible that one of the yeast species will be more suitable for an industrial application. The transformation/expression platforms, pFPMT121 [13,14] and Xplor®2 [15], were used for *H. polymorpha* and *A. adeninivorans*, respectively.

Several methods to entrap live cells to prolong the stability of catalytic activity have been reported in the literature [16] and entrapment of *S. cerevisiae* cells in alginate fibres with double gel layers has shown excellent stability with no leakage of the cells from the matrix [5]. In the present study, calcium alginate and Lentikat® PVA gel were used to immobilize the cells. The Lentikat® matrix is based on polyvinyl alcohol (PVA), which is non-toxic and forms hydrogels with excellent mechanical properties [17]. Stability and relative activity under optimal reaction conditions using these immobilization approaches to entrap *A. adeninivorans* and *H. polymorpha* permeabilized cell biocatalysts were compared.

2. Materials and methods

2.1. Chemicals

1-phenylethanol, 2-phenylethanol, 1-nonanol, 2-nonanone, 2-nonanol, 1,2-butanediol, 1,6-hexanediol, ethyl (R)-4-chloro-3-hydroxybutanoate, ethyl (S)-4-chloro-3-hydroxybutanoate, ethyl-4-chloroacetoacetate, butyraldehyde were purchased from Acros (Germany). Pentanal, 1-hexanol, hexanal, 4-hydroxy-3-butanone and 2-butanol were from Sigma (Germany). Methanol, ethanol, isopropanol, 2-butanone, acetone, NAD^+ were purchased from Roth (Germany) and NADH was purchased from Roche Diagnostics (Germany). 1-Butanol, 1-pentanol and glucose were from Applichem (Germany) and 2,5-hexanedione and 5-chloropentanone were from Merck (Germany). Ethyl benzoylformate, ethyl (R)-mandelate, ethyl (S)-mandelate and ethyl pyruvate were purchased from Sigma (Germany). Ethyl mandelate was from Merck (Germany) and ethyl benzoylacetate was from Alfa Aesar (Germany).

2.2. Strains and culture conditions

E. coli XL1 blue [*recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lac* [F′*proABlacI* q Z DM15 Tn10 (Tetr)]], obtained from Invitrogen (USA), was the host strain for bacterial transformation and plasmid isolation. The cells were grown on LB medium (Sigma, USA) supplemented with 50 mg l^{-1} ampicillin (Applichem, Germany).

The auxotrophic mutants *A. adeninivorans* G1212 (*aleu2 atrp1:ALEU2*) [18], G1216 (*aleu2 aade2:ALEU2*) [19], MS1006 (*aleu2 ALEU2:aade2–ALEU2:atrp1*) [20] and *H. polymorpha* Kla8 (Δ*ura3*, Δ*leu2*) [21] were used as the yeast host organisms to produce the strains which co-expressed *ChADH-6H* and *BmGDH*. *A. adeninivorans* was grown at 30 °C under selective conditions in yeast minimal medium – $NaNO_3$ supplemented with 20 g l^{-1} glucose as carbon source (YMM-glucose-$NaNO_3$) [22,23] or under non-selective conditions in a complex medium (YEPD). *H. polymorpha* was initially grown at 37 °C in YMM-glucose-$NaNO_3$ or in a complex medium (YEPD) and then at 48 h, switched to YMM–$NaNO_3$ or YP (1% yeast extract, 2% peptone), both supplemented with 2% glycerol. After further 48 h incubation, methanol was added at 1% and the cells were cultivated for a further 24 h. Agar plates were prepared by adding 16 g l^{-1} agar to the liquid media.

2.3. Transformation procedures, recovery of stable yeast strains, isolation and characterization of nucleic acids

E. coli was transformed according to a standard protocol [24] and *A. adeninivorans* G1212, G1216 or MS1006 cells were transformed according to Böer et al. [15]. *H. polymorpha* Kla8 was transformed as for *A. adeninivorans,* however, undigested circular plasmids were used for the transformation. Stable yeast transformants were obtained by passaging on selective and non-selective media [25]. Isolation of plasmid DNA and DNA restrictions were carried out as described previously [26].

2.4. Construction of ChADH and ChADH-BmGDH expression plasmids

2.4.1. Construction of plasmid Xplor2.2-ChADH

The Xplor®2 platform has been established as a transformation/expression platform in *A. adeninivorans*. It allows construction of resistance-marker free transformants [15]. The system consists of bacterial vector backbone into which yeast selection and expression modules are integrated between 25S rDNA segments to produce a linear fragment of DNA, which can be transferred into an auxotrophic mutant strain. The advantage of this system is that multiple integrations into the genome are possible and the DNA has expression modules with the *TEF1* promoter, which enables constitutive gene expression.

The open reading frame (ORF) was established for *ChADH* for de novo gene synthesis (GeneArt, Germany) based on the published amino acid sequence of ChADH from *C. hydrogenoformans* [3]. The ORF using optimized codon usage for *A. adeninivorans* and *Eco*RI and *Bam*HI restriction sites at the 5′- and 3′-ends, respectively, was purchased from GeneArt (construct no. 14AER67C). PCR was done with gene specific primers on the GeneArt template to obtain the ORF of *ChADH* gene with a His-tag encoding sequence on 5′ end. The primers used were, primer 1, (5′-GCC**GAATTC**ATGCACCATCATCACCACCACGGATTCAAGGACAAGG-TG-3′, nucleotide positions 1–39, *Eco*RI restriction site in bold type) and primer 2, (5′-GCC**GGATCC**TTAAGGCTCGTAGATCATCTT-3′, nucleotide positions 745–765, *Bam*HI restriction site in bold type). PCR was performed to produce an ORF of *ChADH* gene with the His-tag encoding sequence on 3′ end of primer 3; (5′-GCC**GAATTC**ATGGGATTCAAGGACAAGGTG-3′, nucleotide positions 1–21), and primer 4, (5′-GCC**GGATCC**TTAGTGGTGGTGATGATGGTGAGGCTCGTAGATCATCTT-3′ nucleotide positions 727–765).

The *Eco*RI-*Bam*HI *ChADH-6H, ChADH* and *6H-ChADH* gene fragments, were inserted into the plasmid pBS-TEF1-PHO5-SA (flanked by *Apa*I-*Sal*I restriction sites [12]) between the *A. adeninivorans*-derived *TEF1* promoter and the *S. cerevisiae* derived *PHO5* terminator. Construction of plasmids with ChADH-6H, ChADH and