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Enantioselective enzymatic synthesis of the α-hydroxy ketone (*R*)-acetoin from *meso*-2,3-butanediol

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ABSTRACT

Acetoin (3-hydroxy-2-butanone) is an important flavour compound and is applied in cosmetics, pharmacy and chemical synthesis. In contrast to chemical syntheses or fermentations an enzymatic route facilitates enantioselective acetoin production. The discovery of a (*S*)-selective alcohol dehydrogenase enables a novel production process of (*R*)-acetoin from *meso*-2,3-butanediol. It was shown that the regeneration of oxidised nicotinamide adenine dinucleotide is a key point in preparative application of dehydrogenases for the oxidative route. An electrochemical regeneration system was successful combined with the ADH catalysed reaction. Up to 48 mM (*R*)-acetoin was produced in the reaction system while productivities up to 2 mM h^{-1} were reached. The possibility to apply an electrochemical system in a semi-preparative synthesis will stimulate further research of electroenzymatic processes with oxidoreductases.

1. Introduction

Acetoin (3-hydroxy-2-butanone) is an important flavour compound, widely existing in dairy products and some fruits. Because of its unique butter flavour, it is used as flavour enhancer of butter, cheese, coffee and nut containing food. Furthermore, it was classified as one of the 30 platform chemicals which were given the priority to their development and utilisation by the U.S. Department of Energy [1]. It can be widely applied in cosmetics, pharmacy and chemical synthesis. Many efforts have been made to develop acetoin production methods including chemical synthesis and fermentative technologies. Acetoin can be produced chemically by partial reduction of diacetyl, selective oxidation of 2,3-butanediol and oxidation of 2-butanone followed by basic hydrolysis. Furthermore, acetoin is an important physiological metabolite excreted during fermentation by a lot of microorganisms, such as *Serratia marcescens* [2], *Bacillus* species [3,4], *Paenibacillus polymyxa* [5] and *Lactococcus lactis* [6]. Chemical syntheses or fermentations generally lead to the production of racemic mixtures [7]. For the application as flavour component and as intermediate for the synthesis of building blocks the production of racemic acetoin is often sufficient. Enantiopure acetoin is widely used to synthesise novel optically active α-hydroxy ketone derivatives and liquid crystal composites [8]. Furthermore, it was shown that (*R*)-acetoin is a female sex pheromone of the beetle *Amphimallon solstitiale* [9]. (*R*)-Acetoin is highly attractive to swarming males, whereas neither *rac*-acetoin nor the 2,3-butanediols shows activity.

For this wide range of application it is essential to develop an effective process to produce enantiomeric pure acetoin. Due to the fact that enantioselectivity is one key advantage of enzyme-catalysed oxidation we investigated an enzymatic process to produce acetoin. In general, three different classes of enzymes can be used to produce α-hydroxy ketones (Scheme 1). These α-hydroxy ketones can be used as building blocks for compounds of pharmaceutical interest, e.g. antidepressants, HIV-protease inhibitors and antitumorals [10,11]. The oxidative synthesis of enantiopure acetoin starting from 2,3-butanediol would be a highly attractive route due to the availability and costs of the substrate. Different biotechnological routes to produce (*S*)-acetoin from *meso*-2,3-butanediol and (*R*)-acetoin from (2*R*,3*R*)-butanediol are described in literature [12–14]. Until now, no (*S*)-selective ADH for the conversion of

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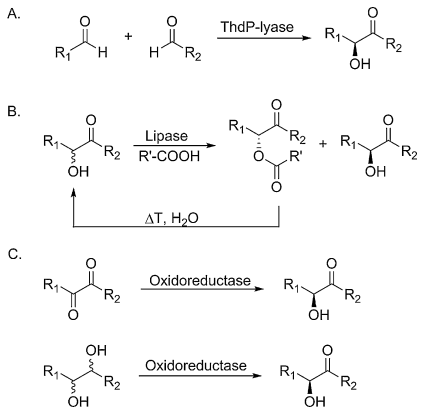

Scheme 1. Enzymatic production of α-hydroxy ketones: A – thiamine diphosphate-dependent lyase (ThdP-lyase) can be used to catalyse the carboligation of aldehydes; acetoin is obtained by self-ligation of acetaldehyde, B – lipases can be applied in dynamic kinetic resolutions. Lipase-catalysed kinetic resolutions are combined with racemisation of the substrate, C – oxidoreductases can be used in reductions of diketones and by selective oxidations of vicinal diols (in the synthesis of acetoin R_1, $R_2 = CH_3$).

the 2,3-butanediol is described. Recently, it was shown that different alcohol dehydrogenases from the proprietary c-LEcta collection exhibit high activity for the enantioselective oxidation of aldehydes. The ADH-9 which was identified from a biodiversity library was shown to have exceptional high oxidative activity as well as (*S*)-specific activity onto the substrates 2-phenyl propionaldehyde, benzyl aldehyde and benzyl alcohol, hexanal and hexanol, propanal and propanol as well as flurbiprofen aldehyde [15]. In the present study the (*S*)-specific ADH-9 was investigated to produce (*R*)-acetoin by an oxidative route starting from butanediol (Scheme 1).

2. Materials and methods

2.1. Chemicals

All chemicals were of analytical grade or higher quality and purchased from Fluka (Buchs, Switzerland) or Sigma–Aldrich (Taufkirchen, Germany).

2.2. Enzyme production

The ADH-9-gene was expressed in *Escherichia coli* BL21 (DE3) as described [15]. In brief, recombinant *E. coli* cells were cultured in ZYM505 medium [16] at 37 °C to an optical density of 0.7 at 600 nm. Expression was induced by addition of 0.1 mM isopropyl-β-D-thiogalactopyranoside and the cultivation temperature was reduced to 30 °C. Cells were harvested after overnight induction and resuspended with 0.05 M Tris/HCl buffer (pH 7.0) containing 2 mM $MgCl_2$. Cells were lysed by sonication, the supernatant was separated from insoluble matter by centrifugation and freeze dried. The specific activity of the enzyme was 7.14 U per mg enzyme determined by using the substrate *meso*-2,3-butanediol.

2.3. Adsorptive immobilisation of ADH-9 onto Amberlite FPA54

100 mg lyophilised ADH-9 was dissolved in 10 mL 0.1 M Tris/HCl buffer pH 9.0. The enzyme solution was added to 7 g Amberlite FPA54 carrier in a crystallisation bowl. The suspension was incubated for 12 h at 23 °C. After incubation, the supernatant was taken and analysed via Bradford assay (Roti-Quant) for calculating the amount of protein. The particles were washed once with 5 mL 0.1 M Tris/HCl pH 9.0 buffer. The immobilisation yield on Amberlite FPA54 was 73%, with 2% protein loss in the wash solution. Consequently an ADH-9 loading of 10 mg ADH-9/g Amberlite FPA54 could be achieved. The specific activity of the immobilised enzyme was 0.01 U per mg carrier determined by using the substrate *meso*-2,3-butanediol.

2.4. Characterisation of the ADH-9

All enzymatic conversions were performed in 0.1 M Tris/HCl (pH 8.0) buffer at 30 °C. NADH-assays were performed measuring absorption of NADH formed during the reaction at 340 nm continuously in a Shimadzu spectrometer or by taking samples after distinct time of incubation and measuring absorption at 340 nm. Experiments measuring the absorption at 340 nm continuously contained 0.01 mg mL^{-1} of soluble ADH-9, 5–100 mM 2,3-butanediol (mix of isomers) and 0.1 mM NAD^+. Assays performed by taking samples contained 0.01–0.1 mg mL^{-1} ADH-9 as soluble form or 10 mg mL^{-1} as immobilised form, 25 mM 2,3-butanediol (mix of isomers), 25 mM NAD^+. Reaction vessels were shaken at 75 rpm (Certomat-R, B. Braun, Biotech International, Melsungen). Experiments regarding the selectivity were performed using same concentrations as before and each of the enantiomer of the substrate. Samples were incubated for 1 h and extracted with ethyl acetate for GC analysis. Investigations for testing the electrochemical stability of the enzyme and substrate were performed in an electrochemical reactor. The reaction solution containing 0.12 mM 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), and 10 mg mL^{-1} enzyme or 50 mM substrate was polarised at 600 mV vs. Ag/AgCl. The electrode set-up consisted of polished glassy carbon ($A = 2.0\ cm^2$, HTW Hochtemperatur-Werkstoffe GmbH, Thierhaupten, Germany) as working electrode, a platinum plate as counter electrode and a Ag/AgCl (3 M KCl) reference electrode (SE 21, Sensortechnik Meinsberg, Waldheim, Germany). After different incubation times samples were taken and analysed in an NADH-assay (enzyme containing samples) or with GC (substrate containing samples, see Section 2.6).

2.5. Electroenzymatic conversion

Electroenzymatic reactions using the immobilised or soluble ADH-9 were performed in the above-mentioned glass reactor with a reaction volume of 20 mL. The reaction solution by using the immobilised enzyme contained 0.1 or 1 mM ABTS, 75 mM *meso*-2,3-butanediol, 0.1 U mL^{-1} immobilisate and 1 mM NAD^+. Reaction solutions with soluble ADH-9 contained 1 mM ABTS, 75 mM *meso*-2,3-butanediol, 0.1 or 10 U mL^{-1} soluble ADH-9 and 1 mM NAD^+. After different time of incubation samples were taken and extracted with ethyl acetate for GC analysis. The potential of the working electrode was 600 mV vs. Ag/AgCl (3 M KCl). Further reactions have been implemented in a 3-dimensional electrochemical cell working as a loop reactor. The reactor consisted of a packed bed working electrode between two cathodes. The packed bed was built up of 7.2 g splintered glassy carbon particles (1000–2000 μm; HTW Hochtemperatur-Werkstoffe GmbH, Thierhaupten, Germany). The cathodes were iridium-based oxide plated titanium net electrodes (Metakem, Usingen, Germany). The electrodes were separated with a cation exchange membrane (THOMAPOR® CMX, Reichelt Chemietechnik, Heidelberg, Germany). Each cathode had a separate catholyte chamber. The Ag/AgCl (3 M KCl) reference electrode was positioned in one of these chambers (for further information see [17]). The reaction solution containing 1 or 5 U mL^{-1} ADH-9, 75 mM *meso*-2,3-butanediol, 1 mM NAD^+ and 1 mM ABTS was pumped vertically upwards through the packed bed anode with a flow rate of 2.5 mL min^{-1}. The total reaction volume was 40 mL. After different time of incubation samples were taken and extracted for GC analysis.