Stereoselective Reduction of 2-Phenylpropionaldehyde by Alcohol Dehydrogenase with Cofactor Regeneration*

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Stereoselective reduction of 2-phenylpropionaldehyde catalyzed by alcohol dehydrogenase was investigated. Because of the water insolubility of the substrate and the target product (2-phenyl-1-propanol) organic solvent was used. The process was intensified by the cofactor (NADH) regeneration in the same media, using ethanol/acetaldehyde reaction as hydrogen carrier system. The aim of the study was to realize the enzymatic process and *in situ* cofactor regeneration, simultaneously.

Laboratory experiments in shake flasks system were carried out to determine the optimal reaction conditions. Gas chromatography was used to follow the conversion and the enantioselectivity of this reaction. Different organic solvents were tested and isopropyl ether was found the most suitable. In water—isopropyl ether solvent system ($\varphi_r(V_W/V_O) = 37/63$), optically pure (min. 95 %) (*S*)-2-phenyl-1-propanol was produced with 25 % yield after 2 h of reaction time.

Nowadays, chiral compounds play an important role in pharmaceutical, agrochemical, cosmetic, environmental, and other industries. 2-Phenyl-1-propanol is valuable raw material for the cosmetic industry. It serves for corresponding esters production used as fragrances (fruity bouquet) [1]. In addition, this substance is an initial material also for other syntheses [2, 3].

The optically active alcohols are produced by enantioselective reduction of prochiral ketones and aldehydes [4]. In these reactions, enzyme catalysts exhibiting high oxidation and reduction activity are applied. The most commonly used oxidoreductase enzymes are the alcohol dehydrogenases [5]. These enzymes are potential industrial catalysts due to their broad substrate specificity [6].

Additional substances, besides the enzyme and the substrate, are often required so that the reaction could be carried out. These compounds, coenzymes, take part in the reaction itself often as carriers of some chemical groups. Therefore, they may be regarded as an essential part of the catalytic mechanism. One of the coenzymes of alcohol dehydrogenases is nicotinamide adenine dinucleotide (NAD). The principal duty of NAD^+/NADH system is the transport of hydrogen atoms. Due to the high cost of cofactors required in stoichiometric amount, the design of economically profitable syntheses comprises the cofactor regeneration [7]. The coenzymes can be readily regenerated by another reaction catalyzed by the same enzyme (the second substrate method) [8].

In this study, the stereoselective reduction of 2-phenylpropionaldehyde to 2-phenyl-1-propanol was investigated. Simultaneously, ethanol, a substrate of the second (regeneration) reaction, was oxidized to acetaldehyde in the same media. Because of the substrate and product water insolubility, an organic solvent is required. Therefore, the investigated reaction was carried out in a two-phase system.

EXPERIMENTAL

All organic solvents (isopropyl ether, *tert*-butyl methyl ether, ethyl acetate, and ethyl alcohol) were of anal. grade, racemic 2-phenylpropionaldehyde (98 %) and (±)-2-phenyl-1-propanol (97 %), as well as trifluoroacetic anhydride (99 %) (derivatizing agent for GC analysis) were purchased from Aldrich. Equine liver alcohol dehydrogenase, content in solid (0.3 units mg^{-1}), and β-nicotinamide adenine dinucleotide were obtained from Sigma. (*S*)-2-Phenyl-1-propanol (99 %) was purchased from Fluka.

The influence of organic solvent, volume of water phase, initial amount of ethanol, reaction temperature, and amount of (±)-2-phenylpropionaldehyde on the reaction was investigated. In all experiments 5 cm^3 of organic solvent were used. The amount of β-NADH was 60 μg = 85 nmol (0.3 cm^3 of freshly made stock so-

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lution containing 0.2 mg cm^{-3} of substance in aqueous buffer solution). The reactions were initiated by addition of 1.2 unit of alcohol dehydrogenase enzyme. The reaction mixtures were shaken in a thermostated incubator G24 type, New Brunswick Scientific Co. (USA). In all the laboratory experiments 160 min^{-1} shaking rate was used. During the kinetic measurement, aliquot samples were taken out and analyzed directly by GC.

Gas chromatography was used to determine the substrate and product concentrations, to measure the concentration of the second substrate, ethanol, and to determine the optical purity of the product. HP 5890 (Hewlett—Packard) gas chromatograph with a flame ionization detector was used. A Permabond OV 1701 type capillary column (0.50 μm × 25 m × 0.2 mm, Macherey—Nagel) was used to measure the 2-phenylpropionaldehyde and 2-phenyl-1-propanol content in the samples (150 °C isothermal) and to determine the ethanol content in certain samples (50 °C isothermal). A chiral capillary column Lipodex E (0.2 μm × 25 m × 0.25 mm, Macherey—Nagel) served for the quantitative determination of (*S*) and (*R*) enantiomers of 2-phenyl-1-propanol product. In the latter case a pre-column derivatization was needed. The trifluoroacetate derivative of the analyte was prepared by the reaction with trifluoroacetic anhydride. In preliminary tests it was found that the unreacted excess of 2-phenylpropionaldehyde remained in an original form during the derivatization process and had no effect on this reaction. The derivatization was carried out in isopropyl ether solvent. 0.5 cm^3 of trifluoroacetic anhydride was added to 2.5 cm^3 of sample solution. The mixture was refluxed for 0.5 h. After cooling it to room temperature, 8 cm^3 of Na_2CO_3 solution (5 mass %) were added to neutralize the mixture. Then, the phases were separated and 0.5 mm^3 of the organic phase was injected into the GC.

RESULTS

Selection of the Suitable Organic Solvent

Because of the substrate and product water insolubility, the reaction was carried out using organic media. In the system studied a small amount of water is essentially needed for the enzyme action. In our previous work focused on investigation of lipase enzyme behaviour [9, 10], important role of the solvent used in the enzymatic synthesis carried out in nonaqueuos media was found. Based on the literature data [5] and our pre-experiments, three different solvents (water immiscible or slightly miscible, nonpolar organic materials) were tested.

Investigation of the organic solvent influence on the 2-phenylpropionaldehyde stereoselective reduction was carried out in the presence of 0.3 cm^3 of buffer solution (it means $\varphi_r = 6/94$), 150 mg (1.12 mmol) of (±)-2-phenylpropionaldehyde, and 460 mg (10.0 mmol) of ethanol. The reaction temperature was 20 °C. The amount of product was determined during the process, the values are shown in Fig. 1.

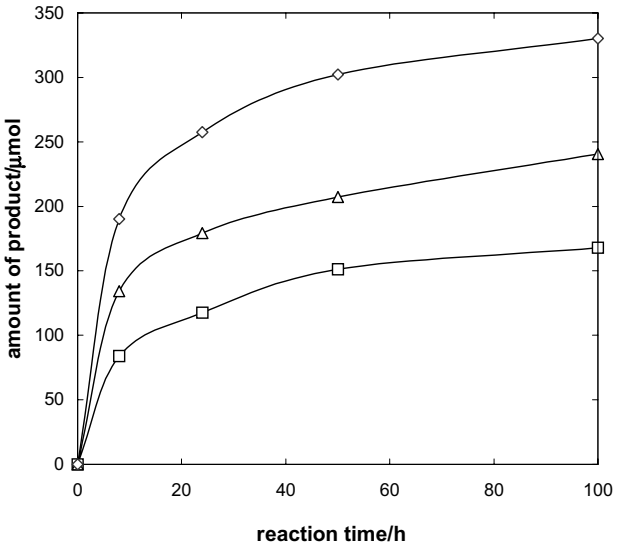

Fig. 1. 2-Phenyl-1-propanol production in the presence of ethyl acetate (□), *tert*-butyl methyl ether (△), and isopropyl ether (◇) solvents at 20 °C.

The best results were achieved in isopropyl ether solvent. After 50 h reaction time 0.30 mmol (26.8 % yield) of product was detected under given reaction conditions. Under the same conditions, lower yield of 2-phenyl-1-propanol was obtained if *tert*-butyl methyl ether or ethyl acetate as the organic solvent were used. Therefore, further experiments were carried out in isopropyl ether—buffer solution reaction media.

Effect of the Water/Organic Solvent Ratio (φ_r) on the Reaction

The activity and stability of enzymes depends on the reaction media composition [11]. In two-phase (ternary mixture) systems, reactions take place in an aqueous phase, being the enzyme and the cofactor insoluble in nonpolar organic media. Moreover, organic phase causes the enzyme denaturation. These effects can be reduced by dilution. Thus, increased volume of water usually enhances the enzyme stability. On the other hand, larger amount of water may decrease the reaction rate since the substrates concentration is reduced.

Kinetic measurements were carried out to study the effect of the φ_r (V_W/V_O ratio). Keeping constant volume of isopropyl ether (5 cm^3), different amount of buffer solution was added (from 0 cm^3 up to 4.7 cm^3). The amount of substrates was: 1.12 mmol of (±)-2-phenylpropionaldehyde and 10.0 mmol of ethanol. The reaction temperature was 20 °C, while the other reaction parameters were not changed. The results after 4 h reaction time are presented in Fig. 2.

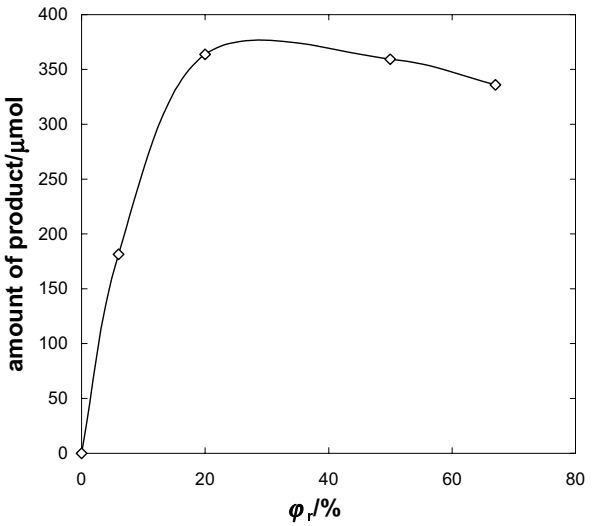

Fig. 2. Effect of the water to solvent ratio, φ_r, on 2-phenyl-1-propanol production after 4 h of reaction time at 20 °C.

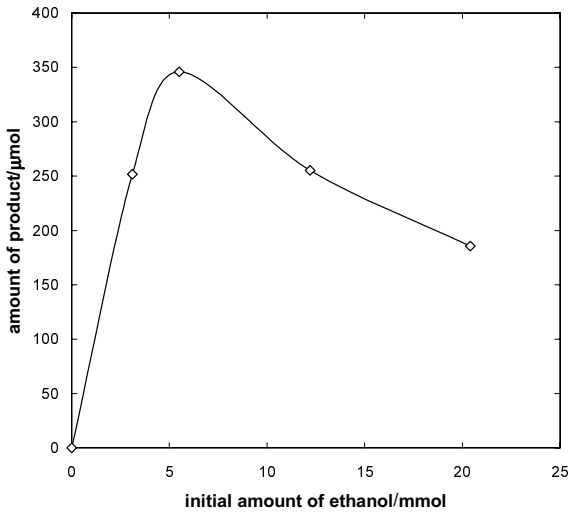

Fig. 3. Effect of the initial amount of ethanol on 2-phenyl-1-propanol production after 8 h of reaction time at 20 °C.

The yield was significantly increased by rising the volume of water phase up to $\varphi_r = 20/80$. The further addition of buffer solution caused only small differences. Over $\varphi_r = 40/60$, the amount of product formed in reaction decreased. Further experiments were carried out with the optimum water/isopropyl ether ratio $\varphi_r = 37/63$, using 2.7 cm^3 of buffer solution and 0.3 cm^3 of stock solution of β-NADH with 5 cm^3 of isopropyl ether.

Effect of Ethanol Concentration

Ethanol has two essential functions in this system. It is the substrate of the cofactor regeneration, and, on the other hand, a compound distributed between the two immiscible phases (water/isopropyl ether). Due to its peculiar character, ethanol plays a special, important role in the substrate transport between the enzyme surface and liquid phases.

Due to the lack of information, ethanol distribution constant in the system investigated (isopropyl ether—aqueous buffer solution) had to be evaluated. The measurements were carried out for $\varphi_r = 37/63$ solvent mixtures. Different amount of ethanol (1.7—40 mmol) was added to this mixture and after 30 min of shaking samples were taken out from the organic phase. The ethanol concentration was determined directly by GC analysis. Constant distribution coefficient value was found for examined concentration range showing that the solubility of ethanol is three times higher in buffer solution compared to the isopropyl ether phase.

The effect of the ethanol amount on the reaction course was determined by the amount of 2-phenyl-1-propanol formed. Besides the most favourable reaction parameters found earlier, different amount of ethanol (in the range of 0—20.4 mmol) was added to the reaction mixtures. For these experiments, 1.12 mmol of (±)-2-phenylpropionaldehyde as the reaction substrate was used. The reaction temperature was 20 °C. After 8 h of reaction time, the samples were taken out and analyzed. The results are presented in Fig. 3.

Increasing the amount of ethanol up to 4.0 mmol, the amount of the desired product was rising significantly. For the initial ethanol concentration range 4.0 mmol to 8.0 mmol, the 2-phenyl-1-propanol production peaked. Further addition of ethanol caused relatively sharp conversion decrease. Further experiments were carried out using optimum, 5.0 mmol, amount of ethanol.

Optimum Reaction Temperature

In the enzyme catalytic processes the reaction temperature has two different effects. Temperature increase enhances the reaction rate, however, at the same time, the higher temperature enables faster destruction of the enzyme structure [12]. In order to determine the optimum reaction temperature value, experiments for different temperatures (20 °C, 30 °C, and 40 °C) were carried out using 1.12 mmol of the substrate. The results are presented in Fig. 4.

The results show that the initial reaction rate increased with the temperature increase. However, during the experiment carried out at 40 °C, the negative influence of increased temperature on the amount of 2-phenyl-1-propanol produced prevailed. The best results were achieved at 30 °C.

Effect of the Substrate Concentration

Optimum initial substrate concentration was determined by the kinetic measurements carried out at the optimum reaction conditions. The data of initial

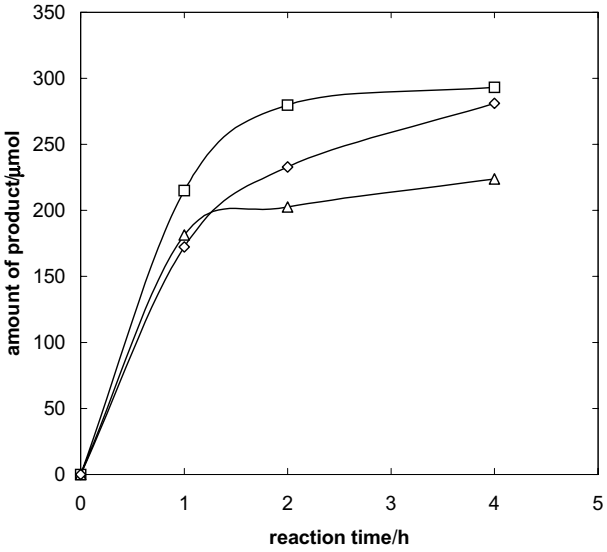

Fig. 4. Effect of the reaction time on 2-phenyl-1-propanol production at 20 °C (◊), 30 °C (□), and 40 °C (△).

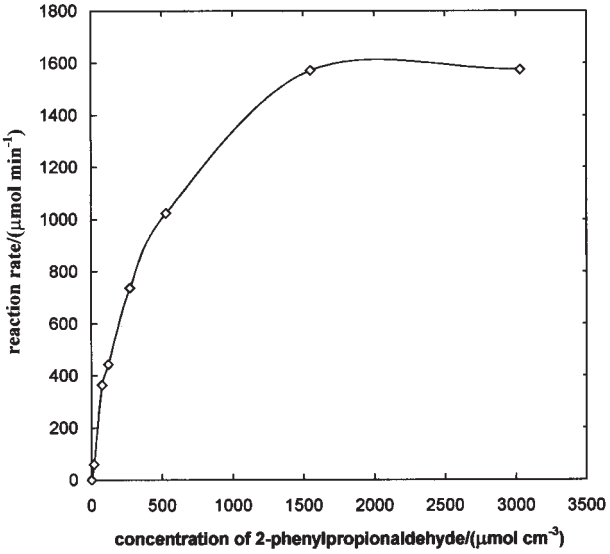

Fig. 5. Effect of the substrate concentration on the reaction rate at 30 °C.

reaction rates obtained for different substrate amounts (in the range of 0.0164—3.0200 mmol cm^{-3}) are presented in Fig. 5.

In the system studied, the β-NADH concentration was maintained constant (as proved by measurement of the reaction mixtures absorbance at 340 nm), thus, the simple Michaelis—Menten kinetics was assumed. From the curve shown in Fig. 5, the apparent kinetic parameters ($V'_{max} = 1834$ μmol min^{-1} and $K'_M = 510$ μmol cm^{-3}) were determined by plotting the reciprocal values of reaction rates and substrate concentrations. Using these parameters, the optimum initial substrate concentration 0.262 mmol cm^{-3} in isopropyl ether was calculated.

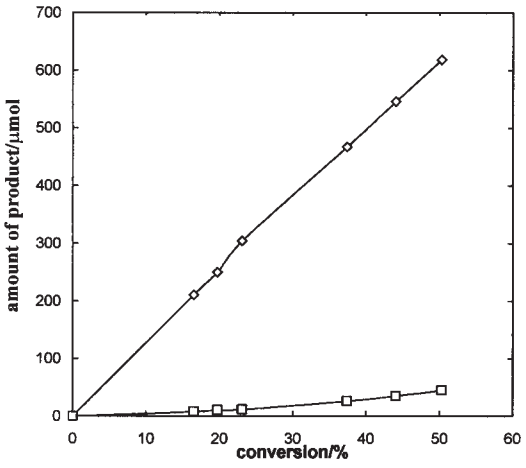

Fig. 6. The amount of (S) (◊) and (R) (□) enantiomers formed in the reaction studied as a function of conversion at 30 °C.

Enantioselectivity of the Reaction

The main purpose of this work was the production of S-enantiomer of 2-phenyl-1-propanol. The stereospecificity of alcohol dehydrogenases was proved by determination of the product optical purity. The optical purity of (S)-2-phenyl-1-propanol was analyzed by GC after a pre-column derivatization. The results obtained for the laboratory experiment performed under the optimal reaction conditions (5 cm^3 of isopropyl ether, 1.32 mmol of 2-phenylpropionaldehyde, 0.3 cm^3 of β-NADH stock solution, 2.7 cm^3 of buffer solution, 5.0 mmol of ethanol, and 1.2 unit of ADH; reaction temperature 30 °C) are presented in Fig. 6. In the first period of the reaction, up to 25 % conversion (about 2 h reaction time), the formation rate of (S) enantiomer is about twenty times higher than the formation of (R) enantiomer. At higher conversions, the product optical purity remained higher than 96 %.

CONCLUSION

The effect of the reaction conditions on the stereoselective reduction of 2-phenylpropionaldehyde by alcohol dehydrogenase with NADH cofactor was studied. The alcohol dehydrogenase used in these experiments showed high enantioselectivity towards (S)-2-phenyl-1-propanol. At 30 °C, after 2 h of reaction time, about 25 % of the initial amount of 2-phenylpropionaldehyde was converted to 2-phenyl-1-propanol. In isopropyl ether/aqueous buffer solution ($\varphi_r = 37/63$) in the presence of alcohol dehydrogenase (1.2 unit) and β-NADH (85 nmol), the optically active target product, (S)-2-phenyl-1-propanol, was produced with 96 % optical purity. During the reaction run, the cofactor was efficiently regenerated by simultaneous transformation of ethanol to acetaldehyde.

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