PRODUCTION OF CHIRAL (S)-2-PHENYL-1-PROPA NOL BY ENANTIOSELECTIVE BIOCATALYSTS

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Enantioselective production of (S)-2-phenyl-1-propanol is important as in order to be applied in industry, a high degree of optical purity is required. Besides organocatalysts and metal complexes, biocatalysts can be used for its synthesis in their isolated form or as whole-cell biocatalysts, both of which have various advantages and disadvantages. In this research, Saccharomyces cerevisiae, as a whole-cell biocatalyst, and recombinant horse-liver alcohol dehydrogenase (ADH), as an isolated enzyme, were investigated in terms of their activity, kinetics and enantioselectivity. In the case of yeast, the rate of cofactor regeneration was twice that of substrate conversion, moreover, the biocatalyst Saccharomyces cerevisiae can be characterised by substrate-limited kinetics and low enantioselectivity. In contrast, the isolated enzyme recombinant horse-liver ADH exhibited biphasic kinetics and cofactor regeneration was the rate-limiting step. The outstanding enantioselectivity of recombinant horse-liver ADH renders it a promising catalyst for the purpose of this synthesis.

Keywords: alcohol dehydrogenase, whole-cell biocatalyst, Saccharomyces cerevisiae

1. Introduction

2-phenyl-1-propanol is a fragrance ingredient that produces the Lila-hyacinth odour commonly used in cosmetics, fine fragrances and household cleaners [1]. Besides, it is the basis of some non-steroidal anti-inflammatory drugs and the precursor of other fragrances [2]. These fields of use require a high degree of optical purity since the enantiomers of the compound, by and large, bring about different biological effects [3].

Asymmetric syntheses are preferentially obtained using enzymes, given the capability of most to catalyse organic reactions with high enantioselectivity under mild conditions [4, 5]. Besides isolated enzymes, whole cells are also being applied more and more often as biocatalysts, given the disparate attributes of both. Whole-cell biocatalysts ensure the optimal environment for the enzyme, thereby providing a quite stable system. Furthermore, they contain cofactors and are able to bring about cofactor regeneration without the necessary addition of any other compounds [6]. However, the presence of a variety of enzymes may lead to side reactions. Also, isoenzymes with different enantiomeric preferences may lower the overall enantiomeric excess into the bargain [7]. Unlike whole-cell biocatalysts, isolated enzymes improve the level of control over the process in the absence of side reactions, thereby enhancing its reproducibility. In addition, inhibition is less likely to occur because of the greater degree of tolerance concerning the concentrations of both the substrate and product. On the other hand, the provision of a cofactor and its regenerating system significantly increases costs. In order to choose the optimal catalyst for a given synthesis, a detailed comparison of their advantages and disadvantages, e.g. attainable yield, productivity, product purity, required downstream processes and costs, should be made [8].

In this research, two types of alcohol dehydrogenases (ADH) were investigated with regard to the conversion of racemic 2-phenylpropionaldehyde to (S)-2-phenyl-1-propanol. For the catalysis, the cofactor nicotinamide adenine dinucleotide (NADH) is required by the enzyme which has to be regenerated in order to ensure continuous product formation. Ethanol was applied as an auxiliary substrate for the regeneration which was catalysed by the ADH. The reaction schemes are presented in Fig. 1. Because of the low solubility of the substrate and the product in aqueous media, a two-phase system was applied.

Saccharomyces cerevisiae was applied in dried form (instant baker’s dry yeast) as a whole-cell biocatalyst. Over recent decades, both wild and genetically modified strains of yeast have been gaining more and more attention as biocatalysts in the production of fine chemicals [7]. Although (S)-alcohol is generally the predominant
enantiomer in the reduction of racemic carboxylic acid compounds by applying yeast [6], this is dependent on the given substrate [9]. Our second catalyst was recombinant horse-liver ADH expressed in E. coli. Isolated recombinant horse-liver ADH is frequently applied for asymmetric syntheses [10–12], moreover, its variant expressed in bacteria can be a cheaper and more accessible alternative.

The aim of this research was to characterise the aforementioned biocatalyst in terms of activity, kinetics and enantioselectivity, thereby enabling their critical comparison. In addition, mass transfer through the organic-water interphase was also investigated in order to characterise the relationship between the rates of each step.

2. Experimental Methods

2.1 Applied chemicals

All chemicals were commercially available and used without further purification. Diisopropyl ether (puriss), ethyl alcohol (a.r.), dodecane (a.r.), trifluoroacetic anhydride (98%), racemic 2-phenylpropanaldehyde (98%) and S-(2)-phenyl-1-propanol (97%) were purchased from Sigma-Aldrich. Recombinant horse-liver alcohol dehydrogenase (expressed in E. coli) as well as lyophilised powder (1.5 U/mg) and β-nicotinamide adenine dinucleotide (sodium salt, 98%) were obtained from Sigma-Aldrich and Thermo Fisher Scientific, respectively. K$_2$HPO$_4$, KH$_2$PO$_4$ and Na$_2$CO$_3$ were purchased from VWR, while instant baker’s dry yeast was purchased from a local store.

2.2 Activity measurements

A spectrophotometric method was used to determine the catalytic activity characteristic of cofactor regeneration. Given that the maximum adsorption of NADH is at 340 nm while that of NAD$^+$ is negligible at this wavelength, conversion of the cofactor can be followed by the change in the wavelength of adsorption. In the case of isolated ADH, 10 µL of enzyme solution (10 mg/mL), 600 µL of NAD$^+$ solution (4 mg/mL) and 1240 µL of buffer solution were mixed in a quartz cuvette. The reaction was initiated by the addition of 150 µL of ethyl alcohol and the change in absorbance was measured at 340 nm by a Shimadzu UV-1800 ultraviolet-visible spectrophotometer. In the case of the whole-cell biocatalyst, 10 µL of an instant yeast suspension (10 mg/mL) was applied instead of an enzyme solution. The catalytic activity was calculated based on the following equation:

$$\text{VA} = \frac{dA}{dV_{	ext{cuvette}}} \cdot \epsilon \cdot V_{	ext{enzyme}} \cdot d \cdot h$$

where VA denotes the volume activity [U/cm$^3$], $\frac{dA}{dV_{	ext{cuvette}}}$ represents the gradient of the line (by plotting absorbance vs. time), $V_{	ext{cuvette}}$ stands for the volume of the mixture [cm$^3$], $\epsilon$ refers to the molar extinction coefficient of NADH at 340 nm [6.22 cm$^2$/mol], $d$ is the width of the cuvette [1 cm], $V_{	ext{enzyme}}$ denotes the volume of the enzyme solution [cm$^3$] and $h$ represents dilution.

The catalytic activity characteristic of the conversion of 2-phenylpropanaldehyde cannot be measured by a spectrophotometer since the reaction mixture consists of two phases. Therefore, the catalytic activity characteristic of the whole process (substrate conversion, cofactor regeneration and mass transfer through the organic-water interface) was calculated from the results gained by measuring the kinetics, as described in Sec. 2.4.

2.3 Mass-transfer rate

Since experimental conditions applied by conversion measurements are unsuitable for determining the mass-transfer rate through organic-water interface, a simplified system was applied for this purpose [13]. 13.4 mg of 2-phenylpropanaldehyde and 18.5 mg of dodecane, which served as an internal standard for gas chromatography (GC) analysis, were dissolved in 10 mL of diisopropyl ether. A Schott glass bottle was filled with 6 L of distilled water and the organic solution carefully poured onto the surface of the water phase. During the following 27 hours, samples were taken from the organic phase and analysed by GC. (The parameters of chromatographic analysis were the same as described in Sec. 2.4).

The ratio of aldehyde to dodecane was plotted as a function of time and a kinetic model was fitted to the measured data in accordance with the following equation:

$$J = d(c_0 - ac_w)$$

where $J$ denotes mass transfer through the interface [mol/(min cm$^2$)], $d$ represents the mass transfer coefficient [cm/min], $c_0$ stands for the initial substrate concentration in the organic phase [mol/cm$^3$], $c_w$ refers to the substrate concentration in the water phase [mol/cm$^3$] and $a$ is the ratio of the activity coefficients. The difference between the measured and calculated data was minimized by the Excel Solver plug-in. Although the water phase was gently mixed by a magnetic stirrer, the water–organic interface was stationary. Therefore, its surface can be regarded as a constant. The mass transfer rate can be calculated by dividing the mass transfer ($J$) by the interfacial area.
2.4 Kinetics

In all the experiments, 7 cm$^3$ of organic solvent and 7 cm$^3$ of phosphate buffer (75 mM, pH 8.0) were used. The molar ratio of 2-phenylpropionaldehyde to ethyl alcohol was set at 3:7, based on data from the literature [14]. In the case of isolated ADH, 60 $\mu$L of NADH solution (7.5 mg/mL, freshly made with a buffer solution) was added to the reaction media. The optimal amount of cofactor was determined during preliminary measurements, however, this data is not shown. In the case of instant baker’s dry yeast, since the cell contained a sufficient amount of cofactor, no further addition of NADH was necessary. The reactions were initiated by adding the catalyst – 300 mg of instant baker’s dry yeast or 500 $\mu$L of enzyme solution (10 mg/mL, freshly made with a buffer solution). The reaction mixtures were shaken in a thermostatic incubator (IKA KS 4000 i control) at 200 rpm and 30°C. To investigate the product formation, samples were taken from the organic phase and analysed by an HP 5890 gas chromatograph (140°C isothermal). The GC was equipped with a DB-FFAP column (1 $\mu$m × 30 m × 0.53 mm, Agilent Technologies) and a flame ionisation detector.

2.5 Enantioselectivity

While measuring the enantioselectivity, the content of the reaction mixture and operational parameters were the same as described in Sec. 2.3. Before GC analysis, preliminary derivatization was required. 1 mL of trifluoroacetic anhydride and 1 mL of diisopropyl ether were added to 500 $\mu$L of the sample while heating the mixture under reflux at 70°C for 30 mins. Once the reaction mixture had been cooled to room temperature, it was neutralized with 4 mL of Na$_2$CO$_3$ (20%) and a sample from the organic phase analysed by a Shimadzu GC-204 gas chromatograph equipped with a LIPODEX E capillary column (0.2 $\mu$m × 25 m × 0.25 mm, Macherey-Nagel) and a flame ionisation detector.

Table 1 contains the parameters of the applied heating program. The peaks were deconvoluted by OriginPro software to fit the Gaussian curves. To identify the peaks of the enantiomers, derivatization and analysis was performed using pure S-(2)-phenyl-1-propanol. The retention time of the derivative of the product was 52.5 mins.

### Table 1: Heating program

| Ramp rate [°C/min] | Temperature [°C] | Hold time [min] |
|-------------------|-----------------|-----------------|
| 0                 | 70              | 25              |
| 1                 | 110             | 0               |
| 20                | 180             | 2               |

3. Results and Evaluation

3.1 Kinetics

By plotting the amount of product as a function of time, a line can be fitted to the initial linear phase of the graph and its gradient is the initial rate of reaction as a result of the given substrate concentration. A kinetic curve results from repeating the method with different initial substrate concentrations, yielding information about the dependence of the reaction rate on it.

In the case of whole-cell biocatalysts, the product formation can be described by the single-substrate Michaelis-Menten model as the amount of cofactor can be considered to be constant due to its fast regeneration (see Sec. 2.3).

In the region of lower substrate concentrations, first-order kinetics was observed as expected (Fig. 2). However, at 0.267 M, the curve peaked followed by a relatively steady decrease instead of phase saturation. As a result, it can be concluded that higher amounts of substrate limit enzymatic conversion, therefore, 0.267 M is the optimal initial concentration to maximise the reaction rate.

In contrast to yeast cells, by applying recombinant ADH, the reaction rate of cofactor regeneration may limit product formation (see Section 3.2), therefore, cannot be neglected. Since two reactions involved multiple substrates catalysed simultaneously by the same enzyme, the kinetics did not follow the Michaelis-Menten model. Alternatively, a biphasic model was applied which divides the curve into two phases: at low substrate concentrations, the enzyme’s affinity is relatively high while the turnover number is low. On the contrary, a low affinity and high turnover number is characteristic of the region of high substrate concentration (Fig. 3).

Biphasic kinetics can be modelled by the following equation [15]:

$$ V = \frac{V_{\text{max}1} [S] + CL_{\text{int}} [S]^2}{K_{\text{M}1} + [S]} $$

where $V_{\text{max}1}$ as well as $K_{\text{M}1}$ describe the first high affinity–low-turnover phase and $CL_{\text{int}}$ – equal to the ratio $V_{\text{max}2} : K_{M2}$ – denotes the second low-affinity–high-turnover phase.

![Figure 2: Kinetics of the whole-cell biocatalyst](image-url)
Apart from one exception (at 0.22 M), since model data calculated using Eq. 3 fitted well to the measured data (Fig. 4), biphasic kinetics is presumably a suitable model to describe the kinetics of an isolated enzyme. Having been minimized by the Excel Solver plug-in, the model parameters were the following:

$$V_{\text{max}1} = 7.523;$$
$$CL_{\text{int}} = 3.458;$$
$$K_{M1} = 0.464.$$

According to Manevski [15], biphasic kinetics may imply the presence of multiple substrate binding sites. However, the applied methods were unsuitable for further investigating the underlying mechanisms of the reactions taking place.

### 3.2 Activity

The activity of the catalysts was measured during both cofactor regeneration and the process as a whole (Table 2). One Unit (U) stands for the amount of catalyst necessary to produce 1 µmol of product in 1 minute under the measurement parameters. (Substrate conversion could not be investigated separately as previously mentioned in Sec. 2.2). Although normally the catalytic activity should be measured in the saturation phase of the kinetics, none of the kinetic curves enabled this. Therefore, measurements were made at a substrate concentration of 0.27 M, which is the substrate concentration at which the kinetic curve of the yeast-cell catalyst is at its maximum. In the case of the isolated enzyme, the activity was measured at the same concentration as that of yeast in order to compare the two catalysts. This was also determined at the highest measured point of the kinetic curve, namely at 1.08 M.

Based on the results, cofactor regeneration is one scale faster when applying a whole-cell biocatalyst instead of an isolated enzyme. Although the same enzyme catalyses both substrate conversion and cofactor regeneration when isolated ADH is used, yeast cells contain several enzymes that are capable of participating in regeneration reactions which can occur more rapidly as a result.

On the other hand, the overall reaction rate using the same substrate concentration was 35 times higher when recombinant ADH was used and further increases in substrate concentration resulted in even higher reaction rates.

### 3.3 Mass transfer through the organic–water interface

In order to determine the rate-limiting step of the whole process, the mass transfer rate through an organic–water interface was investigated. Owing to simplifications of the measurement and the imprecise nature of model fitting, the calculated mass transfer rate may be somewhat inaccurate. Nevertheless, the goal was to estimate its order of magnitude rather than determining its precise value.

Based on the kinetic model fitted to the measured data (Fig. 5), the mass transfer rate was calculated to be $1.33 \cdot 10^{-5}$ mol/min. By comparing this value with the

|               | yeast cell [U/mg] | recombinant ADH [U/mg] |
|---------------|-------------------|------------------------|
| cofactor regeneration | 0.19 | 0.05 |
| complete reaction (0.27 M) | 0.02 | 0.7 |
| complete reaction (1.08 M) | – | 1.64 |
reaction rates of both catalysts, it could be established that the mass transfer rate is two or three times faster than those of product formation or cofactor regeneration. Therefore, the rate-limiting step of the whole process is cofactor regeneration and substrate conversion when an isolated enzyme and whole-cell biocatalyst is applied, respectively.

3.4 Enantioselectivity

The enantioselectivity of the whole-cell biocatalyst was measured at three different initial substrate concentrations: at the maximum of the kinetic curve (0.267 M) as well as at two higher values (0.345 M and 0.746 M) to examine whether increasing the substrate concentration is beneficial as far as achieving optical purity is concerned. The enantiomeric ratio was calculated from the ratio of the peak areas at 0.5, 1.0 and 1.5 hours (Fig. 6).

Changes in the enantiomeric ratio as the reaction progressed were insignificant, moreover, the difference in the reaction time between 0.345 M and 0.746 M was negligible. At 0.267 M, the final result was 0.62, while the best result, that is, 0.68, was achieved at 0.746 M. As a result, it could be established that to achieve an optimal reaction rate and enantioselectivity, different initial substrate concentrations are required.

In the case of the isolated enzyme, the enantioselectivity was measured at the same substrate concentrations as when the whole-cell biocatalyst was applied. However, since only one peak corresponding to the (S)-enantiomer of the derivative was detected, further analysis was unnecessary and the enantioselectivity regarded as 100%.

As in terms of consumption optical purity is a key concern, the results of this work were compared with some data from the literature (Table 3). In the case of whole-cell biocatalysts, the results of Rapp et al. [16] are more favourable than ours. However, since both studies suggest that the maximum degree of conversion and enantiomeric excess cannot be achieved simultaneously, a compromise must be made. In the case of the isolated enzyme, the degree of conversion in this study is promising if the reaction time is also taken into account. As for the enantiomeric excess, our result is clearly outstanding, therefore, recombinant horse-liver ADH provides a satisfactory alternative for catalysing the production of (S)-2-phenyl-1-propanol.

4. Conclusion

In this work, the conversion of racemic 2-phenylpropionaldehyde into (S)-2-phenyl-1-propanol was investigated by applying a whole-cell biocatalyst (Saccharomyces cerevisiae) and an isolated enzyme (recombinant horse-liver ADH expressed in E. coli). Significant differences were observed between the catalysts in terms of all the considered parameters. Firstly, the whole-cell biocatalyst exhibited substrate-limited kinetics, while the isolated enzyme could be described by biphasic kinetics. The yeast cell contained a sufficient amount of cofactor for the reaction, moreover, its regeneration was twice as fast as in the case of the isolated
enzyme. Therefore, the whole-cell biocatalyst is more beneficial from this point of view. On the other hand, the enzymatic activity of the whole process was at least 35 times higher when recombinant horse-liver ADH was applied and could be further enhanced by increasing the initial substrate concentration. Most importantly, the isolated enzyme catalysed the conversion with 100% enantioselectivity, which is also clearly outstanding compared to data from the literature. In conclusion, although recombinant horse-liver ADH is more expensive, it is definitely a more efficient catalyst than yeast as a whole-cell biocatalyst. Therefore, recombinant horse-liver ADH is a promising biocatalyst with regard to the synthesis of (S)-2-phenyl-1-propanol.

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