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Studies on the continuous production of (*R*)-(−)-phenylacetylcarbinol in an enzyme-membrane reactor

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Abstract

The optimization of a continuous enzymatic reaction yielding (*R*)-(−)-phenylacetylcarbinol ((*R*)-PAC), a key intermediate of the (1*R*,2*S*)-(−)-ephedrine synthesis, is presented. We compare the suitability of different mutants of the pyruvate decarboxylase (PDC) from *Zymomonas mobilis* with respect to their application in biotransformation using pyruvate or acetaldehyde and benzaldehyde as substrates, respectively. Starting from 90 mM pyruvate and 30 mM benzaldehyde, (*R*)-PAC was obtained with a space time yield of 27.4 g/(L · day) using purified PDC*W392I* in an enzyme-membrane reactor. Due to the high stability of the mutant enzymes PDC*W392I* and PDC*W392M* towards acetaldehyde, a continuous procedure using acetaldehyde instead of pyruvate was developed. The kinetic results of the enzymatic synthesis starting from acetaldehyde and benzaldehyde demonstrate that the carboligation to (*R*)-PAC is most efficiently performed using a continuous reaction system and feeding both aldehydes in equimolar concentration. Starting from an inlet concentration of 50 mM of both aldehydes, (*R*)-PAC was obtained with a space-time yield of 81 g/(L · day) using the mutant enzyme PDC*W392M*. The new reaction strategy allows the enzymatic synthesis of (*R*)-PAC from cheap substrates free of unwanted by-products with potent mutants of PDC from *Z. mobilis* in an aqueous reaction system.

Keywords: (*R*)-(−)-phenylacetylcarbinol; Enzyme–membrane reactor; Biotransformation; Pyruvate decarboxylase; Site-directed mutagenesis

1. Introduction

(*R*)-(−)-Phenylacetylcarbinol ((*R*)-PAC) is an important key intermediate for the synthesis of (1*R*,2*S*)-(−)-ephedrine and (1*S*,2*S*)-(+)-ephedrine (D-(+)-pseudo-ephedrine), which are major ingredients of several pharmaceutical products used as congestants and anti-asthmatics [1]. It is currently produced by a fermentative process using *Saccharomyces cerevisiae*, glucose and benzaldehyde [2] (Scheme 1). Like many other biotransformations using living cells, this fermentative process is limited by several factors including toxicity of benzaldehyde towards the yeast cells [3–5] and many by-products,

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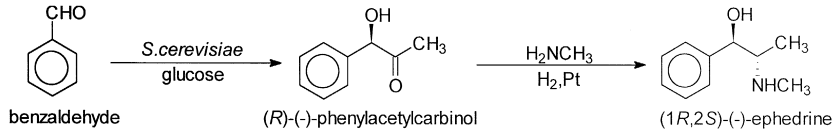


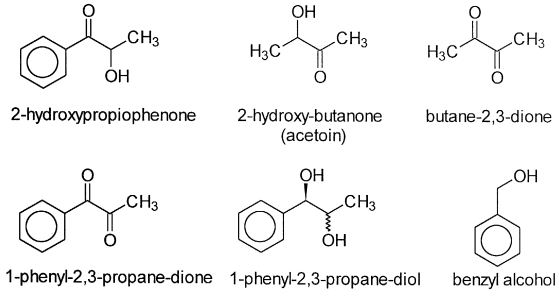
Scheme 1. Chemo-enzymatic synthesis of (1*R*,2*S*)-(−)-ephedrine [2].

especially benzyl alcohol [5–10] (Scheme 2). These factors reduce the yield of the desired product and make an additional purification necessary. A possibility to overcome the disadvantages of whole-cell biotransformations is the application of purified enzymes catalyzing only the desired reaction.

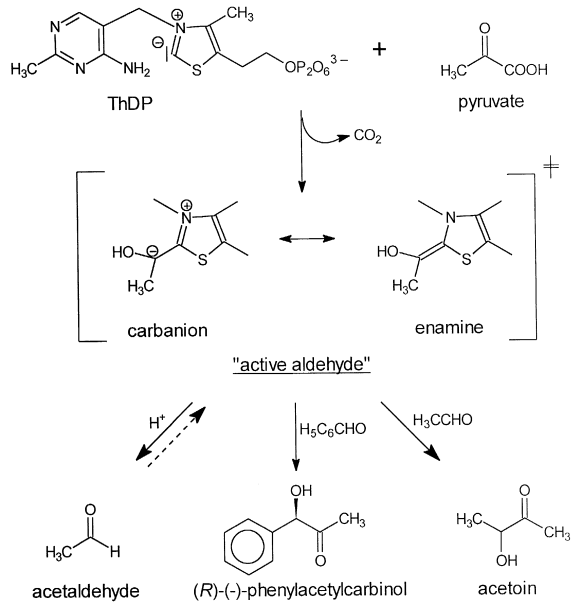
The enzyme responsible for the synthesis of (*R*)-PAC is pyruvate decarboxylase (PDC, E.C.4.1.1.1), which performs the non-oxidative thiamindiphosphate-mediated decarboxylation of pyruvate to acetaldehyde as a main reaction and the carboligation of two aldehydes to 2-hydroxy ketones as a side reaction (Scheme 3). It has to be stressed that decarboxylation of pyruvate is not an essential prestep for carboligation, and the formation of acyloins can be also carried out using acetaldehyde as acyldonor (Scheme 3). However, acetaldehyde was shown to inactivate PDC rapidly [11–13].

The enzymes from *S. cerevisiae* (PDC*S.c.*) and *Zymomonas mobilis* (PDC*Z.m.*) have been characterized in detail with respect to their carboligation potential [14–16].

They differ significantly in their stability and in their potential to catalyze the formation of (*R*)-PAC. PDC*S.c.* shows a high carboligase activity, but exhibits low stability in the isolated state, whereas PDC*Z.m.* is significantly more stable, but less active with respect to the carboligase reaction than the yeast enzyme [14,17]. We have recently reported that the carboligase potential of PDC*Z.m.* to catalyze the formation of (*R*)-PAC could be enhanced by a single amino acid replacement in the deep cleft leading to the active center of the enzyme [13–15,18]. Among the different mutants of the type PDC*W392X*, those containing either methionine or isoleucine in position 392 are best suited as biocatalysts in the isolated form, since they combine maximal carboligase activity (Fig. 1) with maximal stability [14,19]. However, the application of crude extracts or isolated PDC requires pyruvate or acetaldehyde as substrates since the glucose degrading system existing in living yeast cells is no longer active. In comparison to glucose as a cheap source for acetaldehyde, pyruvate is by a



Scheme 2. By-products of the fermentative process.



Scheme 3. Thiamindiphosphate (ThDP)-mediated decarboxylation of pyruvate and formation of 2-hydroxy ketones catalyzed by PDC.

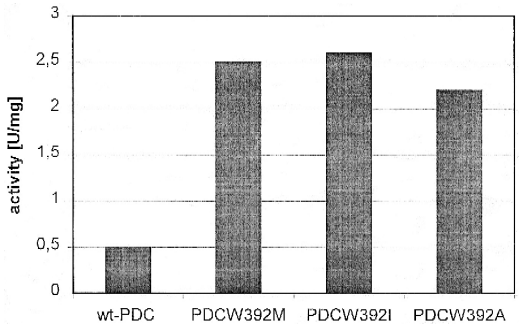


Fig. 1. Carboligase activity of various PDC mutants from *Z. mobilis* (*Z.m.*) compared to the wt enzyme [15].

factor of about 200 more expensive on a molar base. Since the decarboxylase activity of PDC is significantly faster than the carboligase reaction (e.g. PDC*W392M*: decarboxylase activity: 70 U/mg; carboligase activity: 2.5 U/mg) [15], pyruvate is rapidly decarboxylated to acetaldehyde accumulating in the reaction medium and causing enzyme inactivation. For the same reason, the direct application of acetaldehyde was shown to be difficult.

We have recently presented a simple method to reduce excess acetaldehyde in situ in a batch procedure using the mutant enzyme PDC*W392A* and pyruvate and benzaldehyde as substrates by a coupled enzymatic reaction employing alcohol dehydrogenase from *S. cerevisiae* and formate dehydrogenase from *Candida boidinii* for regeneration of the cofactor NADH [13,18]. However, this method is not applicable in large scale.

In the present paper, we present two approaches producing (*R*)-PAC in a continuous operated enzyme–membrane reactor (EMR). In a first approach, we studied a system of pyruvate and benzaldehyde in various molar ratios using the mutant enzyme PDC*W392I* as a catalyst. The second approach was carried out with acetaldehyde and benzaldehyde and PDC*W392M* as a catalyst based on the result that the mutant enzymes show a pronounced higher resistance towards acetaldehyde than the wt enzyme [20]. According to the kinetic data, a reaction model was developed suggesting that both, maximal activity of the enzyme and minimal inactivation are combined, if both aldehydes are used in equimolar concentration. Our results demonstrate that the mutant PDCs are stable for several days under these reaction conditions.

2. Material and methods

2.1. Chemicals

Pyruvate, acetaldehyde, and benzaldehyde were obtained from Sigma. Thiamindiphosphate and magnesium sulfate were purchased from Fluka. NADH was obtained from Boehringer. All chemicals were of analytical grade.

2.2. Strain, cell growth and isolation of biocatalyst

wt PDC*Z.m.*, *PDCW392I*, and PDC*W392M* were obtained as recombinant C-terminal hexahistidine-fusion proteins from an *E. coliK12*-strain as described elsewhere [13,21].

2.3. Assay of decarboxylase activity

The decarboxylation of pyruvate was measured in a coupled photometric assay by monitoring the depletion of NADH as described elsewhere [22]. To determine the pH-optimum of the decarboxylase reaction, a direct photometric assay was used, monitoring the depletion of pyruvate at 320 nm.

One unit of decarboxylase activity corresponds to the amount of enzyme which decarboxylates 1 μmol pyruvate per minute under standard conditions (pH 6.5, 30°C).

2.4. Stability investigation of PDCs

The different PDCs (1 mg/ml) were incubated in 50 mM Mes/KOH buffer, pH 6.5, containing 5 mM magnesium sulfate and 0.1 mM ThDP in the presence of substrates or at different temperatures, respectively. After various time intervals, 50 μl were sampled and directly analyzed in the decarboxylase assay. Residual activity was expressed in terms of residual decarboxylase activity.

2.5. Enzymatic synthesis of (R)-PAC and acetoin

All enzymatic syntheses were performed in 50 mM potassium phosphate buffer, pH 6.5, containing 5 mM $MgSO_4$ and 0.1 mM ThDP. The concentration of substrates was varied depending on the purpose of the respective test: pyruvate (30–90 mM), acetaldehyde (3–90 mM) and benzaldehyde (3–50 mM).

2.6. Analysis of aldehydes and 2-hydroxy ketones

Analysis of (*R*)-PAC and benzaldehyde was performed by reversed phase HPLC using a RP18 Hypersil ODS 5μ column (250 mm × 4.6 mm) (C&S Chromatographie Service, Germany) and an elution buffer consisting of acetic acid (0.5% in water) and acetonitrile (25%) (v/v) (flow: 1.5 ml/min). Detection was performed at 263 nm and 283 nm. Under these conditions, (*R*)-PAC concentrations of 0.5–150 mM (±1 mM) were detectable. Benzaldehyde was detected in the range of 0.2–50 mM (±0.8 mM). Quantification was done according to calibration curves obtained with standard samples.

Acetoin (1–40 mM (±0.5 mM)) was analyzed on a Lichrosorb RP18 5-μm column (120 mm × 4.6 mm) using a linear gradient of 0–25% acetonitrile in 0.5% acetic acid (v:v) (flow: 1 ml/min). Detection was performed at 260 nm.

2.7. Chiral analysis of (R)-PAC

Chiral analysis was performed by gas chromatography as described elsewhere [13]. The enantioselectivity of PDC*W392M* and *PDCW392I* with respect to (*R*)-PAC was > 98%.

2.8. Determination of the protein concentration

The protein concentration was determined according to Bradford [23] using bovine serum albumin for calibration.

2.9. Determination of initial rate velocities in a batch reactor

Initial rate velocities were measured at 25°C in 10-ml vessels equipped with a magnetic stirrer. The substrate concentration varied in the range of 3–90 mM for acetaldehyde and 3–50 mM for benzaldehyde. For product inhibition studies (*R*)-PAC was added in the range of 0–100 mM. Each vessel contained 1 mg/ml purified PDC*W392M*. After several time intervals (0, 3, 6, 10, 15, 20, 25, 30, 60 min, and 24 h) samples (1 ml) were removed from the reaction vessels, transferred in a 1.5-ml tube, and the enzyme was heat inactivated in boiling water for 2 min. Subsequently, the concentration of benzaldehyde, (*R*)-PAC and acetoin were determined using HPLC.

The initial rate velocity was calculated from those values referring to conversions < 10%. The slope of this straight line obtained by linear interpolation was taken as initial velocity.

2.10. Calculation and kinetical analysis

For calculation of the kinetic models the program "Scientist" (MicroMath) was used.

2.11. Continuous synthesis of (R)-PAC using an enzyme-membrane reactor

One significant factor to establish a continuous enzymatic synthesis in an EMR is to find an appropriate membrane material that shows a high stability towards the aldehydes in the reaction mixture. The polyaramide membrane (UF-PA-20H, Hoechst) is stable for more than 3 months under the reaction conditions. A conventional stainless-steel reactor is not suitable for an enzymatic synthesis using PDC*W392I* or PDC*W392M*, since we observed rapid inactivation of the enzyme in this reactor type. The problem can be avoided when a polypropylene reactor is used instead [24]. The EMR (reactor volume: 10 ml) was manufactured by the Institute of Biotechnology 2, Jülich (Germany). The substrates acetaldehyde and benzaldehyde or pyruvate and benzaldehyde, respectively, were premixed in the desired concentrations in 50 mM potassium phosphate buffer, pH 6.5, containing 5 mM $MgSO_4$ and 0.1 mM ThDP. The enzyme concentration was 1 mg PDC as crude extract (PDC*W392M*) or 0.4 mg of purified PDC (PDC*W392I*) per ml reactor volume. The resi-

dence time τ was set to 1 h. A reciprocating pump (P 500, Pharmacia Biotech) was used to pump the substrate solution through the reactor. The outlet of the reactor was connected to a fraction collector 2128 (Bio-Rad). Alternatively, the formation of (*R*)-PAC was detected polarimetrically using a polarimeter 241 (Perkin-Elmer).

3. Results

3.1. Selection of an appropriate biocatalyst

As it has already been pointed out in Section 1, acetaldehyde is unavoidable during biotransformation yielding (*R*)-PAC catalyzed by crude extracts or isolated PDC, independently whether pyruvate or acetaldehyde is used as an educt. The main selection criterion to choose an appropriate biocatalyst for an enzymatic synthesis was the stability of different PDCs towards acetaldehyde at room temperature. Although isolated PDC*S.c.* shows a pronounced higher stability towards acetaldehyde than wt PDC from *Z. mobilis* (PDC*Z.m.*), as well as the earlier described mutant PDC*W392A* [13,18], it is rapidly inactivated at temperatures ≥ 25°C [14] and therefore not suitable for a technical process. The mutant enzymes PDC*W392M* and PDC*W392I* combine both, a high carboligase activity (Fig. 1) and a pronounced higher resistance towards acetaldehyde than the wt enzyme from *Z. mobilis* [18,20]. Consequently, these muteins were chosen as appropriate biocatalysts for an enzymatic synthesis of (*R*)-PAC.

3.2. Investigation of the reaction system

Developing an optimal reaction system requires knowledge of the thermodynamics of the desired reaction. In the case of the formation of (*R*)-PAC, the reaction is quasi-irreversible under the investigated conditions. The experiments were performed in aqueous buffer, pH 6.5, at room temperature. We did not observe any cleavage of (*R*)-PAC into the educts, neither in presence of the enzyme, nor in its absence. A further possible side reaction is the racemization of (*R*)-PAC, which occurs upon base treatment, at elevated temperature, in non-aqueous systems (Iding, unpublished results), and in the presence of traces of metals of the group VIII of the periodic system [25]. However, racemization of (*R*)-PAC has not been observed under the chosen reaction conditions ($t < 24$ h).

In order to investigate all possible enzyme-catalyzed reactions PDC*W392I* and PDC*W392M* were added to pyruvate and benzaldehyde or acetaldehyde and benzaldehyde, respectively. We observed only (*R*)-PAC and acetoin as products.

3.3. Determination of optimal reaction conditions

Potassium phosphate was chosen as a cheap buffer salt appropriate for a technical application. PDC*Z.m.* shows optimal stability and activity in this buffer [22]. In contrast, PDC*S.c.* is inhibited by phosphate ions [26]. The pH-optimum has been determined with respect to decarboxylase- and carboligase activity. As demonstrated in Fig. 2, both optima overlap in the range of pH 6.0–7.5. To ensure maximal stability of the product, pH 6.5 was chosen as an appropriate pH for biocatalysis.

3.4. Biotransformations using pyruvate and benzaldehyde

We first investigated a continuous reaction system using pyruvate and benzaldehyde in various molar rations and isolated PDC*W392I* as a catalyst.

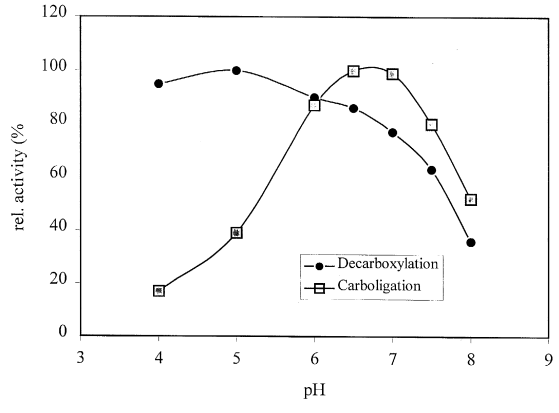


Fig. 2. pH-optima of PDC-catalyzed decarboxylation of pyruvate and carboligation of acetaldehyde and benzaldehyde yielding (*R*)-PAC.

The space–time yield of (*R*)-PAC as function of the molar ratios of pyruvate to benzaldehyde were evaluated at 25°C in an enzyme-membrane reactor. The residence time τ was set to 1 h according to the maximal productivity of the system. The concentration of benzaldehyde was limited by the low solubility of benzaldehyde in aqueous medium (4 g/L = 37.7 mM in water at 20°C). Therefore, we used benzaldehyde in a concentration of 30 mM and varied the pyruvate concentration in the range 30–90 mM. The results presented in Fig. 3 indicate that an increase of the molar ratio (pyruvate/benzaldehyde) was accompanied by a small increase in the space-time yield of (*R*)-PAC from 21.5 g/(L · day) at a molar ratio of 1.5:1 to 27.4 g/(L · day) at 3:1. The lower yields at higher molar ratios (2:1, 3:1) were probably caused by a high temporary concentration of acetaldehyde in the reactor, giving rise to enzyme inactivation. Maximal space-time yields (27.4 g/(L · day)) were obtained using 90 mM pyruvate and 30 mM benzaldehyde. A conversion of less than 10% pyruvate and 25% benzaldehyde was obtained, yielding 7.6 mM (1.1 g/L) (*R*)-PAC in the outlet.

3.5. Biotransformations using acetaldehyde and benzaldehyde

The continuous enzymatic synthesis of (*R*)-PAC starting from pyruvate and benzaldehyde as substrates has some disadvantages: (i) accumulation of acetaldehyde in the biotransformation mixture, resulting in a progressive inactivation of PDC, (ii) the liberation of gaseous CO_2 may cause problems (e.g. enzyme deactivation at liquid–gas-interface), and (iii) pyruvate is a fairly expensive compound. The results of the previous investigations show that the main part of pyruvate is converted to acetaldehyde and not to (*R*)-PAC which reduces the attractiveness of such a biotransformation. As has been demonstrated before, decarboxylation is not essential for carboligation and the formation of 2-hydroxy ketones and (*R*)-PAC can be carried out using only the respective aldehydes as substrates [27–30]. Due to the higher stability of the mutants PDC*W392M* and PDC*W392I* towards acetaldehyde compared to wt-PDC [20], the enzymatic synthesis of (*R*)-PAC starting from acetaldehyde and benzaldehyde has been investigated in a second approach. In contrast to the first system, which includes consecutive (decarboxylation and carboligation) as well as parallel reactions (carboligation to (*R*)-PAC and acetoin), the carboligation of both aldehydes can be more easily described by a kinetic model. Thus, we first investigated the kinetics of the enzymatic synthesis of (*R*)-PAC and then used the results to identify an appropriate reaction system [31].

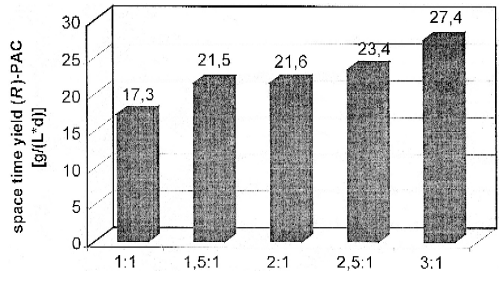


Fig. 3. Space–time yields of different continuous operated enzyme-membrane reactors using various molar ratios of pyruvate to benzaldehyde for the formation of (*R*)-PAC with purified PDC*W392I* (0.4 mg/ml).

3.6. Setup of a kinetic model

The biotransformation of acetaldehyde and benzaldehyde to (*R*)-PAC is a bimolecular reaction that can be described by a double-substrate kinetic. Several parameters may influence the maximal reaction rate. Thus, the correlation of a two-substrate reaction and the maximal reaction rate is described by a plane rather than a curve, as in the case of a one-substrate reaction. To describe this plane, different batch experiments varying both substrate concentrations and investigating the initial velocities of (*R*)-PAC formation, have been carried out to detect the complex influences on V_{max} of the (*R*)-PAC formation.

Fig. 4 shows the experiments that have been carried out to describe the plane between 0 and 90 mM acetaldehyde and 0 and 50 mM benzaldehyde using PDC*W392M* as a catalyst. As a result, V_{max} of the (*R*)-PAC synthesis was found to be maximal in the equimolar substrate range between concentrations of 30 and 40 mM of both aldehydes (Fig. 4). Apart from this small ridge, V_{max} decreases drasti-

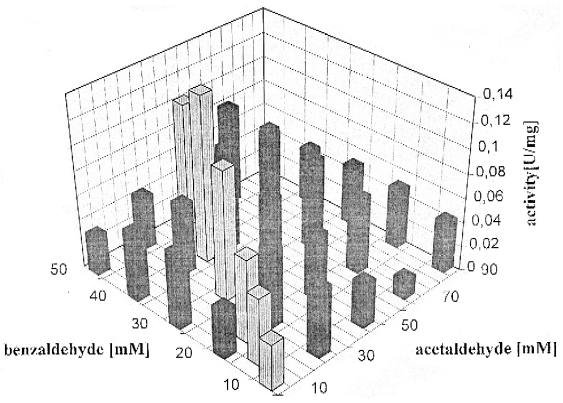


Fig. 4. Initial velocities of (*R*)-PAC formation depending on the concentrations of benzaldehyde and acetaldehyde. Striped columns mark equimolar substrate concentrations.

cally. The depletion of V_{max} in the presence of benzaldehyde concentrations > 40 mM may be described by substrate-excess inhibition. It is important to notice that in the presence of equimolar concentrations of both substrates, (*R*)-PAC was the only product detected. The formation of acetoin is maximal in the presence of excessive acetaldehyde (> 80 mM) and low concentrations of benzaldehyde (about 10 mM), whereas acetoin formation is inhibited by increasing concentration of benzaldehyde (Fig. 5). The kinetic data of the synthesis of both, (*R*)-PAC and acetoin catalyzed by PDC*W392M* were fitted by a double-substrate kinetic according to Michaelis–Menten (Eq. (1)).

$$v = \frac{V_{max} S_1 S_2}{(K_{M1} + S_1)(K_{M2} + S_2)}. \quad (1)$$

S_1 and S_2 are the concentrations of acetaldehyde and benzaldehyde, and K_{M1} and K_{M2} are the corresponding K_M values.

The drop of V_{max} of the (*R*)-PAC formation, as well as of the acetoin formation towards higher concentrations of acetaldehyde and benzaldehyde, suggests an inhibition caused by an excess of substrates or products. For the formation of (*R*)-PAC, a substrate-excess inhibition is assumed for both substrates, which has been implemented into the model in terms of non-competitive inhibition (K_{I1}; K_{I2}). A product inhibition by (*R*)-PAC has not been detected in the range of 20–100 mM (data not shown).

Since the reaction rate of (*R*)-PAC is maximal in the equimolar concentration range (Fig. 4) of both

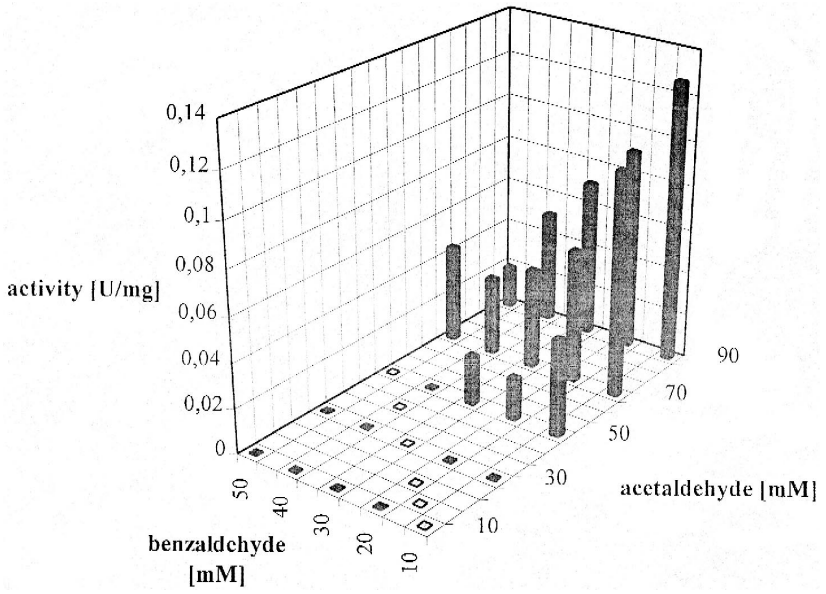


Fig. 5. Initial velocities of acetoin formation depending on the concentrations of benzaldehyde and acetaldehyde. Striped columns mark equimolar substrate concentrations.

aldehydes, the model was reduced to this concentration range. This simplified model (Eq. (2)) is based on the following assumptions:

- The reaction can be described as a double-substrate kinetic with two identical substrates since best results (reaction rates) were obtained with an equimolar mixture of both aldehydes.
- Acetoin has not been detected in batch reactions containing acetaldehyde and benzaldehyde in equimolar concentration; therefore, acetoin formation can be neglected in the reaction model.

$$v_{PAC} = \frac{V_{max\text{-}PAC} S^2}{\left(K_{M1} + S\left(1 + \frac{S}{K_{I1}}\right)\right)\left(K_{M2} + S\left(1 + \frac{S}{K_{I2}}\right)\right)} = \frac{V_{max\text{-}PAC} S^2}{\left(K_{M1} + S + \frac{S^2}{K_{I1}}\right)\left(K_{M2} + S + \frac{S^2}{K_{I2}}\right)} \quad (2)$$

where v_{PAC}: reaction rate for the formation of (*R*)-PAC (μmol min^{-1} mg $(enzyme)^{-1}$), $V_{max\text{-}PAC}$: maximal reaction rate for the formation of (*R*)-PAC (μmol min^{-1} mg $(enzyme)^{-1}$), K_{M1}: Michaelis–Menten constant for benzaldehyde (mmol L^{-1}), K_{M2}: Michaelis–Menten constant for acetaldehyde for the formation of (*R*)-PAC (mmol L^{-1}), *S*: concentration of the substrates (mmol L^{-1}), K_{I1}: uncompetitive inhibition constant for benzaldehyde (mmol L^{-1}), K_{I2}: uncompetitive inhibition constant for acetaldehyde (mmol L^{-1}).

The optimal substrate concentration for the synthesis of (*R*)-PAC calculated from the kinetic model is 32.5 mM for both aldehydes [31].

3.7. Selection of an appropriate bioreactor

The necessity of an exactly adjusted equimolar substrate concentration in the range of 30–40 mM of both aldehydes in the reaction mixture requires a reaction system which guarantees a constant substrate concentration during the reaction time. This can be achieved using a continuously operated stirred tank reactor (CSTR), which was applied in the form of an EMR [32]. In contrast to common batch reactors, where the substrate and product concentrations change during the reaction time, the EMR allows the adjustment of a constant substrate to product ratio, thereby using the catalytic power of the PDC mutants in an optimal manner.

3.8. Evaluation of a kinetic model in an EMR

The following equation describes the continuous synthesis of (*R*)-PAC in an EMR by the simplified kinetic model [31] using acetaldehyde and benzaldehyde:

$$v_{PAC} = \frac{V_{max} S^2}{\left(K_{M1} + S\left(1 + \frac{S}{K_{I1}}\right)\right)\left(K_{M2} + S\left(1 + \frac{S}{K_{I2}}\right)\right)}. \quad (3)$$

The validity of the model was tested with two equimolar concentrations of acetaldehyde and benzaldehyde [31]. Incomplete dissolution of benzaldehyde in aqueous medium may lead to enzyme inhibition due to phase separation. To avoid this, the first experiment was performed with 30 mM of each substrate. This means that the substrate concentration in the reactor (efflux conditions) was about 24 mM, which is about 10 mM lower than the calculated optimum. The second experiment was carried out with 50 mM of each substrate at 27°C, which guaranteed the solubility of the benzaldehyde. The data

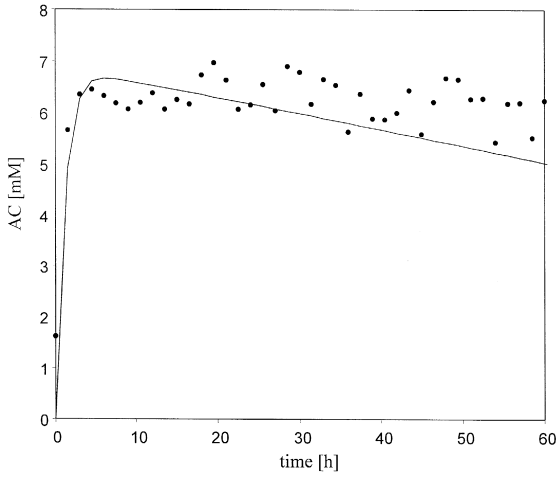


Fig. 6. Concentration–time diagram of a continuous operated EMR using 30 mM of acetaldehyde and benzaldehyde. Experimental data (●) were fitted with the simplified model (Eq. (3)) (line).

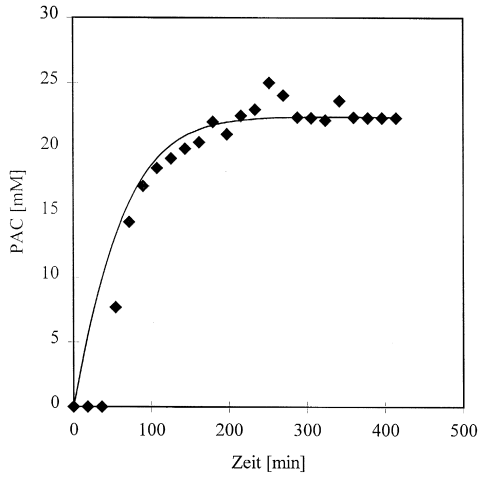


Fig. 7. Concentration–time diagram of a continuous operated EMR using 50 mM of acetaldehyde and benzaldehyde, respectively. Experimental data (♦) were fitted with the simplified model (Eq. (3)) (line).

show that the maximal velocity of both experiments is predicted correctly by the simplified model (Figs. 6 and 7).

4. Discussion

We present here a continuous operated reaction system for the enzymatic synthesis of (*R*)-PAC by two optimized mutants of PDC from *Z. mobilis* using benzaldehyde and pyruvate or benzaldehyde and acetaldehyde as substrates. Using 90 mM pyruvate and 30 mM benzaldehyde as substrates and the purified mutant PDC*W392I* in an EMR, a space-time yield of 27.4 g (*R*)-PAC/(L · day) was obtained (Fig. 1). The reaction was stopped after 18 h; however, the enzyme activity has only decreased by 10% during this time, suggesting that the reaction can be carried out for a longer time. The only detected products were (*R*)-PAC and acetoin. These results are similar to those from Shin and Rogers [12]. These authors obtained the highest yield of (*R*)-PAC (190.6 mM) in a batch reactor using 200 mM benzaldehyde, 2 M ethanol, and a 1.5–2.9 molar excess of semi-purified PDC from *C. utilis* at 4°C [12]. Under these conditions, acetoin was the only by-product detected. The low reaction temperature was chosen to guarantee sufficient stability of the yeast PDC and to reduce the decarboxylase activity and thus the amount of liberated acetaldehyde. Nevertheless, only 20% of the enzyme remained active after 6–8 h, showing the low stability of the enzyme. In contrast, our continuous operated reaction system produced (*R*)-PAC with a space–time yield of 27.4 g/(L · day) using 90 mM pyruvate and 30 mM benzaldehyde at 25°C and does not require any cooling. The activity of the bacterial mutant enzyme decreased only by about 10% in 18 h, showing the considerably higher stability of the bacterial mutant enzyme.

Apart from the significantly higher price for pyruvate compared to acetaldehyde, which is 130-fold higher on a molar base, the reaction using acetaldehyde is easier to handle and to be described in a kinetic model. We investigated the enzymatic synthesis of (*R*)-PAC from acetaldehyde and benzaldehyde using the PDC*Z.m.* mutant PDC*W392M*. The kinetical studies revealed that optimal results with respect to maximal reaction rate, enzyme stability and prevention of acetoin as the only by-product were obtained, employing both aldehydes in equimolar concentration in the range of 30–40 mM [31]. Thus, a continuously operated EMR was chosen to ensure a constant substrate concentration during the reaction. This bioreactor works under efflux conditions, meaning that the substrate and product concentrations affecting the enzyme in the reactor are equal to those measured at the outlet. The effective substrate concentrations during the reaction are a function of the initial substrate concentration, the enzyme concentrations, and the mean residence time (τ). Under unoptimized conditions, a space–time yield of 81 g (*R*)-PAC/(L · day) was obtained, starting from 50 mM of both aldehydes, 1 mg/ml PDC*W392M* as crude extract and choosing a residence time of 1 h and a reaction temperature of 25°C. The reaction was stopped after 40 h, although the enzyme was still active after this time. Apart from economical reasons that make the second process more attractive, our studies show that the enzyme-specific productivities of both processes are identical: 0.019 mmol (*R*)-PAC/(h · mg PDC*W392I*) from pyruvate and benzaldehyde and 0.022 mmol (*R*)-PAC/(h · mg PDC*W392M*) from acetaldehyde and benzaldehyde. The concentration of (*R*)-PAC in the outlet of the EMR can be increased by cascadence of EMRs [31].

In addition, the conversion of the substrates may be increased by variation of the enzyme concentration and the residence time. Our results pave the way for further studies on the enzymatic synthesis of (*R*)-PAC employing water-miscible organic solvents and further improved PDC *Z.m.*-mutants.

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