# Reaction Engineering of Benzaldehyde Lyase from *Pseudomonas fluorescens* Catalyzing Enantioselective C-C Bond Formation

Thomas Stillger,<sup>†,||</sup> Martina Pohl,<sup>‡</sup> Christian Wandrey,<sup>†</sup> and Andreas Liese<sup>§,\*</sup>

Institute of Biotechnology, Research Centre Juelich, D-52425 Juelich, Germany, Institute of Molecular Enzyme Technology, Heinrich Heine University of Duesseldorf, D-52426 Jülich, Germany, and Institute of Technical Biocatalysis, Hamburg University of Technology (TUHH), 21073 Hamburg, Germany

### Abstract:

The reaction engineering of benzaldehyde lyase (BAL, E.C. 4.1.2.38) from Pseudomonas fluorescens catalyzing the enantioselective carboligation of benzaldehyde and acetaldehyde yielding (R)-2-hydroxy-1-phenylpropanone (HPP) is presented. Based on kinetic studies a continuous process is developed. The developed bioreactor allows focusing the complex reaction system on the production of HPP with simultaneous discrimination of the undesired benzoin formation. The application of a continuous process in combination with membrane technology enables high space time yields (1120 g  $L^{-1}$  d<sup>-1</sup>, ee > 99%) of the product as well as high total turnover numbers of the biocatalyst (mol of product/mol of biocatalyst = 188.000). A kinetic model was developed to simulate the continuously operated reactor and to determine optimal production conditions. The synthesis of (R)-(3-chlorophenyl)-2-hydroxy-1-propanone (1214 g  $L^{-1} d^{-1}$ , ee = 99%) in the bioreactor demonstrates a broad applicability of the presented reactor concept for the production HPP derivatives.

#### Introduction

The catalytic asymmetric C–C bond formation is still a challenge in organic catalysis.<sup>1</sup> Classical methods in organic chemistry, e.g., chiral thiazolium and triazolium catalysts, usually afford anhydrous reaction media and the protection of acidic functional groups.<sup>2–4</sup> Thiamine-diphosphate (ThDP)-dependent enzymes could be regarded as a biocatalytical pendent of thiazolium and triazolium catalyst.<sup>5</sup> The application of ThDP-dependent enzymes in organic synthesis enables a convergent reaction pathway in combination with mild aqueous reaction conditions and no need for protecting group chemistry.<sup>6,7</sup> Starting with achiral and cheap aldehydes

- (3) Enders, D.; Breuer, K.; Teles, J. H. Helv. Chim. Acta 1996, 79, 1217.
- (4) Enders, D.; Niemeier, O.; Balensiefer, T. Angew. Chem., Int. Ed. 2006, 45, 1463.
- (5) Pohl, M.; Sprenger, G. A.; Müller, M. Curr. Opin. Biotechnol. 2004, 15, 335.

1172 • Vol. 10, No. 6, 2006 / Organic Process Research & Development Published on Web 11/04/2006



Figure 1. BAL-catalyzed carboligation.

the enzyme benzaldehyde lyase (BAL, E.C. 4.1.2.38) from *Pseudomonas fluorescens* catalyzes the reversible formation of 2-hydroxy ketones with high chemical and optical yields.<sup>8–10</sup> Chiral hydroxy ketones are versatile building blocks in organic chemistry and widespread subunits in biological active molecules.<sup>11</sup> Therefore the valuable synthetic potential of the ThDP-dependent enzymes might be used as a key step to introduce chirality into multistep chemoenzymatic syntheses of pharmaceuticals, agrochemicals, and pheromones.

The crystal structure of BAL has just recently been published.<sup>12</sup> Detailed studies on the substrate spectra of BAL have been described in the past 7 years.<sup>10,13–15</sup> Aromatic as well as aliphatic aldehydes are accepted by the enzyme yielding either benzoins as a product of self-condensation of the aromatic aldehyde or 2-hydroxy ketones derived by carboligation of the aromatic and the aliphatic aldehyde which we focus on in this study (Figure 1).

In this publication we present the reaction engineering of BAL catalyzing the carboligation of benzaldehyde and acetaldehyde yielding (R)-2-hydroxy-1-phenylpropanone ((R)-HPP). In a previous paper we reported the development of

- (9) Gonzales, B.; Merino, A.; Almeida, M.; Vicuna, R. Appl. Mircrobiol. Biotechnol. 1986, 52, 1428.
- (10) Gonzales, B.; Vicuna, R. J. Bacteriol. 1989, 171, 2401.
- (11) Adam, W.; Lazarus, M.; Saha-Möller, C. R.; Schreier, P. Acc. Chem. Res. 1999, 32, 837.
- (12) Mosbacher, T. G.; Müller, M.; Schulz, G. E. Fed. Eur. Biochem. Soc. 2005, 272, 6067.
- (13) Demir, A. S.; Sesenoglu, Ö.; Eren, E.; Hosrik, B.; Pohl, M.; Janzen, E.; Kolter, D.; Feldmann, R.; Dünkelmann, P.; Müller, M. Adv. Synth. Catal. 2002, 344, 96.
- (14) Dünkelmann, P.; Kolter, D.; Nitsche, A.; Demir, A. S.; Siegert, P.; Lingen, B.; Baumann, M.; Pohl, M.; Müller, M. J. Am. Chem. Soc. 2002, 124, 12084.
- (15) Demir, A. S.; Sesenoglu, Ö.; Dünkelmann, P.; Müller, M. Org. Lett. 2003, 5, 2047.

#### 10.1021/op0601316 CCC: \$33.50 © 2006 American Chemical Society

<sup>\*</sup> To whom correspondence should be addressed. Fax: +49-40-42878-2127. E-mail: liese@tuhh.de.

<sup>&</sup>lt;sup>†</sup> Research Centre Juelich.

<sup>&</sup>lt;sup>‡</sup> Heinrich Heine University of Duesseldorf.

<sup>&</sup>lt;sup>§</sup> Hamburg University of Technology (TUHH).

<sup>&</sup>lt;sup>II</sup> Present address: Institute of Pharmaceutical Sciences, University of Freiburg, Albertstrasse 25, D-79104 Freiburg, Germany.

<sup>(1)</sup> Faber, K.; Patel, R. N. Curr. Opin. Biotechnol. 2000, 11, 517.

<sup>(2)</sup> Enders, D.; Balensiefer, T. Acc. Chem. Res. 2004, 37, 534.

<sup>(6)</sup> Pohl, M.; Lingen, B.; Müller, M. Chem.-Eur. J. 2002, 8, 5288.

<sup>(7)</sup> Liese, A.; Seelbach, K.; Wandrey, C. Industrial Biotransformations, 2nd ed.; VCH-Wiley: Weinheim, 2006.

<sup>(8)</sup> Demir, A. S.; Pohl, M.; Janzen, E.; Müller, M. J. Chem. Soc., Perkin Trans. 1 2001, 633.



**Figure 2.** pH-dependent activity and stability of BAL (stability: 10 mM KPi, 0.35 mM ThDP, 0.35 mM MgSO<sub>4</sub>, T = 0 °C; activity: 10 mM KPi, pH = 8, 0.35 mM ThDP, 0.35 mM MgSO<sub>4</sub>, 20 mM benzaldehyde, 30 vol % DMSO, T = 20 °C). The data points are connected for better visualization.

a batch reactor concept.<sup>16</sup> Space time yields of 36 g L<sup>-1</sup> d<sup>-1</sup> (*R*)-HPP were achieved. The aim of this work was to improve the productivity in the synthesis of (*R*)-HPP by employing a continuous process that enables a high yield in combination with economical catalyst consumption. The reaction system was characterized kinetically to identify an appropriate reactor concept. Based on experimental data a kinetic model for the BAL-catalyzed formation of HPP was developed.

#### **Results and Discussion**

**Enzyme Characterization.** In a recent publication we evaluated the industrial applicability of BAL investigating the crucial parameters influencing the enzymatic carboligation.<sup>16</sup> The cofactors thiamine diphosphate and MgSO<sub>4</sub> (0.5 mM of each) are substantial for the stability of BAL. Within a range of 0.015 and 1 mM the enzymatic activity is not dependent on the cofactor concentration. To dissolve high concentrations of the aromatic substrate dimethyl sulfoxide (DMSO, 30 vol %) was added to the aqueous reaction media. Surprisingly, BAL was stabilized significantly in the presence of DMSO.<sup>16</sup>

In view of technical applications the pH-dependency of both the enzyme activity and stability have to be considered. As depicted in Figure 2 the maximum of enzyme stability is achieved at pH 7, whereas the highest carboligation activity is observed at almost pH 9. To obtain a favorable enzymatic overall performance the pH of the reaction media was adjusted to 8 as a compromise. Furthermore the reactions were performed in 35 mM TEA buffer containing 30 vol % of the cosolvent DMSO and 0.35 mM concentrations of the cofactors ThDP and MgSO<sub>4</sub>.

**Characterization of the Reaction System.** The HPP formation starting from benzaldehyde and acetaldehyde was performed in a batch reactor to evaluate the course of the biotransformation. Figure 3 shows a typical time-dependent course of reactant concentrations. To shift the reaction equilibrium towards HPP formation a surplus of varying



*Figure 3.* HPP formation in a batch reactor with intermediate formation of benzoin (20 mM benzaldehyd, 60 mM acetaldehyde, 35 mM TEA, pH = 8, 0.35 mM ThDP, 0.35 mM MgSO<sub>4</sub>, 30 vol % DMSO, 3 U mL<sup>-1</sup> BAL, T = 20 °C, V = 3 mL). The data points are connected for better visualization.



*Figure 4.* Reaction scheme based on the postulated mechanism<sup>9</sup> for BAL-catalyzed acyloin formation and cleavage.

amounts of acetaldehyde were reacted with 20 mM benzaldehyde. Above 60 mM, acetaldehyde conversion<sup>17</sup> exceeds 99% as depicted in Figure 3.

Although benzoin is formed as an intermediate at the beginning of the biotransformation, both benzaldehyde and benzoin are quantitatively converted to HPP at the end of the reaction. The intermediate formation of benzoin is connected with an intermediate precipitation due to the low solubility of benzoin (0.2 mM in KP<sub>i</sub> buffer and 1.2 mM in buffer with 30 vol % DMSO, respectively). The formation of solid benzoin is a crucial effect which has to be considered in the development of a continuous process because the solid gives rise to tube and membrane blocking within the reactor.

**Kinetic Characterization and Reactor Choice.** Demir et al. suggested a mechanism for the BAL-catalyzed acyloin formation and cleavage.<sup>8</sup> Based on the postulated mechanism the HPP formation starting with benzaldehyde and acetaldehyde was divided into reversible subreactions as shown in Figure 4.

Parallel to the cross-coupling of benzaldehyde and acetaldehyde to HPP (Figure 4, reaction 3) the self-condensation of benzaldehyde in terms of the benzoin reaction is observed (Figure 4, reaction 1). In the presence of acetaldehyde BAL catalyzes the cleavage of (R)-benzoin yielding HPP and

<sup>(16)</sup> Domínguez de María, P.; Stillger, T.; Pohl, M.; Wallert, S.; Drauz, K.; Gröger, H.; Trautwein, H.; Liese, A. J. Mol. Catal. B: Enzym. 2006, 38, 43.

<sup>(17)</sup> The conversion was calculated on the consumption of benzaldehyde serving as the key component in view of reaction engineering aspects.

benzaldehyde (Figure 4, reaction 2). The course of the reactant concentrations in a batch reactor shown in Figure 3 is in accordance with the postulated mechanism. Demir et al. showed that the formation of HPP (reaction 2, reaction 3) is quasi irreversible in the presence of a surplus of acetaldehyde.<sup>13</sup> As will be shown below, the cleavage of benzoin in the presence of acetaldehyde always results in C-C bond formation yielding HPP (reaction 2) which led us to assume that the formation of benzoin from benzaldehyde (reaction 1) is, under the conditions applied, irreversible as well.

Considering the irreversibility, the reaction system shown in Figure 4 is reduced to three subreactions which were kinetically characterized: the formation of benzoin starting from benzaldehyde (reaction 1), and the formation of HPP starting from benzaldehyde (reaction 3) and benzoin (reaction 2), respectively.

**Benzoin Formation (Reaction 1).** The rate of benzoin formation as a function of the benzaldehyde concentration is in accord with a Michaelis–Menten-like kinetic. The limited solubility of benzaldehyde in aqueous media ( $\sim$ 50 mM) prevents enzyme saturation.<sup>18</sup> The measured data could be described mathematically using eq 1 considering an inhibition by acetaldehyde and HPP.

$$R_{1} = v_{\max 1} \frac{[BA]}{K_{M,BAAcc} \left(1 + \frac{[HPP]}{K_{I,HPP}}\right) \left(1 + \frac{[AA]}{K_{I,AA}}\right) + [BA]}$$
(1)

**Cleavage of Benzoin to HPP (Reaction 2).** The rate of HPP formation was measured with constant benzoin concentration (1.5 mM) and varied acetaldehyde concentrations as well as vice versa ( $c_{AA} = 60 \text{ mM}$ ).<sup>19</sup> The results could be described mathematically according to a Michelis–Menten-like double substrate kinetic using eq 2.

$$R_2 = v_{\text{max2}} \frac{[\text{BZ}]}{K_{\text{M,BZ}} + [\text{BZ}]} \cdot \frac{[\text{AA}]}{K_{\text{M,AA}} + [\text{AA}]}$$
(2)

Starting from benzoin and acetaldehyde (reaction 2) the formation of HPP is paralleled by an equal amount of benzaldehyde formation. With increasing benzoin concentration the rates of HPP and benzaldehyde formation increase similarly (at a constant acetaldehyde concentration of 60 mM). Supposing that benzoin is additionally cleaved by BAL in terms of the reverse reaction of reaction 1, a stronger acceleration of the benzaldehyde formation compared to the HPP formation would be expected but was not observed.<sup>20</sup> We assume that in the presence of a surplus of acceptor aldehyde the C–C bond formation always is catalyzed by the enzyme as the dominant reaction. As a consequence reaction 1 can be regarded to be irreversible under the conditions applied (surplus of acetaldehyde).

Formation of HPP from Benzaldehyde and Acetaldehyde (Reaction 3). The rates were measured with the same procedure as that described before (see reaction 2), applying

(19) See Figure 11 in the Supporting Information.(20) See Figures 11 (left) and 12 in the Supporting Information.

```
(20) see Figures 11 (left) and 12 in the Supporting information
```

1174 • Vol. 10, No. 6, 2006 / Organic Process Research & Development

**Table 1.** Kinetic Parameters of BAL Catalyzed HPP Formation

parameter	value	unit
K <sub>M BAACC</sub>	$111.9 \pm 16.5$	mM
K <sub>M,BADo</sub>	$2.2 \pm 0.3$	mM
K <sub>M BZ</sub>	$0.2 \pm 0.1$	mM
K <sub>MAA</sub>	$6.0 \pm 1.0$	mM
KLAA	$6.4 \pm 0.4$	mM
KLHPP	$7.2 \pm 0.5$	mM
$v_{\rm max1}$	$916.7 \pm 106.6$	$\rm U~mg^{-1}$
$v_{\rm max2}$	$20.9 \pm 3.0$	$U mg^{-1}$
$v_{\rm max3}$	$14.4 \pm 0.4$	$U mg^{-1}$

benzaldehyde instead of benzoin.<sup>21</sup> Again the substratedependent rate increase could be described with a Michaelis– Menten-type double substrate kinetic (eq 3).

$$R_{3} = v_{\text{max3}} \frac{[\text{BA}]}{K_{\text{M,BADo}} + [\text{BA}]} \frac{[\text{AA}]}{K_{\text{M,AA}} + [\text{AA}]}$$
(3)

In contrast to the formation of benzoin (reaction 1) enzyme saturation was achieved within the solubility of benzaldehyde quite well. A reasonable explanation could be given by the different chemical behavior of benzaldehyde in both reactions. Regarding the formation of benzoin, benzaldehyde has to react as both electron donor and acceptor, whereas, in the HPP formation, benzaldehyde has to react as an electron donor only. These circumstances have been considered by introducing two  $K_{\rm M}$  values for benzaldehyde depending on whether the benzaldehyde reacts to benzoin ( $K_{M,Acc}$ ) or to HPP ( $K_{M,Do}$ ).

The kinetic parameters have been obtained by nonlinear curve fitting of the corresponding kinetic equations to the measured data (Scientist 2.0, Micromath, USA). The results are summarized in Table 1.

In order to describe the time-dependent course of the concentrations in a reactor, the rates of the subreactions were combined to one kinetic model. The reactant concentrations in the simulation experiments were described using the following equations.

$$d[BA]/dt = [BAL](-2v_{R1} + v_{R2} - v_{R3})$$
$$d[BZ]/dt = [BAL](v_{R1} - v_{R2})$$
$$d[HPP]/dt = [BAL](v_{R2} + v_{R3})$$

**Choice of Reactor Type.** The key parameter for the choice of an appropriate reactor type is the difference in the kinetics of benzoin and HPP formation starting from benzaldehyde (Figure 5). The rate of benzoin formation increases within the solubility range of benzaldehyde nearly linear with the benzaldehyde concentration. Regarding the rate of HPP formation, enzyme saturation is achieved above a benzaldehyde concentration of approximately 5 mM. In a batch reactor the concentrations of all reactants vary with the reaction time. Especially at the beginning of the reaction the benzoin formation is kinetically preferred. In contrast to the batch reactor the continuously operated stirred tank reactor

<sup>(18)</sup> See Figure 10 in the Supporting Information.

<sup>(21)</sup> See Figure 13 in the Supporting Information.



*Figure 5.* Rate of HPP and benzoin formation as a function of benzaldehyde concentration.



*Figure 6.* Enzyme membrane reactor (1, pump; 2, mass flow meter; 3, manometer; 4, air remover; 5, reactor vessel with membrane; 6, personal computer; data connections are symbolized as dashed gray lines).

(CSTR) operates at a steady state with constant reactant concentrations over time. In a CSTR reactor it should be possible to establish an operation point with the focus on HPP synthesis and discrimination of benzoin formation (Figure 5).

**Continuous HPP Production.** An enzyme membrane reactor (EMR) shows CSTR characteristics. The advantage of applying the EMR technology in a continuous process is a high space time yield typical of a continuous process in combination with comfortable catalyst retention without the need for enzyme immobilization. The retention of the enzyme within the reactor enables decoupling of the residence times of the enzyme and the substrate. The efficiency of a catalyst employed in a process is quantified by the total turnover number (ttn) (eq 4).

$$ttn = \frac{n_{\text{product}}}{n_{\text{catalyst}}} \tag{4}$$

An appropriate reactor concept, designed to obtain high total turnover numbers as well as high space time yields, is depicted in Figure 6.

The reaction conditions for the continuous HPP production were equal to those developed for the batch reactor. Acetaldehyde was employed in a 3-fold excess (60 mM) relative to benzaldehyde (20 mM). In a first experiment the residence time was set to 1 h and kept constant over the complete reaction time to evaluate the general applicability of the developed reactor concept in combination with BAL. As depicted in Figure 7 the reactor could be operated over a period of 240 h. Due to enzyme deactivation the conversion decreased from 91% to 70% within the reaction time. The course of conversion can be simulated by the kinetic model considering a deactivation constant of  $6.83 \times 10^{-3} h^{-1}$ , which is equivalent to an enzyme consumption of 16% per day.



*Figure 7.* Continuous HPP production in EMR (20 mM benzaldehyde, 60 mM acetaldehyde, 35 mM TEA, pH = 8, 0.35 mM ThDP, 0.35 mM MgSO<sub>4</sub>, 30 vol % DMSO, 30 U mL<sup>-1</sup> BAL, T = 20 °C, V = 10 mL).



*Figure 8.* Continuous HPP production in EMR at varying operating points (20 mM benzaldehyde, 60 mM acetaldehyde, 35 mM TEA, pH = 8, 0.35 mM ThDP, 0.35 mM MgSO<sub>4</sub>, 30 vol % DMSO, 30 U mL<sup>-1</sup> BAL, T = 20 °C, V = 3 mL).

The total turnover number was calculated to be 150 000 with respect to the total amount of employed catalyst and 188 000 just considering the deactivated amount of enzyme. Compared to a batch reactor, the ttn is increased significantly in the continuous process (ttn<sub>batch</sub> = 7200).

In a second reactor run residence times of 1, 0.5, and 0.25 h were applied. As depicted in Figure 8 a steady state was observed for each operating point. The corresponding space time yields were calculated to be 61 (1 h), 112 (0.5 h), and 173 (0.25 h) g  $L^{-1} d^{-1}$  HPP with an enantiomeric excess > 99% (*R*). The benzoin concentration never exceeded 0.4 mM within the reaction time which is less than one-third of the maximum solubility of benzoin in the reaction system (1.5 mM). Again the time-dependent course of concentrations could be simulated based on the kinetic model (comprising the above-mentioned mass balances and kinetic rate equations) in excellent accordance.

To enhance the productivity of the bioreactor, further experiments were performed with strongly reduced residence times down to 3 min. Short residence times are accompanied by a significant decrease of conversion which was compensated by an increased enzyme concentration (up to 300 U mL<sup>-1</sup>). As a consequence, the space time yield of HPP was increased up to 1120 g L<sup>-1</sup> d<sup>-1</sup> (Supporting Information). Compared to the batch reactor the space time yield in a CSTR



*Figure 9.* Continuous production of (*R*)-1-(3-chlorophenyl)-2-hydroxy-1-propanone in EMR (20 mM 3-chlorobenzaldehyde, 60 mM acetaldehyde, 35 mM TEA buffer, pH = 8, 0.35 mM ThDP, 0.35 mM MgSO<sub>4</sub>, 30 vol % DMSO, T = 20 °C, V = 3 mL).

is increased by more than 30-fold. As a result, the CSTR concept turns out to be an appropriate reactor type for the production of HPP ensuring a high productivity, thereby avoiding undesired benzoin formation.

To evaluate a broader applicability of the reactor concept, the synthesis of (*R*)-1-(3-chlorophenyl)-2-hydroxy-1-propanone, which has been used in the synthesis of Bupropion,<sup>22</sup> was performed in the bioreactor starting from 3-chlorobenzaldehyde and acetaldehyde. Figure 9 shows the conversion as a function of the total number of residence times. Starting with a residence time of 6 min a conversion of 90% was achieved. Reducing the residence time to 3 min lowers the conversion to 70%. The corresponding space time yields were calculated to 711 g L<sup>-1</sup> d<sup>-1</sup> (6 min) and 1214 g L<sup>-1</sup> d<sup>-1</sup> (3 min), respectively. The enantiomeric excess was determined as ee = 99% (*R*). Again, no precipitation of the corresponding 3,3'-dichlorobenzoin derivative was observed.

These results encourage us to suggest the CSTR concept as the reactor type of choice for the BAL-catalyzed synthesis of 2-hydroxy-1-phenylpropanone and HPP derivatives. The CSTR concept allows focusing the complex reaction system on the formation of the desired product in combination with a high total turnover number and high space time yields. Further HPP derivatives, e.g., derived by carboligation of substituted aromatic aldehydes with functionalized acetaldehyde derivatives, have been synthesized successfully by application of the developed bioreactor.<sup>23</sup>

#### **Experimental Section**

**Chemicals and Biocatalyst.** All chemicals were commercially available by Sigma-Aldrich. The aldehydes were purchased as redestillates and stored under an inert atmosphere. ThDP was obtained from Fluka. Benzaldehyde lyase was overexpressed in *Escherichia coli* as a recombinant hexahistidine fusion protein (SG13009 BAL<sub>HIS</sub>).<sup>24</sup> The enzyme was purified by nickel-chelate affinity chromatography and employed to biotransformations as a freeze-dried preparation.

**Determination Protein Concentration and Activity.** The protein concentration was determined photometrically using Coomassie-Brilliant-Blue (BioRad, Munich) and bovine serum albumin as a standard.<sup>25</sup>

All activity measurements were performed using standard conditions. 0.06 mmol of benzaldehyde was dissolved in 2.9 mL of TEA buffer (35 mM, pH = 8) containing 0.35 mM ThDP, 0.35 mM MgSO<sub>4</sub>, and 30 vol % DMSO. The reaction was started by adding 0.1 mL enzyme solution (0–0.5  $\mu$ g mL<sup>-1</sup>). Aliquots were taken periodically, and the amount of the benzoin formed was determined by HPLC.

1 U benzaldehyde lyase was defined as the amount of enzyme which catalyzes the formation of one  $\mu$ mol of benzoin per min starting with 20 mM benzaldehyde at 20 °C.

For kinetic measurements the procedure and reaction conditions were used as described above. The reactants and their concentrations were adjusted to the respective reaction. Benzaldehyde and benzoin were employed within the range of their solubility in aqueous media (50 and 1.5 mM). Acetaldehyde was employed up to 160 mM, ensuring enzyme saturation in all reactions. To ensure initial rate conditions only data points referring to a conversion below 10% were used to calculate the kinetic parameters.

**Determination of Enzyme Stability.** 2 U of BAL were dissolved in 2 mL of 10 mM potassium phosphate buffer containing 0.35 mM ThDP and 0.35 mM MgSO<sub>4</sub>. The temperature was kept at 0 °C to avoid thermal deactivation of the enzyme. Aliquots were withdrawn periodically and assayed for residual activity as described above.

**HPLC Analytics.** Quantitative analysis of all reactants was performed by HPLC using a LiChrosphere RP-8 column (250 mm  $\times$  4 mm) and TEA buffer (0.2%, pH = 3)/ acetonitrile (60:40, v/v) as an eluent (flow: 1.0 mL min<sup>-1</sup>, 20 °C). The enantiomeric excess was determined by chiral phase HPLC with a Daicell Chiralcel OD-H column and 2-propanol/*n*-hexane (98:2, v/v) as an eluent (flow: 1.0 mL min<sup>-1</sup>, 20 °C).

Analytical Batch Experiments. 0.06 mmol of aromatic aldehyde and 0.18 mmol of acetaldehyde were dissolved in 3 mL of buffer solution consisting of 35 mM TEA buffer, pH = 8, 0.35 mM ThDP, 0.35 mM MgSO<sub>4</sub>, and 30 vol % DMSO. The reaction vessel was maintained at 20 °C. The reaction was started by adding 2 U BAL. Aliquots were taken periodically, and the concentrations of reactants were measured by HPLC.

**Continuous Experiments.** A solution of 20 mM aromatic aldehyde and 60 mM acetaldehyde in 35 mM TEA buffer, pH = 8, 0.35 mM ThDP, 0.35 mM MgSO<sub>4</sub>, and 30 vol % DMSO was stored under argon atmosphere. The substrate solution was pumped into the reactor using a piston pump (P500, Pharmacia Biotech, Germany). The temperature was

<sup>(22)</sup> Fang, Q. K.; Han, Z.; Grover, P.; Kesser, D.; Seneneyake, C. H.; W. S. A. Tetrahedron: Asymmetry 2000, 11, 3659.

<sup>(23)</sup> Hildebrand, F.; Kühl, S.; Pohl, M.; Vasic-Racki, D.; Müller, M.; Wandrey, C.; Lütz, S. *Biotechnol. Bioeng.* 2006, published online: 7 Oct 2006, DOI: 10.1002/bit.21189.

<sup>(24)</sup> Janzen, E.; Müller, M.; Kolter-Jung, D.; Kneen, M. M.; McLeish, M. J.; Pohl, M. *Bioorg. Chem.* **2006**, DOI: 10.1016/j.bioorg.2006.09.002.
(25) Sedmark, J. J.; Grossberg, S. E. *Anal. Biochem.* **1977**, *79*, 544.

kept at 20 °C. To maintain a constant volumetric flow a thermal mass flow meter (Bronkhorst, Netherlands) was used in combination with a software assisted pump adjustment (LabView). The enzyme was retained in the reactor by a semipermeable membrane (regenerated cellulose, YM10, Millipore, USA). Aliquots were taken periodically at the outlet of the reactor and were analyzed by HPLC.

# ABBREVIATIONS

AA	acetaldehyde
BA	benzaldehyde
BAL	benzaldehyde lyase
BZ	benzoin
CSTR	continuously operated stirred tank reactor
DMSO	dimethyl sulfoxide
EMR	enzyme membrane reactor
HPP	2-hydroxy-1-phenyl-propanone
$K_{\mathrm{I,AA}}$	inhibition constant for acetaldehyde
K <sub>I,HPP</sub>	inhibition constant for (R)-2-hydroxy-1-phenyl-1-pro-
	panone
K <sub>M,AA</sub>	Michaelis-Menten constant for acetaldehyde
K <sub>M,BAAcc</sub>	Michaelis-Menten constant for benzaldehyde as acceptor
$K_{\rm M,BADo}$	Michaelis-Menten constant for benzaldehyde as donor
$K_{\rm M,BZ}$	Michaelis-Menten constant for benzoin
sty	space time yield

TEA	Triethanolamine
ThDP	Thiamine diphosphate
ttn	total turnover number
v	reaction velocity
$v_{\rm max1}$	maximal velocity of the benzoin formation
$v_{\rm max2}$	maximal velocity of the cleavage of benzoin to HPP
$v_{max3}$	maximal velocity of the formation of HPP from benz- aldehyde and acetaldehyde

## Acknowledgment

The authors would like to thank Dr. Elena Janzen, University of Düsseldorf, for cloning and recombinant expression of BAL and Prof. Durda Vasic-Racki, University of Zagreb, for fruitful discussions. Expert technical assistance from Denise Herzog and Lilia Härter is gratefully acknowledged. This work was funded by the Deutsche Forschungsgemeinschaft (SFB 380).

### **Supporting Information Available**

Additional figures. This material is available free of charge via the Internet at http://pubs.acs.org.

Received for review July 3, 2006. OP0601316