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Characterization of benzaldehyde lyase from *Pseudomonas fluorescens*: A versatile enzyme for asymmetric C–C bond formation

Elena Janzen [a], Michael Müller [b], Doris Kolter-Jung [c], Malea M. Kneen [d], Michael J. McLeish [d], Martina Pohl [a,*]

[a] *Institute of Enzyme Technology, Heinrich-Heine University Duesseldorf, Research Centre Juelich, 52426 Juelich, Germany*
[b] *Institute of Pharmaceutical Sciences, Albert-Ludwigs-University Freiburg, Albertstr. 25, D-79104 Freiburg, Germany*
[c] *Institute of Biotechnology 2, Research Centre Juelich, D-52425 Jülich, Germany*
[d] *College of Pharmacy, University of Michigan, Ann Arbor, 428 Church St., MI 48109-1065, USA*

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Abstract

The thiamin-diphosphate-dependent enzyme benzaldehyde lyase is a very import catalyst for chemoenzymatic synthesis catalyzing the formation and cleavage of (*R*)-hydroxy ketones. We have studied the stability of the recombinant enzyme and some enzyme variants with respect to pH, temperature, buffer salt, cofactors and organic cosolvents. Stability of BAL in chemoenzymatic synthesis requires the addition of cofactors to the buffer. Reaction temperature should not exceed 37 °C. The enzyme is stable between pH 6 and 8, with pH 8 being the pH-optimum of both the lyase and the ligase reaction. Potassium phosphate and Tris were identified as optimal reaction buffers and the addition of 20 vol% DMSO is useful to enhance both the solubility of aromatic substrates and products and the stability of BAL. The initial broad product range of BAL-catalyzed reactions has been enlarged to include highly substituted hydroxybutyrophenones and aliphatic acyloins.

* Corresponding author. Fax: +49 2461 612490.
E-mail addresses: michael.mueller@pharmazie.uni-freiburg.de (M. Müller), mcleish@umich.edu (M.J. McLeish), ma.pohl@fz-juelich.de (M. Pohl).

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1. Introduction

Benzaldehyde lyase (BAL, EC 4.1.2.38) has so far been found only in *Pseudomonas fluorescens* Biovar I. This strain is able to utilize lignin-like compounds such as benzoin and anisoin as a sole carbon source. The enzyme responsible for this catabolic activity is BAL which catalyzes the cleavage of the aromatic acyloins to aldehydes, which are further catabolized in the β-ketoadipate pathway. BAL has been first described by Gonzales and Vicuna [1]. These authors established the requirement for thiamin diphosphate (ThDP) and a divalent cation such as Mg^{2+}. Later the coding gene (bzl) was cloned and its DNA sequence has been published [2]. Recently the 3D structure of BAL has been elucidated by some of us [3], and catalytically important residues have been determined [4]. Although BAL was first identified by its lyase activity, we have previously reported that it is also able to catalyze the reverse reaction [5,6]. Subsequently the enzyme has been used to catalyze the synthesis of various aromatic and heteroaromatic 2-hydroxy ketones [7–10]. In contrast to wild-type benzoylformate decarboxylase (wtBFD) [11], BAL accepts aromatic aldehydes substituted in the *ortho*-position as well. Only a few aromatic aldehydes, such as pyridine 3- and 4-carbaldehyde as well as sterically exceedingly demanding aldehydes resulted in either very low yields or in no benzoin condensation at all [5]. Moreover, in addition to acetaldehyde, mono- and dimethoxy acetaldehyde are good acceptor substrates for BAL, providing enantiopure hydroxypropiophenone derivatives [12]. Various ThDP-dependent enzymes, especially 2-keto acid decarboxylases such as pyruvate decarboxylase (PDC) [13] and BFD [11,14,15], have been described to catalyze C–C bond formation and/or cleavage. Of the enzymes of this type, BAL possesses a remarkably broad substrate and reaction range [16–19], making a vast variety of chiral 2-hydroxy ketones accessible [6,20,21]. In addition, since BAL is strictly (*R*)-enantioselective, the enzyme is also useful for kinetic resolution of racemic benzoin [5]. Some typical reactions catalyzed by BAL are shown in Fig. 1.

BAL catalyzes the cleavage and formation of (*R*)-2-hydroxyketones such as benzoin and 2-hydroxypropiophenone via a common intermediate ThDP-bound carbanion-enamine. Conversely, wild-type (wt) BAL does not show any decarboxylase activity towards 2-keto acids. However, this activity can be introduced by a single point mutation (BAL*A28S* [4]). The enantioselectivity and the substrate specificity of BAL have recently been explained by molecular modelling based on the 3D structure of the enzyme [22].

We have re-cloned the BAL gene (*blz*) [2] in order to obtain an efficient expression system for BAL and have corrected some errors in the original DNA sequence [23]. The recombinant enzyme carrying a C-terminal hexahistidine tag was thoroughly characterized with respect to its carboligase and carbolyase activity, its stability, pH- and temperature optima, kinetic parameters, native molecular weight, and isoelectric point. Mutagenesis studies based on sequence and structural comparison with BFD from *Pseudomonas putida* revealed A480 in BAL as a key residue in the carboligase and carbolyase reactions. Further, we describe the BAL catalyzed synthesis of hydroxybutyrophenones, which opens up a new chemoenzymatic access to chiral polyols.

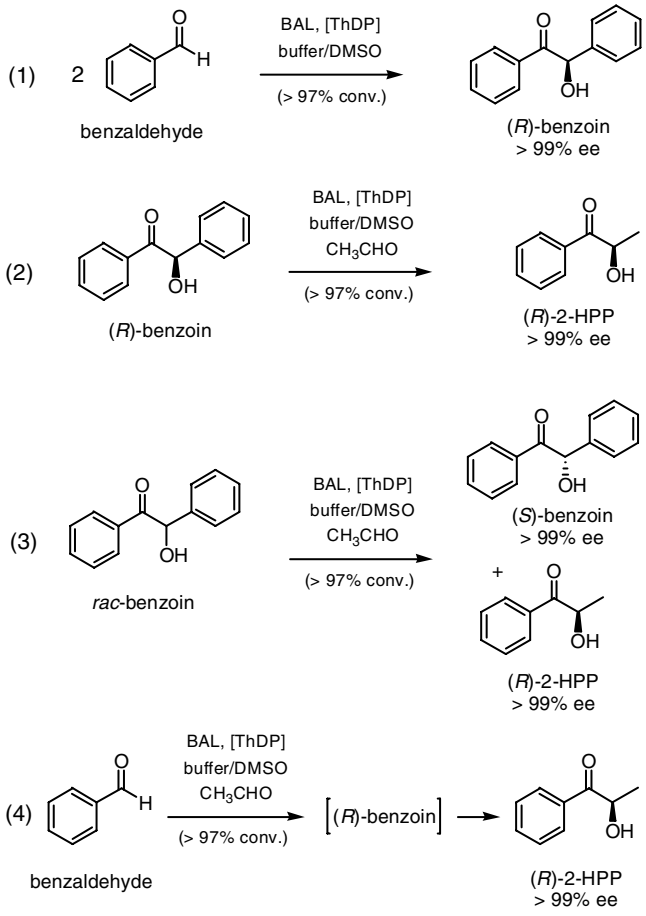

Fig. 1. A variety of reactions catalyzed by BAL [5].

2. Materials and methods

2.1. Re-cloning of BAL

PCR amplification of the BAL gene (blz) (1.7 kb) was performed using the plasmid pUC18/blz [2] as a template and the following primers, which introduce appropriate restriction sites for the subsequent cloning steps:

BAL-s 5′-CCA TGGCGA TGA TTA CAG GCG GCGAA-3′
*Nco*I
BAL-as 5′-GGA TCC GAA GGG GTC CAT GCC GAT CAG AA-3′
*Bam*HI

The BAL gene was first ligated into the vector pUC18, which was linearized with *Sma*I and dephosphorylated prior to ligation, in order to allow digestion with *Nco*I. The resulting plasmid (pBAL) was subsequently linearized with *Bam*HI. Since the BAL gene includes an

internal *Nco*I site, a partial digestion with this restriction enzyme was performed in order to isolate the complete gene, which was then ligated into the vector pKK233-2. The latter was obtained by excision of the BFD gene from the vector pBFD*his* [11], using *Nco*I and *Bgl*II, thereby leaving the His-tag encoding codons in the vector. The BAL gene was then ligated into this vector yielding the vector pBAL*his* (6.3 kb). The corresponding gene product is named wtBAL.

2.2. Construction of mutant enzymes

The BAL*A28S* variant was available from a previous study [4]. The other BAL mutants were prepared using the QuickChange site directed mutagenesis kit (Stratagene, La Jolla, USA) as previously described [4]. The forward primers used for the mutagenesis are shown below with the mutated codons underlined and the lowercase letters indicating a base change from wild-type.

H286A 5′-GGCCTTAACACCGGGgcTGGATCcGGGCAGTTG-3′
A480I 5′-CCAAAGCTGGGGGataACgTTGCATTTCCAGCAATTGG-3′
F484I 5′-GGGGGGCaACgTTGCATaTCCAGCAATTGGCCGTC-3′

For A28S this adds a *Bam*HI restriction site which allows for ready screening of transformants. For A480I and F484I the mutation results in the loss of a *Bsr*DI site. Following mutagenesis, the template DNA was removed by treatment with *Dpn*1 and the remaining PCR products transformed into *Escherichia coli* strain XL1-Blue (Stratagene, La Jolla, CA). Single colonies were picked and their DNA isolated and screened for the desired mutation by restriction analysis. The fidelity of the PCR amplification and the presence of the mutation were then confirmed by sequencing. Like wtBAL all variants were expressed as C-terminal hexahistidine fusion proteins.

2.3. Expression and purification

Several *E. coli* strains, such as HB101, AD494, M15, JM109, and SG13009 were transformed with pBAL*his*. Cells were grown in 1.5 L shaking flasks and protein expression was induced with 1 mM IPTG after 3.5 h at 37 °C (OD 0.7–1.0). Cells were harvested after 5 h at 37 °C. Typical cell yields were about 3.5 g/L. The best results were obtained using *E. coli* strain SG13009, which was then used for high density fed-batch cultivation according to the method of Korz et al. [24]. Fed-batch cultivation was performed in a 40 L Techfors fermenter (Infors AG, CH) at 30 °C. Protein expression was induced by the addition of 1.5 mM IPTG at an $OD_{600} \sim 60$. A 15 L culture typically yielded 2–3 kg *E. coli* cells.

Purification of BAL to homogeneity (>95%) was performed by immobilized metal ion chromatography using a purification protocol previously developed for benzoylformate decarboxylase [11]. After purification the enzyme was stored freeze dried.

2.4. Activity assays

One unit of lyase activity is defined as the amount of BAL which catalyzes the cleavage of 1 μmol of benzoin per minute under standard conditions (30 °C, pH 8).

2.4.1. Lyase reaction (direct assay)

The direct assay is based on the cleavage of benzoin into two molecules of benzaldehyde [1]. In 50 mM Tris-buffer, pH 8, 15% (v/v) PEG-400, both benzoin and benzaldehyde have absorption maxima at 250 nm, with very similar extinction coefficients (benzoin: 11.1 L $mmol^{-1}$ cm^{-1}, benzaldehyde: 10.9 L $mmol^{-1}$ cm^{-1}).

The assay mixture contained 950 μL of a 0.1 mM benzoin solution in 50 mM Tris buffer, pH 8, 15% (v/v) PEG-400 with 2.5 mM $MgSO_4$ and 0.1 mM ThDP and 50 μL BAL solution. The reaction was followed for 1 min at 30 °C. Activity was calculated using the following equation:

$$\frac{U}{\mathrm{mL}} = \frac{\Delta E}{\mathrm{min}} \cdot \frac{V}{v \cdot d \cdot (2\varepsilon_p - \varepsilon_s)} = \frac{\Delta E}{\mathrm{min}} \cdot 1.84 \tag{1}$$

where U, Units (μmol/min); ΔE/min, change of absorption at 250 nm per min; V, total volume (1 mL), v, volume of enzyme sample (50 μL); ε_s, extinction coefficient of benzoin [L mol^{-1} cm^{-1}]; ε_p, extinction coefficient of benzaldehyde [L mol^{-1} cm^{-1}].

2.4.2. Lyase reaction (coupled assay)

The coupled assay is also based on the cleavage of benzoin to benzaldehyde. The benzaldehyde is subsequently reduced by horse liver alcohol dehydrogenase (ADH). The reaction is followed by measuring the decrease of NADH at 340 nm ($\varepsilon = 6.31$ L $mmol^{-1}$ cm^{-1}). The assay mixture contained 850 μL benzoin solution (1.5 mM benzoin) in assay buffer (50 mM potassium phosphate buffer, 15% (v/v) PEG-400, pH 8, 2.5 mM $MgSO_4$, 0.1 mM ThDP), 50 μL NADH (2.5 mM) assay buffer, 50 μL horse liver ADH (5 U/mL) in assay buffer, and 50 μL BAL. Taking into account that one benzoin molecule is cleaved into two molecules of benzaldehyde, activity was calculated using the following equation:

$$\frac{U}{\mathrm{mL}} = \frac{\Delta E}{\mathrm{min}} \cdot 1.6077 \tag{2}$$

2.4.3. Ligase reaction

One unit of ligase activity is defined as the amount of BAL which catalyzes the formation of 1 μmol of benzoin per minute under standard conditions (30 °C, pH 8).

Initial rates of BAL-catalyzed benzoin formation were determined using HPLC. Samples containing 10–60 mM benzaldehyde in 50 mM potassium phosphate buffer, pH 8, 2.5 mM $MgSO_4$, 0.1 mM ThDP were incubated with 0.05–0.1 μg/mL BAL at 30 °C in glass vessels. At appropriate time intervals, samples were withdrawn to measure the amount of benzoin.

HPLC conditions:
Column: C18 Hypersil ODS 5 μm, 250 mm × 4.6 mm
Eluent: 40% acetonitrile, 0.5% acetic acid, 59.5% water (v/v/v)
Flowrate: 1.1 mL/min
Detection wavelength: 250 and 263 nm
Retention times: benzaldehyde: 7.2 min; benzoin 9.8 min

2.4.4. Decarboxylase activity

Decarboxylase activity of the BAL*A28S* variant was assayed as previously described [11].

2.5. Protein determination

Protein determination was performed according to Bradford [25] using BSA as a standard.

2.6. Determination of molecular mass and isoelectric point

The molecular mass of BAL was determined using MALDI-TOF-MS (ALADIM, Institute of Laser Medicine, University of Duesseldorf), N_2-Laser, $\lambda = 337$ nm, using a 2,5-dihydroxybenzoic acid matrix (9 mg/mL) in 20% acetonitrile/79.9% water/0.1% trifluoroacetic acid (v/v/v). The system was calibrated with 1.6 mg BSA/mL (MW 66430 Da) in the same solvent.

Size-exclusion chromatography was performed using a Superdex G200 column (total volume 123.6 mL) (∅ 1.6 cm) (Amersham-Pharmacia) and 50 mM potassium phosphate buffer, pH 6.5, including 5 mM $MgCl_2$, 0.1 mM ThDP and 150 mM KCl. Calibration was performed using ribonuclease A (13.7 kDa), chymotrypsinogen A (25 kDa), ovalbumin (43 kDa), BSA (67 kDa), aldolase (158 kDa), catalase (232 kDa), ferritin (440 kDa), and thyroglobulin (669 kDa).

The isoelectric point was determined according to the method of Westmeier [26] using an ampholyte containing agarose gel. The following proteins were used for calibration: cytochrome *c* (p*I* 10.65), ribonuclease A (p*I* 9.45), lectin (p*I* 8.3, 8.0, 7.75), myoglobin (p*I* 7.35, 6.9), carboanhydrase (p*I* 6.0), β-lactoglobulin (p*I* 5.3, 5.15), trypsin inhibitor (p*I* 4.5), glucose oxidase (p*I* 4.2).

2.7. Determination of pH and temperature optima

pH and temperature optima were determined using the direct lyase assay or the ligase assay at different pHs and temperatures, respectively. For determination of the midpoint of thermal inactivation (T_m), BAL was incubated in assay buffer for 5 min at the required temperature in a ThermoStat Plus (Eppendorf, Germany) before 50 μL samples were withdrawn to measure the residual activity in the coupled benzoin lyase assay under standard conditions. A second sample was removed to assay the concentration of soluble protein.

2.8. Stability investigations

For investigation of pH, temperature, and cofactor binding stability BAL or BAL variants were incubated under the reaction conditions given in the figure legends and residual activity was assayed with the coupled benzoin lyase assay.

2.9. Biotransformations

Enzymatic syntheses were performed in standard buffer consisting of potassium phosphate (50 mM, pH 7.0) containing $MgSO_4$ (2.5 mM) and ThDP (0.15 mM). NMR spectra were recorded on a Bruker AMX 300. Chemical shifts δ are reported in ppm relative to $CHCl_3$ (^{1}H: $\delta = 7.27$) and $CDCl_3$ (^{13}C: $\delta = 77.0$) as internal standard. Column chromatography was conducted on silica gel 60 (40–63 μm). GC–MS spectra were determined on a HP 6890 series GC-system fitted with a HP 5973 mass selective detector (Hewlett Packard;

column HP-5MS, 30 m · 250 μm; T_{GC}(injector) = 250 °C, T_{MS} (ion source) = 200 °C, time program (oven): $T_{0\ min} = 60$ °C, $T_{3\ min} = 60$ °C, $T_{14\ min} = 280$ °C (heating rate 20 °C min^{-1}), $T_{19\ min} = 280$ °C). Optical rotations were measured with a Perkin-Elmer 241 polarimeter.

*2.9.1. Synthesis of (2R,3R)-2-(2,2-dimethyl-[1,3]dioxolan-4-yl)-2-hydroxy-1-phenyl-ethanone (**2**) on a preparative scale*

1.0 g (7.7 mmol) (*R*)-2,2-dimethyl-1,3-dioxolan-4-carbaldehyde (**1**) (Fig. 7) was dissolved in 20 mL DMSO, heated at 150 °C for 5 min and cooled immediately to room temperature. 0.8 g (7.7 mmol) benzaldehyde and 80 mL buffer containing 30 mg BAL were added. The mixture was stirred in a closed vessel for 5 d at room temperature. Extraction with dichloromethane (5× 50 ml) was carried out and the collected organic phase was washed with water (3× 30 mL). After drying the collected organic phase over sodium sulfate, removal of the solvent under reduced pressure gave the crude product. Column chromatography (isohexane/ethylacetate) afforded 0.3 g (30%) of the pure product; $[\alpha]_D^{20}$ −28.2 (*c* 1.0, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 8.02$ (d, $J = 6.3$ Hz, 2H), 7.54 (dd, $J = 7.4$ Hz, 1H), 7.50 (dd, $J = 7.4$, 6.3 Hz, 2H), 5.18 (d, $J = 5.6$ Hz, 1H), 4.19 (m, 1H), 4.05 (m, 2H), 3.64 (br, s, OH), 1.53 (s, 3H), 1.42 (s, 3H); ^{13}C NMR (75.5 MHz, $CDCl_3$): $\delta = 200.6$, 134.8, 129.7, 129.1, 110.6, 77.6, 73.9, 66.2, 26.6, 25.5; GC–MS: $t_R = 11.15$ min; *m*/*z* (%): 221 (15) $[M-CH_3]^+$, 160 (5), 136 (5), 105 (100), 101 (40), 77 (40). Traces of the *syn* diastereomer were detected (GC–MS): $t_R = 11.27$ min.

3. Results and discussion

3.1. Cloning and expression

In order to improve the expression yield and to simplify purification of the enzyme a vector, pBAL*his*, was prepared. This allows the expression of BAL with a C-terminal His-tag upon induction with 1-isopropyl-β-thiogalactoside (IPTG). Sequencing of the BAL gene in pBAL*his* yielded significant differences to the published sequence. Overall, three insertions, three deletions and two base exchanges were found in the range of nucleotides 210–299. These resulted in changes to the identity of 29 amino acids from residues 71–99, inclusive. Sequencing of the BAL-gene in pUC18/bzl gave identical results, demonstrating that the differences to the published sequence [2] are not due to PCR errors. The corrected protein sequence has been published under GenBank entries AY4007242 and AX349268.

In order to obtain an optimal expression system, a variety of host *E. coli* strains were tested. Best results were obtained using the SG13009 strain (Qiagen) (data not shown). Typical yields from shaking cultures (1–1.5 L) were 2.4 kU/g cells with a specific activity of 19 U/mg BAL in the crude extract, which calculates to about 27% BAL in the soluble protein fraction. High cell density cultivation (15 L) was used to obtain large amounts of enzyme [24]. Using immobilized metal affinity chromatography about 44 g BAL were purified from 3 kg cells.

3.2. Molecular weight and isoelectric point

The calculated molecular weight of 59.8 kDa for His-tagged BAL was confirmed by SDS–PAGE and MALDI-TOF-MS. The native molecular weight was additionally determined by

size-exclusion chromatography as 216 kDa, suggesting a tetrameric structure. This result is in strong contrast to the 80 kDa previously published as native molecular weight [1] but does correspond well to the size of similar enzymes like BFD from *P. putida* [11] and PDC from *Zymomonas mobilis* [27]. While this manuscript was in preparation the 3D-structure of BAL was solved which confirmed the tetrameric structure of the enzyme [3].

The isoelectric point of His-tagged BAL was determined at pH 4.6 by isoelectric focusing (data not shown).

3.3. Stability investigations

3.3.1. Optimal cofactor concentration

As in all ThDP-dependent enzymes the cofactors in BAL are bound non-covalently in the active site. The main binding interaction is the coordinate binding of the diphosphate moiety via Mg^{2+} to the protein as well as hydrophobic and ionic interactions between protein side chains and the thiazol- and pyrimidine ring of ThDP [3].

The stability of most ThDP-dependent enzymes requires the addition of cofactors to the buffer. After immobilized metal affinity chromatography, which was used to purify His-tagged BAL, the enzyme shows only 35–45% of its activity. However, activity is completely regained after incubation in the presence of Mg^{2+} and ThDP.

In order to determine the optimal cofactor concentration, initial velocities were determined under conditions wherein one cofactor concentration was varied and the other kept constant. In potassium phosphate buffer, pH 7.0, at room temperature the optimal concentrations are in the range of 0.1–5 mM for ThDP, and 1–5 mM for $MgSO_4$, whereas >7.5 mM $MgSO_4$ cause inhibition of the enzyme. The previously described inhibition with excess ThDP [1] was not observed up to 5 mM. The results also demonstrate that the binding of Mg^{2+} to the enzyme is stronger than the binding of ThDP, since we did not observe any loss of activity in the presence of low concentrations of $MgSO_4$ (0.025 mM) but a significant loss of activity with a ThDP concentration below 0.01 mM. While inactivation due to short-term (<2 h) incubation in cofactor-free buffer is completely reversible in the presence of added cofactors, the activity is not completely recovered if incubation without cofactors is prolonged over several hours.

3.3.2. Temperature stability

Temperature stability is an important consideration for the application of enzymes to chemoenzymatic synthesis. With BAL the time- and temperature-dependent inactivation was investigated in the presence of two different concentrations of ThDP and $MgSO_4$ in the incubation buffer. As demonstrated in Fig. 2, thermostability is a function of the cofactor concentration in the buffer, which is clearly visible in the inactivation profiles at 37 °C.

In the presence of 5 mM $MgSO_4$ and 0.1 mM ThDP the enzyme's activity was reduced by only 15% over 3 h at 37 °C. However, a cofactor concentration of 1 mM $MgSO_4$ and 0.01 mM ThDP, which is sufficient to keep BAL stable at 25 °C, leads to >40% inactivation within 3 h at 37 °C. These results suggest that thermal inactivation is probably connected with a decrease in the stability of cofactor binding.

In a second study the midpoint of thermal inactivation (T_m) was determined as 45.5 °C. As demonstrated in Fig. 3A the loss of activity is not directly paralleled by the loss of soluble protein (i.e., aggregation) in the sample. In order to investigate whether the differences between the loss of activity and the loss of soluble protein is a function of protein

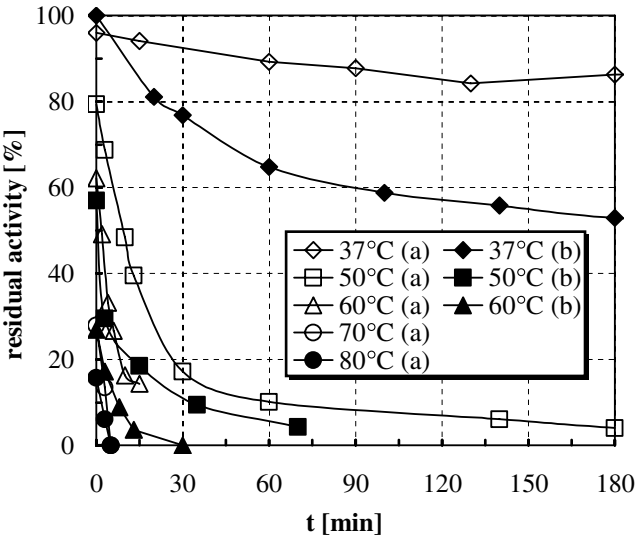

Fig. 2. Thermal stability of wt BAL as a function of temperature and cofactor content of the buffer. Studies were performed in 50 mM potassium phosphate buffer with 5 μg/mL BAL. (a) 5 mM $MgSO_4$, 0.1 mM ThDP (open symbols), (b) 1 mM $MgSO_4$, 0.01 mM ThDP (closed symbols).

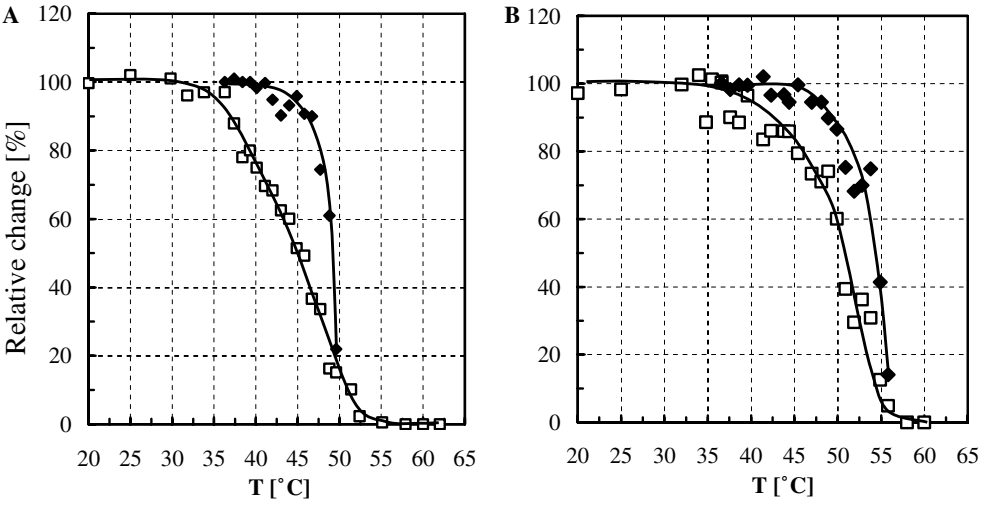

Fig. 3. Determination of the midpoint of thermal inactivation (T_m) (□) and aggregation (♦) of (A) BAL (6 μg/mL) and (B) the variant BAL*A28S* (40 μg/mL). Buffer: 50 mM potassium phosphate, pH 6.5, 5 mM $MgSO_4$, 0.1 mM ThDP. Each temperature was kept for 5 min before samples were withdrawn to determine the residual activity and the soluble protein content.

concentration experiments have been performed with two different protein concentrations (6 and 40 μg/mL), without any observable difference. Again, this suggests that loss of bound cofactors is the main cause of thermal inactivation.

3.3.3. pH and buffer stability

The stability of BAL was investigated in a variety of buffers including potassium phosphate, imidazole, Tris, Mes, and sodium citrate (Fig. 4). Optimum stability at pH 6–8 was observed in potassium phosphate, Tris, and imidazole buffers. pH values below 6 lead to complete inactivation after about one day, while the enzyme shows some activity when kept above pH 8. Citrate and Mes buffers are not appropriate for BAL. The low stability in citrate buffer is probably due to the chelation of magnesium ions by citrate. The effects are partially mediated by the destabilization of the cofactor binding especially at alkaline pH. Overall, potassium phosphate and Tris were selected for the further studies.

3.3.4. Organic solvents

Since biotransformations with BAL often encompass aromatic aldehydes which are only poorly soluble in water, two water-miscible organic solvents (PEG-400 and DMSO) were tested with respect to their influence on the stability of the enzyme. The possibility to use DMSO as a cosolvent for BAL-catalyzed biotransformations has been suggested by Prof. Demir, Ankara [20]. In this study the influences of the two cosolvents on the stability and activity of BAL were determined in detail. As demonstrated in Fig. 5, the activity of BAL in the presence of 20% (v/v) DMSO is up to 20% higher compared to PEG-400 and potassium phosphate buffer, which is most probably due to a higher solubility of benzaldehyde in presence of the organic cosolvent and due to a higher stability of BAL in the presence of DMSO [21]. In the presence of 20% (v/v) DMSO, a concentration of 50 mM benzaldehyde is readily achieved directly after mixing, and this reaction system has successfully been used to obtain various benzoin derivatives in excellent yields and with high enantiomeric excess [5,6,8,12,20,21,28–30].

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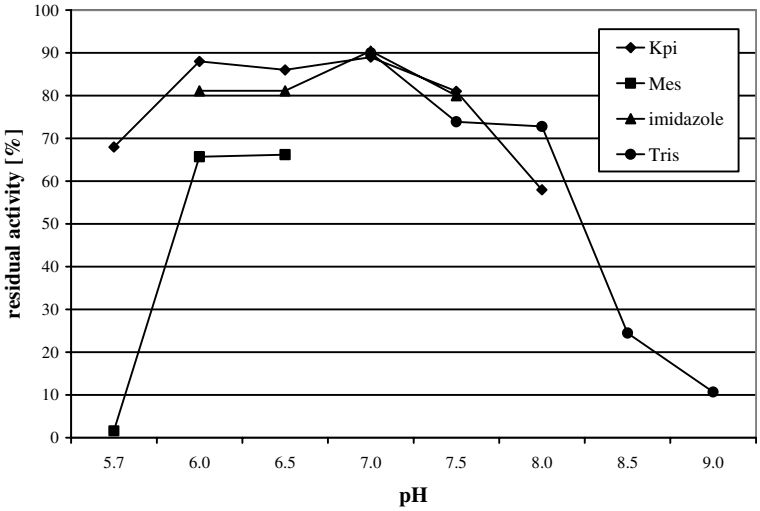


Fig. 4. Stability of wtBAL in the presence of different buffer salts (50 mM, containing 5 mM $MgSO_4$ and 0.1 mM ThDP). Residual activity was measured in the coupled benzoin cleavage assay after 27 h.

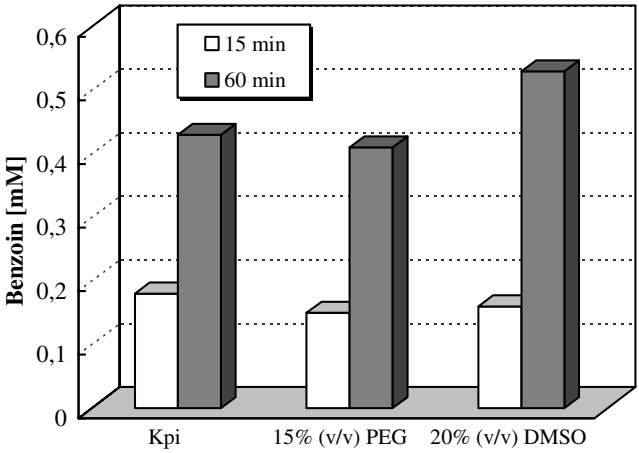


Fig. 5. Investigation of the ligase activity of wtBAL (0.1 μg/mL) in 50 mM potassium phosphate buffer (KPi), pH 7.0, 5 mM Mg^{2+}, 0.1 mM ThDP; plus 15% PEG-400 (v/v); and plus 20% DMSO (v/v) at 30 °C. Benzoin concentration was measured using HPLC.

3.4. Characterization of the catalytic activities

Kinetic properties of the lyase and ligase activity have been characterized based on 2-hydroxy propiophenone and benzoin as model substrates.

3.4.1. Lyase activity

BAL has a sharp pH-optimum at pH 8 for its cleavage of benzoin (Fig. 6).

Kinetic constants were measured in the presence of 15% (v/v) PEG-400 in order to ensure sufficient solubility of benzoin (max. 1.5 mM). Under these conditions V_{max} was calculated, from the hyperbolic v/[S]-plot, to be 74 ± 1 U/mg, and K_M was 48 ± 3 μM. The temperature dependence of the benzoin lyase reaction was investigated between 16 and 55 °C. The maximal activity was obtained at 35 °C. The activation energy of the reaction was calculated as 17 kJ/mol according to the Arrhenius equation.

Studies using enantiopure (*R*)- and (*S*)-benzoin demonstrated that only the (*R*)-enantiomer is accepted by BAL, a property which has already been shown to be useful for kinetic resolution of racemic benzoin [5]. Thus, the true K_M-value for (*R*)-benzoin may be about 24 μM in 50 mM Tris/HCl buffer, 15% (v/v) PEG-400, pH 8.

Apart from benzoin, BAL is also able to cleave other 2-hydroxy ketones such as 2-hydroxypropiophenone (2-HPP). The hyperbolic v/[S]-plot was analyzed up to 16 mM (*R*)-2-HPP and the kinetic constants K_M and V_{max} values were calculated as 0.3 ± 0.02 mM and 3.6 ± 0.1 U/mg, respectively. Conversely, BAL was unable to cleave acetoin under standard conditions, suggesting that BAL prefers substrates with at least one aromatic ring.

3.4.2. Ligase activity

We have investigated the kinetic constants of the benzoin formation in more detail. Further, we describe the formation of novel 2-hydroxyketones using dioxolane **1** as the acceptor aldehyde.

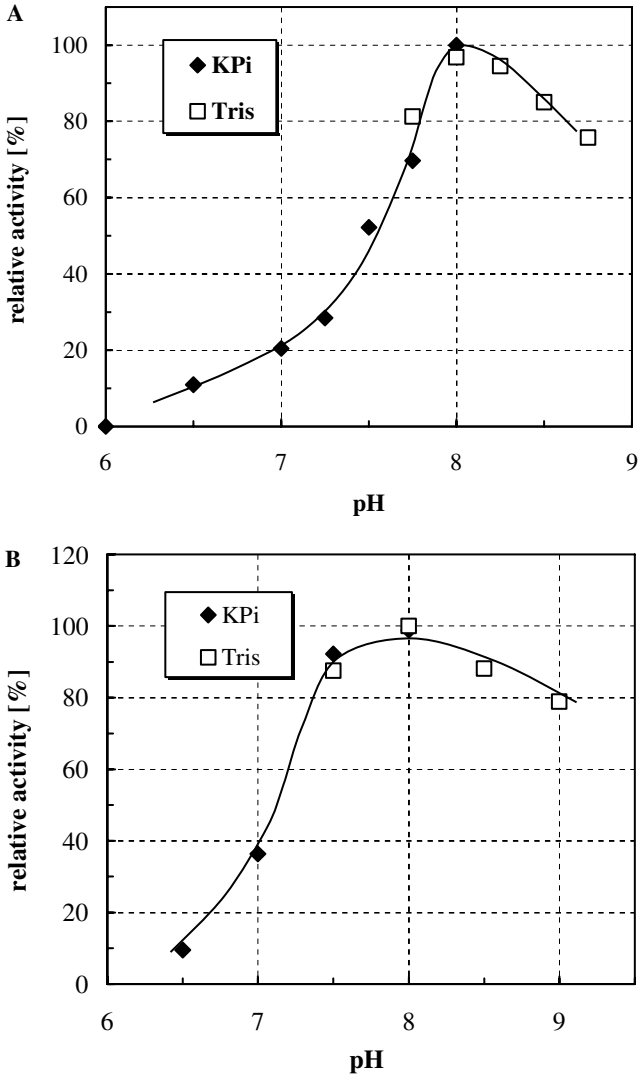


Fig. 6. pH-optima of the BAL-catalyzed lyase (A) and the ligase (B) reaction of benzoin have been determined from initial rate measurements using the direct benzoin lyase assay (A) and HPLC analysis (B). Absolute activities in both buffers are similar. Reactions were performed at 30 °C in 50 mM Tris and 50 mM potassium phosphate (KPi), respectively, including 2.5 mM $MgSO_4$ and 0.1 mM ThDP, 30 mM benzaldehyde, and 0.2 μg/mL BAL.

3.4.3. Benzoin synthesis

The benzoin-forming ligase activity was measured up to 50 mM benzaldehyde in 50 mM potassium phosphate buffer, pH 7.5, at 30 °C. Initial rates were measured using HPLC and, over this concentration range, a hyperbolic v/[S]-plot was obtained. V_{max} of the ligase reaction is 320 ± 5 U/mg, a value about 3-fold higher than the maximal catalytic activity of the reverse reaction. The K_M value for benzaldehyde was found to be 10 ± 1.5 mM. However, it should be noted that the rate of reaction was essentially

constant at benzaldehyde concentrations greater than 30 mM. Although 50 mM benzaldehyde seemed to be completely soluble in the buffer after stirring for about 1 h at 30 °C, a microheterogeneity can not be excluded. This would explain the lack of any observable increase in activity above 30 mM benzaldehyde. This is especially true as studies in the presence of 30% (v/v) DMSO show that activity increases if the solubility of benzaldehyde in the aqueous system is enhanced [31].

3.4.4. pH-optimum

The pH-optimum of the benzoin reaction is somewhat broader (pH 7.5–8.5) than the optimum determined for the lyase reaction (pH 8.0, see above). The pH dependence of benzoin synthesis was examined in both Tris and potassium phosphate, without any observable difference (Fig. 6).

3.4.5. 2-HPP synthesis

In addition to benzoin, BAL is able to catalyze the formation of 2-HPP. (*R*)-2-HPP is obtained either starting from benzaldehyde and acetaldehyde as substrates or if benzoin is cleaved in the presence of acetaldehyde (Fig. 1). The latter is not surprising as an HPLC investigation of the BAL-catalyzed formation of 2-HPP from benzaldehyde and acetaldehyde identified benzoin as an intermediate product. A detailed characterization of the BAL-catalyzed 2-HPP formation has been published elsewhere [31].

*3.4.6. Synthesis of hydroxybutyrophenone **2** and acyloin **4***

To date, the chiral acyloins produced by BFD and BAL catalysis are benzoin and hydroxypropiophenone derivatives. However, by using dioxolane **1** we have been able to enlarge the product range towards highly substituted hydroxybutyrophenones. If benzaldehyde is reacted with equimolar amounts of (*R*)-2,2-dimethyl-1,3-dioxolan-4-carbaldehyde (**1**) in the presence of BAL the acyloin **2** is produced as the major product, with small amounts of benzoin also being detected (Fig. 7).

In order to obtain good yields of the desired product it is important to heat the dioxolane for several minutes at 150 °C prior to the enzymatic transformation in order to cleave the predominant stable dimeric structure. Although the reaction proceeds somewhat slower than the benzoin formation in absence of dioxolane, complete conversion is reached after several days stirring at room temperature. Reduction of the carbonyl group and hydrolysis of the acetonide will result in amphiphilic tetrols, which represents a new chemoenzymatic entry into chiral polyols.

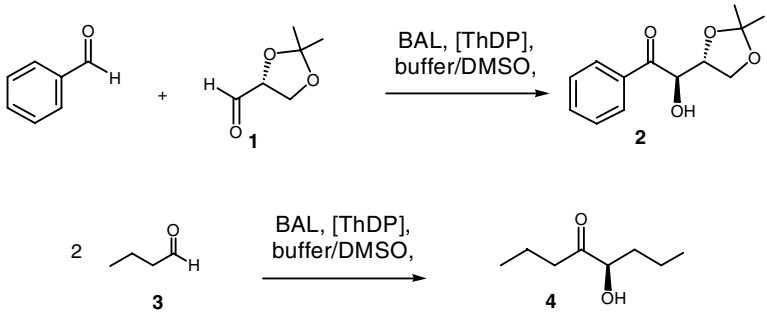


Fig. 7. Application of dioxolane **1** and butyraldehyde **3** in BAL-catalyzed C–C bond formation reactions.

Starting from butyraldehyde (**3**) the aliphatic acyloin **4** is quantitatively formed within 22 h reaction time (data not shown).

3.5. Mutagenesis studies

BAL is similar to BFD from *P. putida*. The enzymes share 24% sequence identity, a similar subunit MW, preference for aromatic substrates, and the potential to catalyze the asymmetric formation of chiral 2-hydroxy ketones. However, the catalytic activity of the two enzymes differs significantly in that wild-type BAL does not decarboxylate benzoylformate, which is the main reaction catalyzed by BFD. Conversely, BFD is unable to catalyze the cleavage of benzoin. A structural comparison explaining these differences has recently been published [22].

In BFD, the residues A460, F464, and H281 have been shown to influence the substrate range and the enantioselectivity of the carboligation reaction [31]. Based on a sequence alignment and the 3D-structures of BAL [3] and BFD [32] the counterparts of these in BAL, were identified as A480, F484, and H286, respectively, These were selected for mutagenesis as was A28. In BFD S26 has been shown to be catalytically important [31]. The BAL*A28S* variant was prepared, as this introduces a serine residue into the corresponding position in BAL.

The BAL variants were tested with respect to catalysis of the cleavage of benzoin and (*R*)-2-HPP, and all showed typical hyperbolic *v*/[S]-plots. The data (Table 1) clearly demonstrate that the K_M values for both substrates are only marginally influenced by the mutations. The most pronounced effects with respect to the K_M value were observed with the variant BAL*A480I*, which exhibits a value nearly 17-fold higher for (*R*)-2-HPP than that of the wt enzyme. However, catalytic efficiency is reduced in all cases with the A480I mutation having the greatest impact.

The ligase activity of all variants was also examined. Unfortunately, the enzymes were not saturated with 30 mM benzaldehyde; therefore the calculation of K_M values was not possible. Apparent k_{cat} values obtained under these conditions decreased in the order wtBAL (335 s^{-1}) > BAL*H*286*A* (151 s^{-1}) > BAL*A28S* (58 s^{-1}) > BAL*F484I* (50 s^{-1}), i.e., the ability to form (*R*)-benzoin is reduced in all cases. Again, the A480I mutation had the most deleterious effect and, with this variant only traces of benzoin could be detected. In the presence of benzaldehyde and acetaldehyde the variants BAL*H*286*A*, BAL*A28S*

Table 1
Lyase activity with racemic benzoin and (*R*)-2-HPP[a]

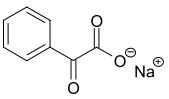
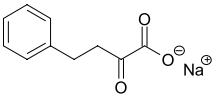
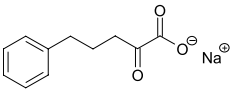
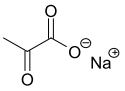
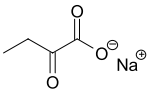
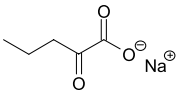
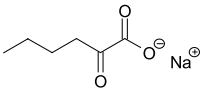
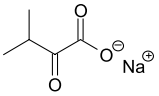
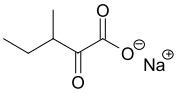
	Benzoin			(*R*)-2-HPP		
	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M (s^{-1} μM^{-1})[b]	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M (s^{-1} μM^{-1})[b]
wtBAL	48	66.9	1394 (100)	0.3	3.4	11.3(100)
H286A	55	56.4	1025 (74)	0.9	2.8	3.1 (27)
F484I	67	22	328 (24)	1.2	4.0	3.3 (29)
A28S	47	9.5	202 (14)	0.6	1.6	2.7 (24)
A480I	45	6.6	147 (11)	5.0	4.9	1(9)

[a] Reactions were carried out in phosphate buffer, pH 8.0, at 30 °C as described in Section 2.
[b] In parentheses is the percentage k_{cat}/K_M compared to the wt value.

and BAL*F484I* catalyze the synthesis of (*R*)-2-HPP. None of the mutations influences the enantioselectivity of BAL.

As previously reported [4], the A28S mutation introduces a significant decarboxylase activity into BAL, an activity which is not measurable with the wild-type enzyme. The pH-optimum of the decarboxylation of benzoylformate was found to be 7.5, which is between the pH optimum of the reaction catalyzed by BFD (pH 6.0) [11] and the optimum of the lyase reaction (pH 8.0). In addition to benzoylformate the substrate range of BAL*A28S* was found to encompass several aliphatic 2-ketoacids as well as 4-phenyl-2-oxobutanic acid and 5-phenyl-2-oxopentanoic acid (Table 2). Thus, overall, the substrate specificity of the decarboxylation reaction of BAL*A28S* very much resembles that of BFD from *P. putida* [11].

Table 2
Substrate range of the decarboxylase reaction catalyzed by BAL*A28S*

Substrate		Specific activity (U/mg)	Rel. activity (%)
	Benzoylformate	2.8	100
	4-Phenyl-2-oxobutanoic acid	0.06[a]	2.1
	5-Phenyl-2-oxopentanoic acid	0.16[b]	5.7
	2-Oxopropanoic acid (pyruvate)	n.d.	n.d.
	2-Oxobutanoic acid	0.07	2.5
	2-Oxopentanoic acid	0.07	2.5
	2-Oxohexanoic acid	0.25	8.9
	2-Oxo-3-methyl butanoic acid	n.d.	n.d.
	2-Oxo-3-methyl pentanoic acid	n.d.	n.d.

Studies were performed in 50 mM potassium phosphate buffer, pH 6.0, 0.1 mM ThDP and 2.5 mM $MgSO_4$, 20 mM of the respective 2-keto acid (unless otherwise stated); n.d., not detected.

[a] Final concentration 5 mM.

[b] Final concentration 10 mM.

3.5.1. Stability investigations of BAL variants

It is of interest to note that the BAL*A28S* variant retains about 50% of its ThDP and $MgSO_4$ after 3 h incubation of the holoenzyme in cofactor-free potassium phosphate buffer, pH 7, at 30 °C. Under these conditions wtBAL shows 35% residual activity, BAL*H*286*A* retains only 10%, and both BAL*A480I* and BAL*F484I* are completely inactivated. Reactivation studies in the presence of both cofactors led to recovery of 80% activity in all cases. The increased stability of BAL*A28S* is also visible in the thermostability of the variant. As demonstrated in Fig. 3 both, the T_m value and thermal induced protein aggregation of the variant are 5 °C higher compared to the wt enzyme.

4. Conclusions

In this study we have investigated optimal reaction conditions for BAL in more detail.

The application of BAL in chemoenzymatic synthesis requires the addition of cofactors to the buffer: 2.5 mM $MgSO_4$ and 0.1 mM ThDP are sufficient to keep the enzyme stable at up to maximal 37 °C (Fig. 3). The enzyme is stable between pH 6 and 8, with pH 8 being the pH-optima of the lyase and ligase reaction (Fig. 6). Optimal buffers are potassium phosphate and Tris (Fig. 5).

As water-miscible organic cosolvents both, PEG-400 as well as DMSO are useful additives to enhance the solubility of aromatic substrates and products, however the enzyme is significantly more stable in DMSO (Fig. 5).

BAL is an extremely useful enzyme for biocatalysis. We here demonstrate that we can enlarge the already broad product range to highly substituted hydroxybutyrophenones and aliphatic acyloins. As an example dioxolan **1** was ligated as a donor aldehyde to benzaldehyde as an acceptor (Fig. 6). Aliphatic acyloins like **4** are easily formed in an enantioselective manner from readily available aldehydes applying BAL as a catalyst (Fig. 6).

From the viewpoint of the organic-preparative chemist, it is important to mention that crude cell extracts of the recombinant *E. coli* strain overexpressing the BAL gene are sufficient for catalysis, hence, purification of the enzyme is not necessary.

Mutagenesis of wt BAL was directed to positions which have shown to have a significant impact on the selectivity and activity of BFD-catalyzed carboligation reactions. The A480I mutation has the greatest influence on the enzyme activity and kinetic constants of the lyase reaction (Table 1). The variant BAL*A28S* was investigated with respect to its stability and substrate range of the decarboxylase reaction. Surprisingly, the mutation does not only introduce a decarboxylase activity into BAL, it also increases the thermostability, which is visible from a 5 °C higher T_m value (Fig. 3). The substrate range of BAL*A28S* indicates a strong similarity to BFD, which can be taken as a further proof for the close relationship between both enzymes.

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