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Review

Application of α-keto acid decarboxylases in biotransformations

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Abstract

The advantages of using enzymes in the synthesis of organic compounds relate to their versatility, high reaction rates, and regio- and stereospecificity and the relatively mild reaction conditions involved. Stereospecificity is especially important in the synthesis of bioactive molecules, as only one of the enantiomeric forms usually manifests bioactivity, whereas the other is often toxic. Although enzymes which catalyze asymmetric carbon-carbon bond formation are of great importance in bioorganic chemistry, only a few examples are known for thiamin diphosphate (ThDP)-dependent enzymes, whereas transformations using e.g. aldolases, lipases and lyases are well documented already. The present review surveys recent work on the application of pyruvate decarboxylase and benzoylformate decarboxylase in organic synthesis. These enzymes catalyze the synthesis of chiral α-hydroxy ketones which are versatile building blocks for organic and pharmaceutical chemistry. Besides the substrate spectra of both enzymes amino acid residues relevant for substrate specificity and enantioselectivity of pyruvate decarboxylase have been investigated by site-directed mutagenesis.

Keywords: Acetoin; Biotransformation; Benzoylformate decarboxylase; α-Hydroxy ketone; α-Keto acid decarboxylase; Phenylacetyl carbinol; Pyruvate decarboxylase; Site-directed mutagenesis; Thiamin diphosphate

Contents

Abbreviations: BFD*Ps.p.*, benzoylformate decarboxylase from *Pseudomonas putida*; 2-HPP, 2-hydroxypropiophenone; InDC, indole-3-pyruvate decarboxylase; PDC*Z.m.*, pyruvate decarboxylase from *Zymomonas mobilis*; PDC*S.c.*, pyruvate decarboxylase from *Saccharomyces cerevisiae*; PDC*S.u.*, pyruvate decarboxylase from *Saccharomyces uvarum*; *S.* sp., *Saccharomyces* species; PhDC, phenylpyruvate decarboxylase; (*R*)-PAC, (*R*)-1-hydroxy-1-phenylpropan-2-one; ThDP, thiamin diphosphate; wt, wild-type; 3D, three-dimensional

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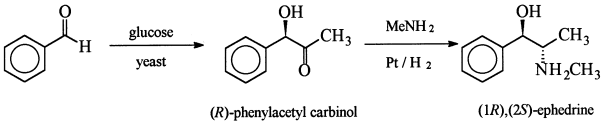
1. Introduction

Thiamin diphosphate (ThDP)-dependent enzymes include the potential of both breaking and formation of C-C bonds [1–3] and both reaction types are catalyzed by enzymes like α-keto acid decarboxylases, transketolases, acetolactate synthase and ThDP-dependent dehydrogenases (for a detailed discussion see the contribution of Schörken and Sprenger, this issue). By contrast, benzaldehyde lyase exhibits exclusively lyase activity [4,5].

A common feature of ThDP-dependent enzymes is the formation of a ThDP-bound carbanionic intermediate upon cleavage of a C-C bond (e.g. decarboxylation). The resulting intermediate was named 'active aldehyde' [2,6].

The enzymes of this family differ widely, however, with regard to the fate of the ThDP-bound 'active aldehyde'. α-Keto acid decarboxylases allow protonation of the intermediate, and their main products are the respective aldehydes. However, these enzymes are also capable of performing an acyloin-type condensation reaction leading to chiral α-hydroxy ketones (Scheme 2). This makes them interesting as catalysts for biotransformations.

Chiral α-hydroxy ketones are versatile building blocks for the organic and pharmaceutical chemistry, e.g. for the synthesis of vitamin E [7] and antifungals [8]. Another well known example is (*R*)-phenylacetyl carbinol (PAC). For many decades, this pre-step for the synthesis of (1*R*,2*S*)-ephedrine has been obtained by biotransformation of benzaldehyde using fermenting yeast [9]. The optimization of this process is still a matter of research (Scheme 1).



(*R*)-phenylacetyl carbinol (1*R*),(2*S*)-ephedrine

Scheme 1. Synthesis of (1*R*,2*S*)-ephedrine.

The present review surveys recent work concerning investigations of the substrate spectra and the mechanistic background of the carboligation reactions of pyruvate decarboxylases (PDC) (EC 4.1.1.1), and benzoylformate decarboxylase (BFD) (EC 4.1.1.7).

2. α-Keto acid decarboxylases

Among the ThDP-dependent α-keto acid decarboxylases which catalyze the decarboxylation of α-keto acids as a main reaction are pyruvate decarboxylases (PDC), benzoylformate decarboxylases (BFD), phenylpyruvate decarboxylases (PhDC) and indole-3-pyruvate decarboxylases (InDC) (Table 1). Whereas the formation of C-C bonds has been observed as a side-reaction of some of these enzymes, other α-keto acid decarboxylases like acetolactate synthase (EC 4.1.3.18) or α-keto glutarate synthase (EC 4.1.1.71) combine both decarboxylation and carboligation in their main reaction path (for a detailed discussion see the contribution of Schörken and Sprenger, this issue).

2.1. Pyruvate decarboxylases

PDC plays an important role in glycolysis and ethanol fermentation and was first detected in yeast.

The enzyme is widely distributed in plants, but has only a very limited occurrence in bacteria such as *Zymomonas mobilis*.

The decarboxylation of pyruvate to acetaldehyde by fermenting yeast was first described by Neuberg and coworkers [21,22].

The same group detected the potential of fermenting yeast to form C-C bonds in 1921 during their studies of 'phytochemical reductions' of various aldehydes by fermenting yeast [23]. Neuberg named the new enzyme 'carboligase', and assumed it to exist apart from 'α-carboxylase' (PDC) in yeast. First indications that the formation of α-hydroxy ketones is a side-reaction of PDC were obtained 10 years later [24,25]. These studies were confirmed by the work of Singer and Pensky, who detected the production of acetoin by PDC from wheat germ using either acetaldehyde or pyruvate as precursors [26,27], and Juni, who was unable to separate PDC from the acetoin-forming system of brewer's yeast, wheat germ and bacteria [28,29]. Unequivocal proofs for PDC as the origin of both the carboligase and decarboxylase activity were obtained by Hanc and Kakac [30], who incubated an enriched fraction of PDC from yeast with pyruvate and benzaldehyde and obtained (*R*)-PAC.

Genes of pyruvate decarboxylases have been isolated from yeasts and fungi such as *Saccharomyces cerevisiae* [10,31–33], *Hanseniaspora uvarum* [34], *Kluyveromyces marxianus* [35], *Kluyveromyces lactis* [36], *Neurospora crassa* [37], *Aspergillus parasiticus* [38], *Aspergillus nidulans* [39], plants like maize (*Zea mays*) [40], rice (*Oryza sativa*) [41,42], tomato (*Lycopersicon esculentum*) [43,44], pea (*Pisum sativum*) [45], tobacco (*Nicotiana tabacum*) [46], and from the bacterium *Zymomonas mobilis* [11,12,47,48].

Despite the large number of available PDC genes, only the enzymes from *Saccharomyces* sp. and *Zymomonas mobilis* have been intensively studied with respect to the reaction mechanism and the substrate spectra of both the decarboxylase and carboligase reaction [49–64] (for a review see [65]). Some data are also available from PDC from wheat germ [66–68], although this enzyme has not yet been cloned and sequence information is not available.

The three-dimensional structures have been determined from PDC*S.u.* [69], PDC*S.c.* [70] in the non-activated state. Recently, Lu et al. [71] published the X-ray-structure of pyruvamide-activated PDC*S.c.*

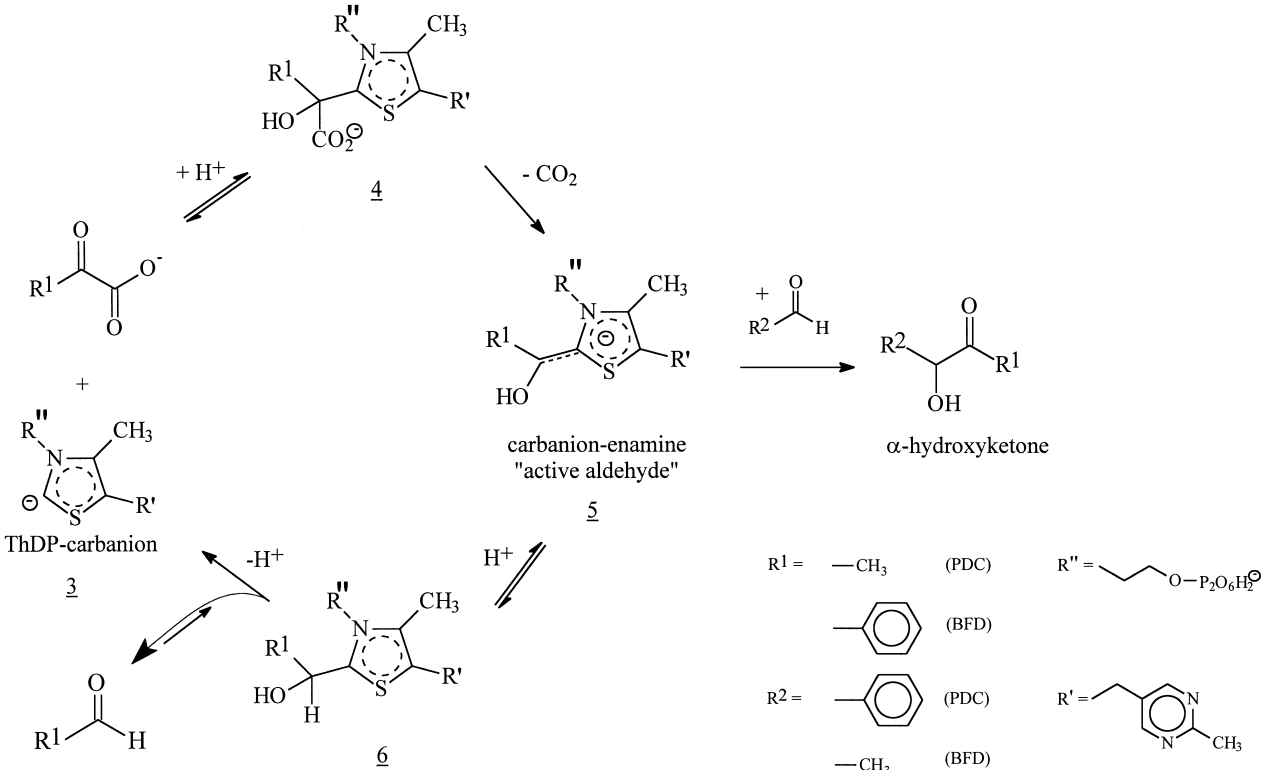
2.2. Benzoylformate decarboxylases

BFD is a component of the mandelate pathway, which allows bacteria to utilize (*R*)-mandelic acid as a sole carbon source by converting it to benzoic acid, which is than metabolized by the β-ketoadipate pathway and citric cycle. BFD activity has been found in *Pseudomonas putida* [72–74], *Pseudomonas aeruginosa* [75], and *Acinetobacter calcoaceticus* [17] (Table 1). Only the enzyme from *Ps. putida* has been cloned so far [16], and the crystal structure is under investigation [76,77]. Like PDC, BFD is a tetrameric enzyme of similar size (Table 1). The X-ray data [77] suggest that the structure of BFD*Ps.p.* is more compact than that obtained from PDC from yeast in the non-activated state [69,70]. BFD was found to exhibit a syn-

Table 1
ThDP-dependent α-keto acid decarboxylases

Enzyme	EC number/SwissProt accession number	Species	Sequence size (number of amino acids)/MW (Da)	Reference
Pyruvate decarboxylase	4.1.1.1 P 06169	*Saccharomyces cerevisiae*[1]	562/61 350	[10]
Pyruvate decarboxylase	4.1.1.1 P 06627	*Zymomonas mobilis*	568/60 925	[11]
Pyruvate decarboxylase	4.1.1.1 (not cloned)	*Triticum vulgare* (wheat germ)	n.d./α: 61.5; β: 64.0	[13–15]
Benzoylformate decarboxylase	4.1.1.7 P 20906	*Pseudomonas putida*	499/53 614	[16]
Benzoylformate decarboxylase	4.1.1.7 (not cloned)	*Acinetobacter calcoaceticus*	n.d./58 000 ± 650	[17]
Indole-3-pyruvate decarboxylase	4.1.1.74 P 23234	*Enterobacter cloacae*	552/60 023	[18]
Indole-3-pyruvate decarboxylase	4.1.1.74 P 51852	*Azospirillum brasiliense*	554/57 980	[19]
Phenylpyruvate decarboxylase	4.1.1.43 (not cloned)	*Achromobacter eurydice*	n.d.	[20]
Phenylpyruvate decarboxylase	4.1.1.43 (not cloned)	*Acinetobacter calcoaceticus*	n.d./56 800 ± 2200	[17]

[1]PDC from *S. cerevisiae* is given as an example of the various enzymes that have been isolated from yeasts and fungi (see text).



Scheme 2. Reaction pathway of enzymatic α-keto acid decarboxylation and formation of α-hydroxy ketones by PDC and BFD.

thetical potential to form α-hydroxy ketones similar to PDCs [78–82].

2.3. Other ThDP-dependent α-keto acid decarboxylases

Two further ThDP-dependent α-ketoacid decarboxylases have been found in the metabolic pathway of the aromatic amino acids tryptophan and phenylalanine.

Indole-3-pyruvate decarboxylase (InDC) (EC 4.1.1.74) (Table 1) is a key enzyme in the biosynthetic path of tryptophan to indole-3-acetic acid. When studying this pathway in bacteria, Koga and coworkers isolated a gene from *Enterobacter cloacae* coding for an enzyme with significant sequence similarity to PDC*S.c.* and PDC*Z.m.* [18]. A similar enzyme has been cloned from *Azospirillum brasiliense* [19].

PhDC (EC 4.1.1.43) (Table 1) is part of the L-phenylalanine metabolism to phenylacetic acid. Such enzymes have been isolated from *Achromobacter eurydice* [20] and *Acinetobacter calcoaceticus* [17]. Although some protein chemical data have been published, these enzymes have not yet been cloned and protein sequence information is not available.

Despite the differences in substrate specificity, all known α-keto acid decarboxylases have been described to be tetrameric enzymes of about 240 kDa (Table 1). But the comparison of the amino acid sequences revealed sequence similarities of only about 30% among PDC*S.c.*, PDC*Z.m.*, InDC and BFD [18,83]. Additionally, no antigenic cross-reaction between different BFDs of *Ps. putida, Ps. aeruginosa* and *A. calcoaceticus* could be detected, and no common epitopes were found amongst BFD and PhDC of *A. calcoaceticus* and PDC from yeast [75].

3. Mechanism of decarboxylation and carboligation

The capacity of ThDP to catalyze the decarboxylation of α-keto acids depends mainly on two properties of the thiazolium ring of ThDP: (a) its capacity

to ionize to form a nucleophilic anion and thus bind to the α-carbonyl group of α-keto acids, and (b) its ability to stabilize the negative charge upon cleavage of the carbon dioxide [84]. The different steps which are relevant for thiamin-catalyzed decarboxylation and the formation of α-hydroxy ketones are summarized in Scheme 2. The reaction cycle is started with activation of ThDP **3** by the enzyme. This initial deprotonation step has recently been elucidated for PDC*S.c.*, PDC*Z.m.* and transketolase by Kern et al. [85]. Subsequently, the negatively charged C2-ThDP performs a nucleophilic attack on the α-carbonyl group of the keto acid. The resulting double negatively charged species is stabilized by proton transfer to the former carbonyl group to give **4**. The decarboxylation of **4** results in the formation of an α-carbanion-enamine **5**. This step has been intensively studied with PDC from yeast using conjugated α-keto acids with strong electron-withdrawing substituents on the phenyl ring. PDC [86–88] converts such compounds to an α-carbanion-enamine that is a visible chromophore with a discrete life-time. A series of [*p*-(halomethyl)]benzoylformates have been investigated as substrates for BFD. These analogues vary from acting as normal substrates to acting as competitive inhibitors [78,89].

The carbanionic intermediate **5** is also known as 'active aldehyde' [6] and is probably present in the mechanism of all ThDP-dependent enzymes, which decarboxylate α-keto acids as a first step. The main reaction path of α-keto acid decarboxylases is the generation of the respective aldehydes. Alternatively, the formation of α-hydroxy ketones have been described for PDCs and BFD. It is important to mention that the last step of the reaction cycle is reversible in PDC [90], and probably also in BFD [81]. Thus **5** may also be generated by addition of an aldehyde to give **6** and subsequent deprotonation. The latter reaction path allows the formation of α-hydroxy ketones from aldehydes as precursors.

Table 2
Comparison of the substrate spectra of PDC from yeast (*Saccharomyces sp.*) [49], PDC from *Z. mobilis* and BFD from *Ps. putida* [74,79]

α-Keto acid	Enzyme (relative activity, %)		
R-CO-COO$^-$	PDC yeast	PDC*Z.m.1*	BFD*Ps.p.*
-CH_3	100	100	0
-C_2H_5	57	70	0
-n-C_3H_7	54	11	n.d.
-n-C_4H_9	49.5	<1	n.d.
$(CH_3)_2CH$-	20.0	0	n.d.
cyclo-C_6H_{11}-	7.0	0	n.d.
$(CH_3)_2CH(CH_2)_2$-	19.6	0	n.d.
$(C_2H_5)(CH_3)$-CH-	51.3	0	n.d.
$(CH_3)_2CH$-CH_2-	5.8	0.3	n.d.
$(C_2H_5)_2$-CH-	8.0	n.d.	n.d.
$(n\text{-}C_3H_7)_2$CH-	3.0	n.d.	n.d.
$C_2H_5OOC(CH_2)_2$-	25.7	n.d.	n.d.
phenyl-CH_2-	n.d.	0	0
phenyl-	n.d.	0	100
R-phenyl-CO-COO$^-$			
-H	100[a]	0	100
3-Br	57.9[a]	n.d.	n.d.
3-F	n.d.	n.d.	43
4-Br	134.0[a]	n.d.	n.d.
4-Cl	128.5[a]	n.d.	52
4-F	114.3[a]	n.d.	n.d.
4-CH_3	67[a]	n.d.	110
4-OH	n.d.	n.d.	100
4-OCH_3	44.3[a]	n.d.	23
4-C_2H_5	75.0[a]	n.d.	n.d.
4-$C(CH_3)_3$	0	n.d.	n.d.

[a]Relative activities of aromatic α-keto acids refer to benzoylformate. Activities related to pyruvate are not available for PDC from yeast (according to [49]).

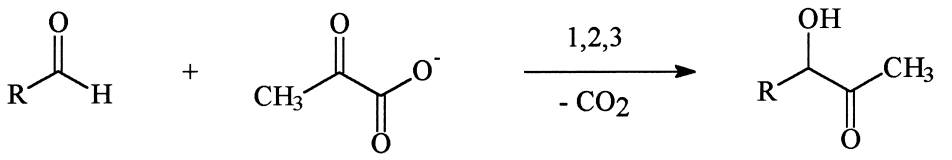
4. Substrate spectra of the decarboxylation reaction

PDC from *Saccharomyces* sp. decarboxylates a very broad spectrum of α-keto acids. A survey is given in Table 2. Besides pyruvate, yeast PDC accepts longer aliphatic α-keto acids, like α-keto butanoic acid, α-keto pentanoic acid, branched aliphatic α-keto acids, as well as α-keto-phenylpropanoic acid (benzoylformate) and various phenyl-substituted derivatives of the latter [49,91,92].

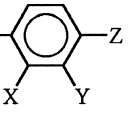
Compared to PDC from yeast the substrate spectra of PDC*Z.m.* and BFD*Ps.p.* are limited to unbranched aliphatic and aromatic substrates, respectively (Table 2). Besides pyruvate, only the C4 and C5-keto acids are substrates for PDC*Z.m.* [50] (Table 5). BFD*Ps.p.* requires substrates with an aromatic ring directly connected to the α-carbonyl group. Phenyl pyruvate is not a substrate for BFD*Ps.p.* [74,79]. Solvent deuterium and ^{13}C kinetic isotope effects indicate that the rate-determining step in the BFD*Ps.p.* reaction rate changes with the change in electronic nature of the substituent. Those analogues contain-

Table 3
α-Hydroxy ketones obtained by (A) decarboxylative incorporation of pyruvate and (B) decarboxylative introduction of various aliphatic α-keto acids, using fermenting yeast (*Saccharomyces* sp.) (1), or isolated enzymes PDC*S.sp.* (2) and PDC*Z.m.* (3)

A



aliphatic α-hydroxy ketones	$R = C_nH_{2n+1}$	
residue R	catalyst	reference
n = 1 - 12	1	[90,115]
n = 1,2	2	[51]
n = 1,2	3	[50,55]

aromatic α-hydroxyketones R =  (benzaldehyde derivatives)

residue	catalyst	references
X, Y ,Z = -H	1	[117-142]
	2	[50,51,53]
	3	[50,55,58,59,65]
monosubstituted derivatives		
X, Y ,Z = -F, -Cl, -Br	1	[101,114,120]
	2	[52,53]
	3	[55]
X, Y ,Z = -OH, $-CH_3$, $-OCH_3$, $-CF_3$	1	[114,115,120]
Z = $-CH(CH_3)_2$, $-N(CH_3)_2$, $-C_6H_5$	1	[115]
disubstituted derivatives		
X,Y = -di-F	1,2	[52,53]
X,X' = -di-F	1,2	[52,53]

ing electron-donating substituents exhibited an increased rate dependence as evidenced by a larger ^{13}C kinetic isotope effect compared to those substrates with electron-withdrawing substituents [79].

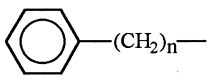
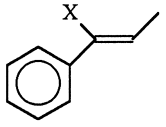
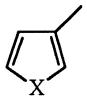
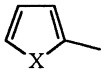
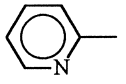
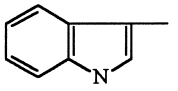
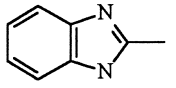
4.1. Unusual decarboxylation reactions

PDC has been reported to decarboxylate and simultaneously dehalogenate various 3-halopyruvate derivatives yielding acetate, carbon dioxide and a halogenide ion as the sole reaction products [94,95]. A similar cleavage reaction may occur upon enzymatic decarboxylation of 3-hydroxypyruvate, which is an alternative substrate and a strong competitive inhibitor of PDC [96].

The decarboxylation of glyoxylate results in the formation of 'active formaldehyde'. Since the release of formaldehyde is very slow, glyoxylate is an uncompetitive inhibitor of PDC [97].

A similar fragmentation reaction has also been reported for BFD*Ps.p.*, with 4-(bromomethyl)-benzoylformate as a substrate. The decarboxylation of

Table 3 (*continued*)

aromatic and heterocyclic α-hydroxy ketones		
residue	catalyst	references
R =  n = 1, 2	1	[115]
 X = H, -CH_3	1	[92,93]
 X = O, S	2 3	[51] [55]
 X = O, S	1 2 3	[115] [51] [55]
	1	[115]
	1	[115]
	1	[115]

this substrate is concomitant by the elimination of bromide and results in the formation of 4-methylbenzoate [89].

5. Formation of α-hydroxy ketones

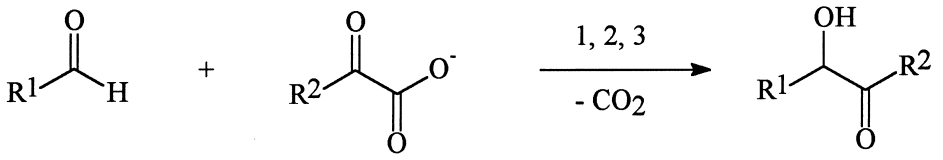
5.1. *Carboligations mediated by pyruvate decarboxylases*

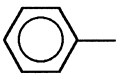
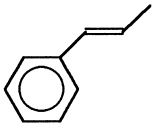
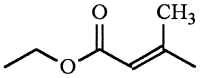
As demonstrated in Scheme 2, the ThDP-bound 'active aldehyde' **5** as C2 donor may be added to a second aldehyde cosubstrate (acceptor) in an acyloin-type condensation reaction. This carboligase reaction has been intensively studied with acetaldehyde as donor, which may be condensed either to a further acetaldehyde molecule yielding acetoin (3-hydroxybutan-2-one) [21,26,27,50,54,55,68,90,98–100] or to a wide range of various aliphatic, aromatic, heterocyclic, and α,β-unsaturated aldehydes [23,50–53,55,91–93,101–115] (Table 3A). Most of these studies have been performed with fermenting yeast and only a few transformations using isolated enzymes were described (Table 3A).

Compared with the broad range of products which is accessible by introduction of a C2 unit via decarboxylative incorporation of pyruvate, only a few examples of decarboxylative incorporation of linear C3, C4, and C5 α-keto acids have been reported (Table 3B). Fuganti and coworkers [92] described the transformation of benzaldehyde and cinnamaldehyde in the presence of α-keto butanoic acid and α-keto pentanoic acid to the corresponding α-hydroxy ketones using fermenting yeast, and Soumalainen

Table 3 (*continued*)

B



R^1	R^2	catalyst	references
	$-CH_3$ $-C_2H_5$ $-C_3H_7$	1	[92]
	$-CH_3$ $-C_2H_5$	1	[92, 107]
	$-CH_3$ $-C_2H_5$	1	[92]
$-CH_3$ and $-C_2H_5$	$-CH_3$ and $-C_2H_5$	1 2	[91] [51]
$-C_2H_5$	$-CH_3$ and $-C_2H_5$	3	[50,55]

and Linnehalme [91] observed the formation of acetoin, propioin (3-hydroxy-4-hexanone), 3-hydroxy-2-pentanone and 2-hydroxy-3-pentanone upon mixed decarboxylation of pyruvate and of α-keto butanoic acid (Table 3B). A similar experiment was carried out by Bornemann and coworkers [55] using PDC*Z.m.* as a catalyst. They reported the formation of 3-hydroxy-2-pentanone and 2-hydroxy-3-pentanone as products of the transformation of propanal and pyruvate. The formation of both products indicates that PDC*Z.m.* is capable of forming an 'active propanal'. In contrast to the yeast enzyme [91], propioin has not been observed as a reaction product.

5.1.1. Stereo-control

A detailed investigation of the carboligase reaction mediated by PDC from yeast, wheat germ and *Z. mobilis* revealed that the stereo-control of this reaction is only strict with aromatic or heterocyclic aldehydes as acceptors, while the formation of acetoin resulted in mixtures of the (*R*)- and (*S*)-enantiomers. In contrast to PDC from wheat germ and PDC*S.c.*, which produce an enantiomeric excess of about 50% (*R*)-(+)-acetoin [26,27,68,90], PDC*Z.m.* was described to synthesize predominantly the (*S*)-(−)-enantiomer [54]. Similar differences in the product stereochemistry have been observed by enzymatic synthesis of lactaldehyde which is formed by carboligation of formaldehyde (from glyoxalate) and acetaldehyde. Again the yeast enzyme forms predominantly (*R*)-lactaldehyde, whereas the (*S*)-enantiomer is predominantly synthesized by the bacterial enzymes [54].

However, the acylation of aromatic and heterocyclic aldehydes by either PDC from yeast or *Z. mobilis* yields exclusively the (*R*)-2-hydroxy ketones with high enantiomeric excesses [3,52].

These differences in the control of the product stereochemistry have recently been investigated by molecular modeling techniques [55,116]. From these

studies, the relevance of the side-chain of isoleucine 476 (PDC*S.c.*), which is conserved in all PDCs (Fig. 1), for the stereo-control during the formation of aromatic α-hydroxy ketones has become likely, since this side-chain may protect one site of the carbanion-enamine **5** (Scheme 2) against the bulky aromatic cosubstrate. In the case of acetoin and lactaldehyde formation the smaller methyl group of acetaldehyde can bind to both sites of **5**. The preference for one of the two acetoin enantiomers has been interpreted in terms of different Boltzmann distributions between the two binding modes of the bound acetaldehyde [51].

5.1.2. Synthesis of (R)-phenylacetyl carbinol

It has to be emphasized that most of the α-hydroxy ketones given in Table 3A,B have been obtained in analytical scale only. Among these, the formation of PAC by biotransformation of benzaldehyde has been studied most intensively with regard to the improvement of yield and the stability of the microbial or enzymatic catalysts in presence of benzaldehyde (Scheme 1). Most of these investigations have been performed using yeasts of different species, mainly *Saccharomyces* sp. and *Candida* sp.

5.1.2.1. Production of (R)-PAC by yeast cells. The current fermentative process is largely limited by side-reactions due to various enzymes existing in living yeast cells and by instability of the cells in presence of benzaldehyde and the fermentative products (for a review of yeast mediated transformations see [3]). Besides the desired fermentative product PAC, up to 16–50% of the benzaldehyde are reduced to benzyl alcohol [101,102,117,118], due to the activity of alcohol dehydrogenases and other oxidoreductases in yeast [119–121]. Further by-products are acetoin, 2-hydroxypropiophenone, benzoin, benzoic acid, butan-2,3-dione (diketone), 1-phenyl-propan-2,3-dione (acetylbenzoyl), *trans*-cinnamaldehyde [122,123]. Additionally, PAC is enzymatically reduced to (1*R*,2*S*)-1-phenyl-1,2-propanediol [117].

The viability of the yeast cells is seriously reduced if the concentration of benzaldehyde exceeds 16 mM, and beyond 20 mM (*R*)-PAC production was completely inhibited [124]. When the residual benzaldehyde declined below 4 mM (0.4 g/l), the formation of benzyl alcohol was predominant over (*R*)-PAC [124].

The productivity and the stability of the microbial catalysts have been optimized by strain selection [125–129], and immobilization of the yeast cells [118,130–135].

Several approaches concerned the reduction of benzyl alcohol formation during the microbial biotransformation. The addition of acetaldehyde suggested by Becvarova et al. [122] and Gröger et al. [117] was successfully applied in small scale fermentations but had no positive effect in a 1000-l scale [137]. Voets and coworkers [123] reported the application of acetone-dried yeast which did not reduce benzaldehyde to benzyl alcohol. Similar effects were achieved by immobilization of *S. cerevisiae* in two polymer matrices. The range of PAC and benzyl alcohol production was found to be dependent on the hydrophobicity of the matrix [135].

Another way to limit the undesired reduction of benzaldehyde was the use of pyruvate instead of hexose. Vojtisek and Netraval [136] observed a significantly increased overall production of PAC during biotransformations using *Saccharomyces carlsbergensis*, whereas the initial rate of the PAC production was not influenced by addition of pyruvate. This effect was explained by preventing generation of a supply of NADH produced by glyceraldehyde-3-phosphate dehydrogenase reaction, necessary for al-

```
                    ****   **  *       *   **** **   * #      *     *
PDC Z.m.      439 (GDGSFQLTAQEVAQMVRLKLPVIIFLI-NN)YGYTIEVMIHDGP---YNN 483
PDC S.c.      443 (GDGSLQLTVQEISTMIRWGLKPYLFVL-NN)DGYTIEKLIH-GPKAQYNE 489
BFD Ps.p.     427 (GDGSANYSISALWTAAQYNIPT-IFVIMNN)GTYGALRWFA-GVLEAENV 473
                  THDP-binding motif
```

Fig. 1. Partial sequence alignment of PDC*Z.m.*, PDC*S.c.* and BFD*Ps.p.* The ThDP-binding motif [143] is shown in parentheses. Identical and similar amino acids in all sequences are marked with asterisks. The isoleucine residue which has been identified as relevant for substrate specificity and enantioselectivity of the carboligation of PDC is marked with #.

dehyde reduction to alcohol, in the absence of hexose [113].

5.1.2.2. Production of (R)-PAC by isolated pyruvate decarboxylases. The application of isolated enzymes in biotransformations is often superior to the use of whole cell, since the problem of undesired by-product formation is reduced significantly by avoiding complex catalytic systems. However, the isolation of catalytically important enzymes from their natural environment in the cell is often connected with impaired stability of the catalyst.

The evaluation of isolated PDCs as catalysts for the synthesis of (*R*)-PAC has only recently been started with studies on PDC from *C. utilis* [138], *S. cerevisiae* and *Z. mobilis* [58,59,65,139,140]. Although Shin and Rogers reported high yields of (*R*)-PAC in 8 h reaction time using partially purified PDC from *C. utilis* [138], it has to be mentioned that the purified yeast enzyme is only stable at 4°C, which makes the application of the yeast enzyme inefficient for the technical production of (*R*)-PAC. Similar results have been obtained with PDC from *S. cerevisiae* [58,65], which was shown to lose activity rapidly at 25°C ($t_{1/2} = 20$ h) and 30°C ($t_{1/2} = 10$ h), respectively. Additionally, the yeast enzyme is sensitive to benzaldehyde [50,141] and acetaldehyde [138].

By contrast, the enzyme from *Z. mobilis* is significantly more stable at room temperature ($t_{1/2} \gg 100$ h). Although PDC*Z.m.* is sensitive towards acetaldehyde, too, we found no negative influence of benzaldehyde up to concentrations of 70 mM [139]. The superior stability makes PDC*Z.m.* well suited for the application in biotransformations. The lower carboligase activity of wt-PDC*Z.m.* [50] was successfully enhanced by site-directed mutagenesis [58,59,65,140]. An exchange of tryptophan 392 which is located at the dimer-dimer interface in the channel leading to the active center of PDC*Z.m.* for alanine enhanced the carboligase activity by a factor of 3–4 [59,140]. This mutant enzyme PDC*W392A* was shown to be a useful catalyst for the synthesis of (*R*)-PAC, if the acetaldehyde which is continuously formed by decarboxylation of pyruvate is removed enzymatically [59,140]. However, the conformational stability of the mutant enzyme was reduced compared to the wt enzyme, obviously due to the loss of hydrophobic stabilization at the dimer-dimer interface.

In order to optimize this enzymatic catalyst, a set of nine further amino acids (Phe, Met, Ile, Val, Gly, His, Asn, Glu, Gln) has been introduced into position 392 [142].

Common features of these mutant enzymes are a reduced decarboxylase activity (about 50–23% of the wt activity), variable K_m values for pyruvate (1.1 mM wt-PDC, 6.8 mM PDC*W392M*) (Table 4), hyperbolic *v*/*S* plots and, most importantly, high enantioselectivity with respect to the formation of (*R*)-PAC (> 98% determined by chiral gas chromatography).

The mutants PDC*W392I* and PDC*W392M* have been selected as the most stable and most active en-

Table 4
Kinetic constants of the decarboxylase and carboligase reaction of various PDC mutants PDC*W392X* compared to the wt enzyme

Enzyme	Decarboxylation of pyruvate		Carboligation to (*R*)-PAC
	activity (U/mg)	K_m (pyruvate) (mM)	activity[a] (U/mg)
wt-PDC	150–180	1.1 ± 0.10	0.5
PDC*W392M*	50–75	6.7 ± 0.15	2.5
PDC*W392I*	50–70	4.0 ± 0.12	2.6
PDC*W392V*	47–55	4.0 ± 0.12	2.3
PDC*W392A*	50–65	3.8 ± 0.10	2.2
PDC*W392G*	55–70	1.6 ± 0.10	2.2
PDC*W392H*	50–60	3.1 ± 0.15	1.2
PDC*W392F*	65–75	5.4 ± 0.15	0.5
PDC*W392Q*	60–80	5.6 ± 0.15	1.0
PDC*W392E*	30–35	4.9 ± 0.15	0.5
PDC*W392N*	45–60	3.9 ± 0.10	0.5

All enzymes were expressed as C-terminal hexa-histidine fusion proteins [142].
[a]Calculated from analytical scale biotransformations.

zymes of the series. The initial rate of (*R*)-PAC formation of these mutant enzymes is increased by a factor of 5–6 compared to the wt enzyme [65].

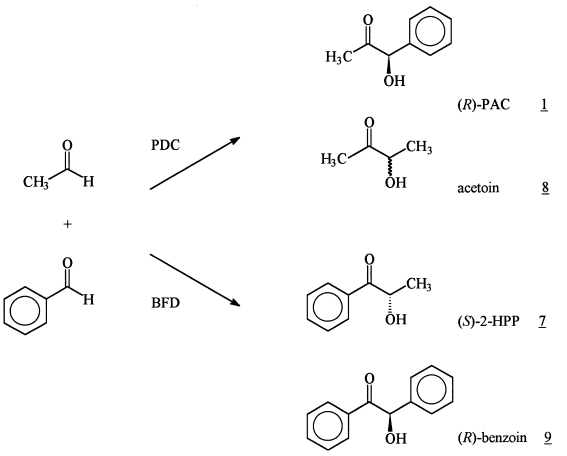
The carboligase activity of the mutant enzymes has an optimum at pH 6.5 at 37°C. But the low stability of the α-hydroxy ketone (*R*)-PAC requires enzymatic synthesis at pH 6.0 and 25°C. Recent studies of our group have demonstrated that (*R*)-PAC is stable for several days at 4°C at pH 6.0, whereas racemization increases significantly with increasing pH and increasing temperatures (H. Iding, M. Pohl, unpublished data).

5.2. Carboligations mediated by benzoylformate decarboxylase

The formation of α-hydroxy ketones by BFD*Ps.p.* was first described by Wilcocks and coworkers [81] using whole cells and cell extracts of *Ps. putida*, which formed (*S*)-2-hydroxypropiophenone (2-HPP) **7**, a tautomer of PAC **1**, from benzoylformate and acetaldehyde (Scheme 3). The enantiomeric excess of **7** was determined by ^{1}H-NMR and was found to be 91–92%. Under the reaction conditions tested, the initial rate of benzaldehyde production was higher than the rate of 2-HPP **7** production, but the benzaldehyde concentration declined again after reaching a maximum. This may be explained with the reversibility of the last step in the catalytic cycle (Scheme 2), allowing the binding of the released aldehyde to ThDP to form the 'active aldehyde' **5** and subsequent addition of acetaldehyde [81]. (*S*)-2-HPP with a higher enantiomeric excess of $>98\%$ has been obtained using whole cells and cell-free extracts of *Acinetobacter calcoaceticus* [82]. A detailed investigation of the reaction conditions optimal for the production of 2-HPP revealed similar conditions which are also optimal for the production of PAC by PDC*Z.m.* (pH 6, 30°C, *Acinetobacter calcoaceticus*) [82]. Factors affecting 2-HPP formation by BFD*Ps.p.* have been investigated by Wilcocks and Ward [80]. They found broad pH (pH 5–8) and temperature optima (20–40°C).

We have recently detected that BFD*Ps.p.* also produces (*R*)-benzoin with high enantiomeric excess (H. Iding, M. Pohl, unpublished data).

Thus, BFD and PDC may complement each other as catalysts for the organic synthesis of symmetric



Scheme 3. Reaction products of the biotransformation of pyruvate and benzaldehyde with PDC, and benzoylformate and acetaldehyde with BFD, respectively.

and mixed α-hydroxy ketones. This is for instance demonstrated by the product spectrum which is accessible by carboligation of benzaldehyde and acetaldehyde with both enzymes in Scheme 3. In the case of PDC, the formation of acetoin **8** occurs upon incubation with either pyruvate or acetaldehyde. The determination of the enantiomeric excess by our group using chiral gas chromatography showed that acetoin synthesized by PDC*Z.m.* is racemic, contradicting results from Crout and coworkers, who found an enantiomeric excess of (*S*)-acetoin [54]. In presence of benzaldehyde and acetaldehyde the formation of the symmetric product acetoin is reduced and the production of (*R*)-PAC **1** is predominant. With wt-PDC*Z.m.* only traces of benzoin have been observed [139]. In the case of BFD, the formation of (*R*)-benzoin **9** occurs using either benzoylformate or benzaldehyde as a substrate, whereas the mixed product (*S*)-2-HPP **7** is a result generating an 'active benzaldehyde' at ThDP (Scheme 2) followed by addition of acetaldehyde.

6. Elucidating differences between pyruvate decarboxylase and benzoylformate decarboxylase by site-directed mutagenesis

PDC and BFD perform very similar enzymatic reactions but with opposite substrate and stereoselectivity. Two aspects have to be taken into account: the initially formed 'active aldehyde' **5** (Scheme 2)

and the influence of the second aldehyde on the stereoselectivity of the carboligation. Carboligation reactions observed with PDC are initiated by the formation of ThDP-bound aliphatic aldehydes, predominantly acetaldehyde (Table 3A,B), whereas reactions mediated by BFD are characterized by the formation of exclusively aromatic 'active aldehydes' **5** (Scheme 2). The stereoselectivity of the carboligation is significantly effected by the size of the second aldehyde. Thus, PDC synthesis racemic acetoin but (*R*)-PAC with high enantiomeric excess (>98%). In the case of BFD this effect is more characteristic, since the change of the second aldehyde from acetaldehyde to benzaldehyde switched the stereoselectivity of the enzymatic reaction completely. Both products, (*S*)-2-HPP and (*R*)-benzoin, are formed with high enantiomeric excesses (Scheme 3).

The differences observed between both enzymes are probably a result of different amino acid residues in the environment of the active site. Since the X-ray structure of BFD is still under investigation [77], a direct comparison of the 3D structures is not yet possible. A comparison of the sequences of both enzymes revealed that the highly conserved isoleucine residue, which had been predicted to be involved in the stereo-control of the carboligation mediated by PDC [55,116] is replaced by alanine in BFD (Fig. 1), a difference which might result in different substrate specificities and/or altered stereospecificity. Ile^{472} in PCD*Z.m.* has a distance of about 5 Å to C2-ThDP and thus is assumed to interact with transition states during catalysis [55,116].

The role of this conserved Ile residue (Fig. 1) in PDC*Z.m.* was recently studied by our group using site-directed mutagenesis (M. Pohl et al., unpublished results). An exchange of Ile^{472} for Ala reduced the decarboxylase activity to about 30% of the wt activity concomitant with an 8-fold increase of K_m for pyruvate. The specific carboligase activity with respect to the formation of PAC was reduced to 60%, and, most remarkably, the resulting PAC showed significant amounts of the (*S*)-enantiomer, reducing the enantiomeric excess of (*R*)-PAC to 60%. Additionally, the formation of PAC is connected with the production of significant amounts of the tautomeric product 2-HPP **7** (Scheme 3), a by-product which is not observed with biotransformations using the wt enzyme or other mutant enzymes of *Z. mobilis* [142]. An investigation of the substrate spectrum of the decarboxylase reaction confirms the significant function of the conserved Ile residue for both the substrate and the stereospecificity of PDC (Table 5). In contrast to the wt enzyme, the mutant enzyme PDC*I472A* is capable of

Table 5
Comparison of the substrate spectra of wt-PDC*Z.m.* and two mutants [93]

	wt-PDC rel. activity (%)	PDC*I472A* rel. activity (%)	PDC*W392A* rel. activity (%)
Linear α-keto carbonic acids			
α-Keto-propanoic acid (pyruvate)	100	100	100
α-Keto-butanoic acid	70	120	34
α-Keto-pentanoic acid	11	108	5
α-Keto-hexanoic acid	<1	80	2
C3-branched α-keto carbonic acids			
α-Keto-3-methylbutanoic acid	<0.1	<0.1	0.12
α-Keto-3-methylpentanoic acid	0	0.3	0.04
α-Keto-3,3-dimethylpentanoic acid	0	0	0
C4-branched α-keto carbonic acids			
α-Keto-4-methylpentanoic acid	0.3	1.8	0.24
α-Keto-4,4-dimethylpentanoic acid	0	0	0
α-Keto-4-methylhexanoic acid	0	3	1.7
Cyclic and aromatic α-keto carbonic acid			
α-Keto-3-cyclohexylpropanoic acid	0	0	0.06
α-Keto-3-phenylpropanoic acid (phenylpyruvate)	0	0.4	1.45
α-Keto-phenylglyoxylic acid (benzoylformate)	0	3	n.d.[a]

[a]n.d. = not determined.

decarboxylating extended aliphatic as well as aromatic α-keto acids, and α-keto butanoic acid is superior to pyruvate as a substrate for this mutant.

Compared to Ile^{472} which is in direct interaction distance of C2-ThDP (5Å), Trp^{392} has a distance of about 20 Å to the active center. Thus, Trp^{392} (Section 5.1.2.2) is unable to influence transition states at C2-ThDP, directly. Consequently, the modification of this side-chain has only little effect on the substrate range of the enzyme relative to wt-PDC [139].

7. Conclusions

The potential of enzymes to form chiral synthones with high enantioselectivity under mild conditions has led to increasing application of biocatalysts in organic synthesis. However, the synthetic application of enzymes is often limited due to a narrow substrate range as well as low stabilities under synthetic conditions. These disadvantages can be overcome by tailoring enzymes for the application in organic synthesis. Versatile tools are offered by modern molecular biology, including methods based on random mutagenesis as well as the so-called 'rational' approach. A well-known example of the 'random' method is the so-called directed evolution which combines 'error-prone' PCR, exon shuffling with appropriate selection and screening parameters (for reviews see [144,145]).

The 'rational' approach requires detailed knowledge of the parameters which should be investigated or altered by directed mutagenesis. An essential prerequisite is structural information as a basis for computer modeling to design appropriate mutants. In the case of PDC the detailed investigations of the reaction mechanism performed by many groups enabled the rational design of mutants to elucidate the predicted function of certain amino acid residues as well as to alter the catalytic properties, e.g. improving the carboligase activity by mutation of Trp^{392} (Section 5.1.2.2) [59,65]. So far, only X-ray structures from PDC*S.sp*. [69–71] are available, which allowed the design of mutants also for the related enzyme from *Z. mobilis*. Up to now the investigation of the synthetic potential of α-keto acid decarboxylases has been limited to PDC and BFD. The X-ray structure of BFD, which will soon be available (M. Hasson, personal communication), will increase the knowledge of the function of ThDP-dependent α-keto acid decarboxylases and enable the design of mutant enzymes with altered substrate specificity and enantioselectivity of both PDC and BFD, to increase the field of application for these interesting enzymes.

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References

[1] K. Drzua, H. Waldmann (Eds.), Enzyme Catalysis in Organic Synthesis, VCH, Weinheim, 1996.
[2] R. Kluger, Enzymes 10 (1992) 271–315.
[3] R. Csuk, B.I. Glänzer, Chem. Rev. 91 (1991) 49–91.
[4] P. Hinrichsen, I. Gomez, R. Vicuna, Gene 144 (1994) 137–138.
[5] B. Gonzales, R. Vicuna, J. Bacteriol. 171 (1989) 2401–2405.
[6] A. Schellenberger, Angew. Chem. 79 (1967) 1050–1061.
[7] C. Fuganti, P. Grasselli, J. Chem. Soc. Chem. Commun. 4 (1982) 205–206.
[8] D. Gala, D.J. DiBedetto, J.E. Clark, B.L. Murphy, D. Schuhmacher, M. Steinman, Tetrahedron Lett. 37 (1996) 611–614.
[9] G. Hildebrandt, W. Klavehn, German Patent No. 548 459, 1932.
[10] E. Kellermann, P.G. Seeboth, C.P. Hollenberg, Nucleic Acids Res. 14 (1987) 8963–8977.
[11] B. Bräu, H. Sahm, Arch. Mircobiol. 144 (1986) 296–301.
[12] M. Reynen, H. Sahm, J. Bacteriol. 170 (1988) 3310–3313.
[13] J. Ullrich, Ann. NY Acad. Sci. 378 (1982) 287–305.
[14] H. Zehender, J. Ullrich, FEBS Lett. 180 (1985) 51–54.
[15] H. Zehender, D. Trescher, J. Ullrich, Eur. J. Biochem. 167 (1987) 149–154.
[16] A.Y. Tsou, S.C. Ransom, J.A. Gerlt, D.D. Buechter, P.C. Babbitt, G.L. Kenyon, Biochemistry 29 (1990) 9856–9862.
[17] M.M. Barrowman, C.A. Fewson, Curr. Microbiol. 12 (1985) 235–240.
[18] J. Koga, T. Adachi, H. Hidaka, Mol. Gen. Genet. 226, (10) (1991) 1–6.
[19] A. Costacurta, V. Keijers, J. Vanderleyden, Mol. Gen. Genet. 243 (1994) 463–472.
[20] T. Asakawa, H. Wada, T. Yamano, Biochim. Biophys. Acta 170 (1968) 375–391.
[21] C. Neuberg, L. Karczag, Biochem. Zeitschr. 36 (1911) 68–81.

[22] C. Neuberg, P. Rosenthal, Biochem. Zeitschr. 51 (1913) 128–142.
[23] C. Neuberg, J. Hirsch, Biochem. Zeitschr. 115 (1921) 282–310.
[24] W. Dischert, Hoppe-Seyler's Z. Physiol. Chem. 201 (1931) 47–77.
[25] W. Langenbeck, H. Wrede, W. Schlockermann, Hoppe-Seyler's Z. Physiol. Chem. 227 (1934) 263–276.
[26] T.P. Singer, J. Pensky, Arch. Biochem. Biophys. 31 (1951) 457–459.
[27] T.P. Singer, J. Pensky, Biochim. Biophys. Acta 9 (1952) 316–327.
[28] E. Juni, J. Biol. Chem. 195 (1952) 715–726.
[29] E. Juni, J. Biol. Chem. 195 (1952) 727–734.
[30] O. Hanc, B. Karac, Naturwissenschaften 43 (1956) 498.
[31] S. Hohmann, J. Bacteriol. 173 (1991) 7963–7969.
[32] S. Hohmann, Curr. Genet. 20 (1991) 373–378.
[33] S. Hohmann, H. Cederberg, Eur. J. Biochem. 188 (1990) 615–621.
[34] P. Holloway, R.E. Subden, Yeast 10 (1994) 1581–1589.
[35] P. Holloway, R.E. Subden, Curr. Genet. 24 (1993) 274–277.
[36] M.M. Bianchi, L. Tizzani, M. Destruelle, L. Frontali, M. Wesolowski-Louvel, Mol. Microbiol. 19 (1996) 27–36.
[37] M.E. Alvarez, A.L. Rosa, E.D. Temporini, A. Wolstenholme, G. Panzetta, L. Patrito, H.J.F. Maccioni, Gene 130 (1993) 253–258.
[38] V. Sanchis, I. Vinas, I.N. Roberts, D.J. Jeenes, A.J. Watson, D.B. Archer, FEMS Microbiol. Lett. 117 (1994) 207–210.
[39] R.A. Lockington, G.N. Borlace, J.M. Kelly, Gene 191 (1997) 61–67.
[40] P.M. Kelley, K. Godfrey, S.K. Lal, M. Alleman, Plant Mol. Biol. 17 (1991) 1259–1261.
[41] M.A. Hossain, E. Hug, T.K. Hodges, Plant Physiol. 106 (1994) 799–800.
[42] M.A. Hossain, E. Huq, A. Grover, E.S. Dennis, W.J. Peacock, T.K. Hodges, Plant Mol. Biol. 31 (1996) 761–770.
[43] A. Foster, T. Chase, FASEB J. 9 (1995) A1292.
[44] A. Foster, T. Chase, Plant Physiol. 108, (Suppl. 2) (1995) 75.
[45] U. Mücke, T. Wohlfarth, U. Fiedler, H. Bäumlein, K.P. Rücknagel, S. König, Eur. J. Biochem. 237 (1996) 373–382.
[46] M. Bucher, K.A. Brander, S. Sbicego, T. Mandel, C. Kuhlemeier, Plant Mol. Biol. 28 (1995) 739–750.
[47] A.D. Neale, R.K. Scopes, R.E.H. Wettenhall, N.J. Hoogenraad, Nucleic Acids Res. 15 (1987) 1753–1761.
[48] T. Conway, Y.A. Osman, J.I. Konnan, E.M. Hoffmann, L.O. Ingram, J. Bacteriol. 169 (1987) 949–954.
[49] H. Lehmann, G. Fischer, G. Hübner, K.D. Kohnert, A. Schellenberger, Eur. J. Biochem. 32 (1973) 83–87.
[50] S. Bringer-Meyer, H. Sahm, Biocatalysis 1 (1988) 321–331.
[51] D.H.G. Crout, H. Dalton, D.W. Hutchinson, M. Miyagoshi, J. Chem. Soc. Perkin Trans. I (1991) 1329–1332.
[52] D.H.G. Crout, S. Davies, R.J. Heath, C.O. Miles, D.R. Rathbone, B.E.P. Swoboda, Biocatalysis 9 (1994) 1–30.
[53] V. Kren, D.H.G. Crout, H. Dalton, D.W. Hutchinson, W. König, M.M. Turner, G. Dean, N. Thomson, J. Chem. Soc. Chem. Commun. 1993 (1993) 341–343.
[54] S. Bornemann, D.H.G. Crout, H. Dalton, D.W. Hutchinson, G. Dean, N. Thomson, M.M. Turner, J. Chem. Soc. Perkin Trans. I (1993) 309–311.
[55] S. Bornemann, D.H.G. Crout, H. Dalton, V. Kren, M. Lobell, G. Dean, N. Thomson, M.M. Turner, J. Chem. Soc. Perkin Trans. I (1996) 425–430.
[56] M. Pohl, J. Grötzinger, A. Wollmer, M.R. Kula, Eur. J. Biochem. 224 (1994) 651–661.
[57] M. Pohl, K. Mesch, A. Rodenbrock, M.R. Kula, Biotechnol. Appl. Biochem. 22 (1995) 95–105.
[58] M. Pohl, K. Mesch, H. Bruhn, G. Goetz, H. Iding, M.R. Kula, M.R., in H. Bisswanger, A. Schellenberger (Eds.), Biochemistry and Physiology of Thiamine Diphosphate Enzymes, A. u. C. Intemann Wissenschaftlicher Verlag, Prien, 1996, pp. 151–159.
[59] H. Bruhn, M. Pohl, J. Grötzinger, M.R. Kula, Eur. J. Biochem. 234 (1995) 650–655.
[60] J.M. Candy, R.G. Duggleby, Biochem. J. 330 (1994) 7–13.
[61] J.M. Candy, J. Koga, P.F. Nixon, R.G. Duggleby, Biochem. J. 315 (1996) 745–751.
[62] R.J. Diefenbach, R.G. Duggleby, Biochem. J. 276 (1991) 439–445.
[63] R.J. Diefenbach, J.M. Candy, J.S. Mattick, R.G. Duggleby, FEBS Lett. 296 (1992) 95–98.
[64] M. Killenberg-Jabs, S. König, I. Eberhardt, S. Hohmann, G. Hübner, Biochemistry 36 (1997) 1900–1901.
[65] M. Pohl, Adv. Biochem. Eng. Biotechnol. 58 (1997) 16–43.
[66] D.E. Green, D. Herbert, V. Subrahmanyan, J. Biol. Chem. 138 (1941) 327–339.
[67] T.P. Singer, J. Pensky, J. Biol. Chem. 196 (1952) 375–388.
[68] D.H.G. Crout, J. Littlechild, S.M. Morrey, J. Chem. Soc. Perkin Trans. I (1986) 105–108.
[69] F. Dyda, W. Furey, S. Swaminathan, M. Sax, B.C. Farrenkopf, F. Jordan, Biochemistry 32 (1993) 6165–6170.
[70] P. Arjunan, T. Umland, F. Dyda, S. Swaminathan, W. Furey, M. Sax, B. Farrenkopf, Y. Gao, D. Zhang, F. Jordan, J. Mol. Biol. 256 (1996) 590–600.
[71] G. Lu, D. Dobritzsch, S. König, G. Schneider, FEBS Lett. 403 (1997) 249–253.
[72] G.D. Hegeman, J. Bacteriol. 91 (1966) 1155–1160.
[73] G.D. Hegeman, J. Bacteriol. 91 (1966) 1161–1167.
[74] G.D. Hegeman, Methods Enzymol. 17 (1970) 674–678.
[75] M.M. Barrowman, W. Harnett, A.J. Scott, C.A. Fewson, J.R. Kusel, FEMS Microbiol. Lett. 34 (1986) 57–60.
[76] M.S. Hasson, A. Muscate, G.T.M. Henehan, P.F. Guidinger, G.A. Petsko, D. Ringe, G.L. Kenyon, Protein Sci. 4 (1995) 955–959.
[77] M.S. Hasson, A. Muscate, G.L. Kenyon, G.A. Petsko, D. Ringe, in H. Bisswanger, A. Schellenberger (Eds.), Biochemistry and Physiology of Thiamin Diphosphate Dependent Enzymes, A. u. C. Intemann Wissenschaftlicher Verlag, Prien, 1996, pp. 174–176.

[78] L.J. Reynolds, G.A. Garcia, J.W. Kozarich, G.L. Kenyon, Biochemistry 27 (1988) 5530–5538.
[79] P.M. Weiss, G.A. Garcia, G.L. Kenyon, W.W. Cleland, P.F. Cook, Biochemistry 27 (1988) 2197–2205.
[80] R. Wilcocks, O.P. Ward, Biotechnol. Bioeng. 39 (1992) 1058–1063.
[81] R. Wilcocks, O.P. Ward, S. Collins, N.J. Dewdney, Y. Hong, E. Prosen, Appl. Environ. Microbiol. 58 (1992) 1699–1704.
[82] E. Prosen, O.P. Ward, J. Ind. Microbiol. 13 (1994) 287–291.
[83] G. Miczka, J. Vernau, M.R. Kula, B. Hofman, D. Schomberg, Biotechnol. Appl. Biochem. 15 (1992) 192–206.
[84] R. Kluger, Chem. Rev. 87 (1987) 863–876.
[85] D. Kern, G. Kern, H. Neef, K. Tittmann, M. Killenberg-Jabs, C. Wikner, G. Schneider, G. Hübner, Science 275 (1997) 67–70.
[86] D.J. Kuo, F. Jordan, J. Biol. Chem. 225 (1983) 13415–13417.
[87] X. Zeng, A. Chung, M. Haran, F. Jordan, J. Am. Chem. Soc. 113 (1991) 5842–5849.
[88] S. Menon-Rudolph, S. Nishikawa, X. Zeng, F. Jordan, J. Am. Chem. Soc. 114 (1992) 10110–10112.
[89] L.J. Dirmaier, G.A. Garcia, J.W. Kozarich, G.L. Kenyon, J. Am. Chem. Soc. 108 (1986) 3149–3150.
[90] G.C. Chen, F. Jordan, Biochemistry 23 (1984) 3582–3589.
[91] H. Suomalainen, T. Linnahalme, Arch. Biochem. Biophys. 114 (1966) 502–513.
[92] C. Funganti, P. Grasselli, G. Poli, S. Servi, A. Zorzella, J. Chem. Soc. Chem. Commun. 1988 (1988) 1619–1621.
[93] C. Funganti, P. Grasselli, S. Servi, F. Spraefico, C. Zirotti, P. Casati, J. Org. Chem. 49 (1984) 4089–4091.
[94] A. Schellenberger, G. Hübner, M. Atanassowa, M. Sieber, S. König, Wissensch. Zeitschr. Univ. Halle 3 (1986) 37–49.
[95] G. Gish, T. Smyth, R. Kluger, J. Am. Chem. Soc. 110 (1988) 6230–6234.
[96] G. Thomas, R.J. Diefenbach, R.G. Duggleby, Biochem. J. 266 (1990) 305–308.
[97] H. Uhlemann, A. Schellenberger, FEBS Lett. 63 (1976) 37–39.
[98] E. Juni, J. Biol. Chem. 236 (1961) 2302–2308.
[99] N.H. Gross, C.H. Werkman, Arch. Biochem. 15 (1947) 125–131.
[100] J.T. Stivers, M.W. Washabaugh, Biochemistry 32 (1993) 13472–13482.
[101] C. Neuberg, L. Liebermann, Biochem. Zeitschr. 121 (1921) 311–325.
[102] C. Neuberg, H. Ohle, Biochem. Zeitschr. 127 (1922) 327–339.
[103] C. Neuberg, H. Ohle, Biochem. Zeitschr. 128 (1922) 611–618.
[104] R. Cardillo, S.t. Servi, C. Tinti, Appl. Microbiol. Biotechnol. 36 (1991) 300–303.
[105] C. Ebert, T. Gardossi, T. Gianferrara, P. Linda, C. Morandini, Biocatalysis 10 (1994) 15–23.
[106] G. Fronza, C. Fuganti, P. Grasselli, G. Pedrocchi-Fantoni, S. Servi, Pure Appl. Chem. 64 (1992) 1099–1101.
[107] C. Fuganti, in Organic Synthesis, IUPAC Symposium 0005, Blackwell, Oxford, 1985, pp. 227–236.
[108] C. Fuganti, P. Grasselli, Chem. Ind. 17 (1977) 983.
[109] C. Fuganti, P. Grasselli, in Enzymes in Organic Synthesis; Ciba Foundation Symposium III, Pitman, London, 1985, pp. 112–127.
[110] C. Fuganti, P. Grasselli, S.t. Servi, F. Spreafico, C. Zirotti, J. Org. Chem. 49 (1984) 4087–4089.
[111] C. Fuganti, P. Grasselli, F. Spreafico, C. Zirotti, J. Org. Chem. 49 (1984) 543–546.
[112] C. Funganti, P. Grasselli, S. Servi, H.E. Högberg, J. Chem. Soc. Perkin Trans. I (1988) 3061–3064.
[113] A. Long, O.P. Ward, J. Ind. Microbiol. 4 (1989) 49–53.
[114] H. Ohta, K. Ozaki, J. Konishi, G. Tsuchihashi, Agric. Biol. Chem. 50 (1986) 1261–1266.
[115] H.P. Schmauder, D. Gröger, Pharmazie 23 (1968) 320–331.
[116] M. Lobell, D.H.G. Crout, J. Am. Chem. Soc. 118 (1996) 1867–1873.
[117] G. Gröger, H.P. Schmauder, K. Mothes, Zeitschr. Allg. Mikrobiol. 6 (1966) 275–287.
[118] W.M. Mahmoud, A.H.M.M. El-Sayed, R.W. Coughlin, Biotechnol. Bioeng. 36 (1990) 47–54.
[119] P.F. Smith, D. Hendlin, J. Bacteriol. 65 (1953) 440–445.
[120] A. Long, P. James, O.P. Ward, Biotechnol. Bioeng. 33 (1989) 657–660.
[121] P. Nikolova, O.P. Ward, Biotechnol. Bioeng. 20 (1991) 493–498.
[122] H. Becvarova, O. Hanc, K. Macek, Folia Microbiol. 8 (1963) 165–169.
[123] J.P. Voets, E.J. Vandamme, C. Vlerick, Zeitschr. Allg. Mikrobiol. 13 (1973) 355–366.
[124] S.C. Agarwal, S.K. Basu, V.C. Vora, J.R. Mason, S.J. Pirt, Biotechnol. Bioeng. 29 (1987) 783–785.
[125] H. Becvarova, O. Hanc, Folia Microbiol. 8 (1963) 42–46.
[126] K.G. Gupta, J. Singh, G. Sahni, S. Dhawan, Biotechnol. Bioeng. 21 (1979) 1085–1089.
[127] J. Netrval, V. Vojtisek, Eur. J. Appl. Microbiol. Biotechnol. 16 (1982) 35–38.
[128] R.J. Seely, D.L. Heefner, R.V. Hageman, M.J. Yarus, S.A. Sullivan, U.S. Patent No. 5,079,145, 1992.
[129] R.J. Seely, D.L. Heefner, R.V. Hageman, M.J. Yarus, S.A. Sullivan, U.S. Patent No. 5,312,742, 1994.
[130] R.J. Seely, D.L. Heefner, R.V. Hageman, M.J. Yarus, S.A. Sullivan, U.S. Patent No. WO 90/04639, 1990.
[131] W.M. Mahmoud, A.B.M.M. El-Sayed, R.W. Coughlin, Biotechnol. Bioeng. 36 (1990) 256–262.
[132] W.M. Mahmoud, A.H.M.M. El-Sayed, R.W. Coughlin, Biotechnol. Bioeng. 36 (1990) 55–63.
[133] H.S. Shin, P.L. Rogers, Appl. Microbiol. Biotechnol. 44 (1995) 7–14.
[134] H.S. Shin, P.L. Rogers, Biotechnol. Bioeng. 49 (1996) 429–436.
[135] P. Nikolova, O.P. Ward, Biotechnol. Lett. 16 (1994) 7–10.

[136] V. Vojtisek, J. Netraval, Folia Microbiol. 27 (1982) 173–177.
[137] R. Zeman, J. Netrval, H. Bulantova, M. Vodnansky, Pharmazie 47 (1992) 291–294.
[138] H.S. Shin, P.L. Rogers, Biotechnol. Bioeng. 49 (1996) 52–62.
[139] H. Bruhn, Dissertation, Heinrich-Heine Universität Düsseldorf, 1995.
[140] H. Bruhn, M. Pohl, K. Mesch, M.R. Kula, German Patent No. 195 23 269. 0-41, 1995.
[141] Y.S. Chow, H.S. Shin, A.A. Adesina, P.L. Rogers, Biotechnol. Lett. 17 (1995) 1201–1206.
[142] K. Mesch, Dissertation, Universität Düsseldorf, 1997.
[143] C.F. Hawkins, A. Borges, R.N. Perham, FEBS Lett. 255 (1989) 77–82.
[144] Z. Shao, F.H. Arnold, Curr. Opin. Struct. Biol. 6 (1996) 513–518.
[145] P.A. Patten, R.J. Howard, W.P.C. Stemmer, Curr. Opin. Biotechnol. 8 (1998) 724–733.