

Short Communication

## Purification, Characterisation and cDNA Sequencing of Pyruvate Decarboxylase from *Zygosaccharomyces bisporus*

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Cells of the wild-type yeast strain *Zygosaccharomyces bisporus* CBS 702 form  $\alpha$ -hydroxy ketones from aromatic amino acid precursors during fermentation. Pyruvate decarboxylase (PDC, E.C. 4.1.1.1), the key enzyme of this biotransformation catalysing the non-oxidative decarboxylation of pyruvate and other 2-oxo-acids, was purified and characterised. The active enzyme is homotetrameric ( $\alpha_4$ ) with a molecular mass of about 244 kDa. Activation of PDC by its substrate pyruvate results in a sigmoidal dependence of the reaction rate from substrate concentration (apparent  $K_m$  value 1.73 mM; Hill coefficient 2.10).

A cDNA library was screened using a PCR-based procedure, and a 1856 bp cDNA of PDC was identified and sequenced. The cDNA encodes a polypeptide of 563 amino acid residues (monomeric unit). Sequence alignments demonstrate high homologies (> 80%) to PDC genes from *Saccharomyces cerevisiae*, *Kluyveromyces lactis* and *Kluyveromyces marxianus*.

**Key words:** Acyloin formation / Enzyme purification /  $\alpha$ -Hydroxy ketones / Non-conventional yeast / Sequence alignment.

Wild-type and non-conventional yeasts are considered to have a more versatile secondary metabolism than *Saccharomyces cerevisiae* strains (baker's or brewer's yeast), which have been cultivated over decades to optimize product yield (*i. e.* ethanol and carbon dioxide) at the expense of product diversity. During a screen of different yeast species and strains for the formation of volatile compounds from added amino acids, *Zygosaccharomyces bisporus* CBS 702 was observed to produce significant quantities of aromatic  $\alpha$ -hydroxy ketones (acyloins) from L-phenylalanine (Neuser *et al.*, 1999). Other strains tested (among them two wine yeasts) yielded smaller or no detectable product amounts at all (Table 1).

We assumed that (*S*)-3-hydroxy-1-phenyl-butan-2-one (**1**) (84% ee; the enantiomeric distribution was determined by capillary GC after derivatisation of enantiomers to the

**Table 1** Production of 3-Hydroxy-1-Phenyl-Butan-2-One (**1**) and 3-Hydroxy-4-Phenyl-Butan-2-One (**2**) from L-Phenylalanine by Different Yeast Species.

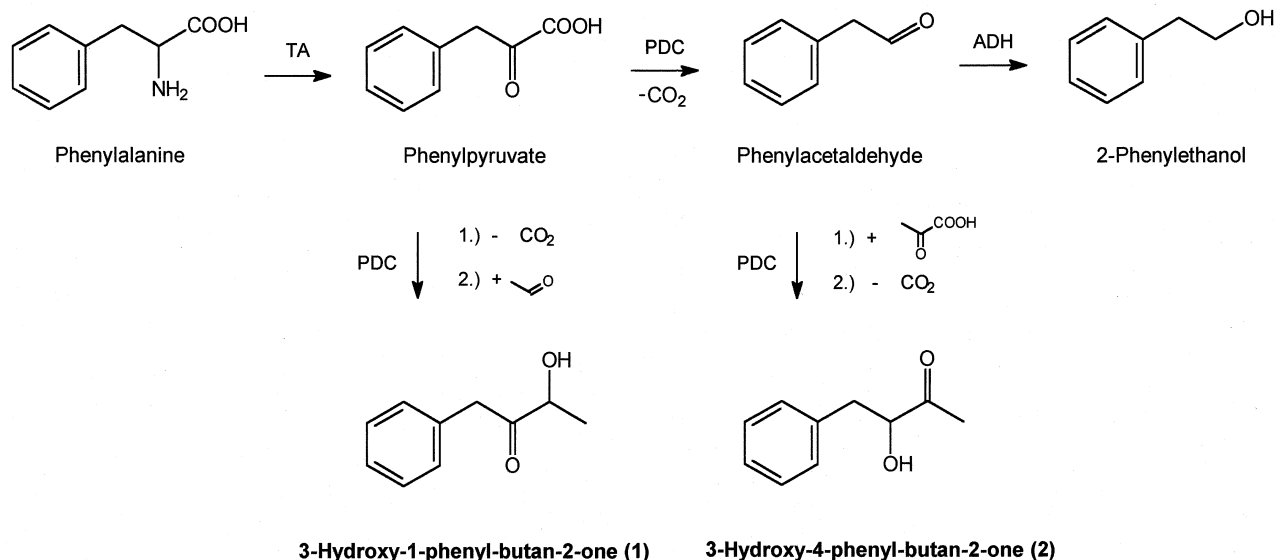
Yeast strain (CBS strain nr.)	$\alpha$ -Hydroxy ketones ( <b>1</b> ) + ( <b>2</b> ) [mg $\times$ l <sup>-1</sup> ]
<i>Zygosaccharomyces bisporus</i> 702	36.0
<i>Zygosaccharomyces baillii</i> 680	5.9
<i>Zygosaccharomyces rouxii</i> 681	1.0
<i>Saccharomyces cerevisiae</i> 457	14.7
<i>Saccharomyces cerevisiae</i> 1250 <sup>a</sup>	2.9
<i>Saccharomyces cerevisiae</i> 5155	0.8
<i>Saccharomyces cerevisiae</i> 6203	0.0
<i>Saccharomyces bayanus</i> 3008 <sup>a</sup>	0.2
<i>Torulaspota delbrueckii</i> 1090	0.3
<i>Candida etchellsii</i> 5008	0.6
<i>Candida versatilis</i> 5007	0.8

Transformations with yeast cells were carried out for 7 days at 24 °C in a medium containing 1% (w/v) glucose, 8% (v/v) ethanol and L-phenylalanine as the sole source of nitrogen (2 g  $\times$  l<sup>-1</sup>).

<sup>a</sup> Wine yeasts.

diastereomeric MTPA-esters; Dale *et al.*, 1969) and (*R*)-3-hydroxy-4-phenyl-butan-2-one (**2**) (58% ee) were formed from phenylalanine after transamination to phenylpyruvate. Two distinct pathways, the addition of either acetaldehyde and phenylpyruvate, or pyruvate and phenylacetaldehyde, will lead to the production of the two isomers of apparently opposite configuration. Both the decarboxylating and the C-C bond forming steps are catalysed by PDC (Figure 1). In fermenting cultures of *Z. bisporus* compound (**1**) proved to be the predominant of the two possible acyloins formed (95%). 2-Phenylethanol, resulting from reduction of phenylacetaldehyde by alcohol dehydrogenase, was found to be the main volatile product of all yeast species examined after supplementation with phenylalanine. There is a single report on the production of the hydroxy ketone (**2**) from phenylacetaldehyde by fermenting cultures of *S. cerevisiae*; however, the isomer (**1**) was not detected (Wanner, 1996).

Pyruvate decarboxylase (PDC, E.C. 4.1.1.1) is a key enzyme in alcoholic fermentation and catalyses the non-oxidative decarboxylation of pyruvate to acetaldehyde. Thiamine diphosphate (ThDP) and Mg<sup>2+</sup> ions are required as cofactors, which dissociate from the protein at pH values above 7.5 (Gounaris *et al.*, 1975). The enzyme was first detected in yeast extracts by Neuberger and Karczag (1911) and has been found in many other fungi, plants, and bac-

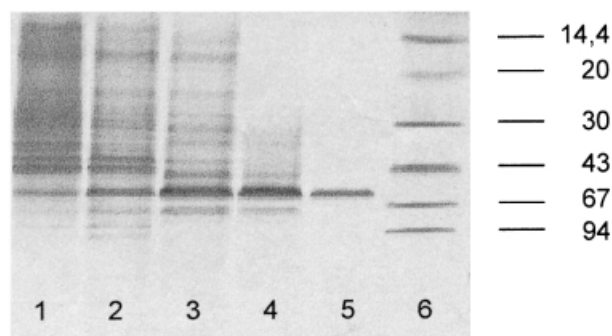


**Fig. 1** Proposed Formation of Aromatic  $\alpha$ -Hydroxy Ketones from Phenylalanine by Yeasts. TA, transaminase; PDC, pyruvate decarboxylase; ADH, alcohol dehydrogenase.

teria. The catalytically active form is tetrameric (dimer of dimers) and consists of either one (*Zymomonas mobilis*, haploid yeast) or two (brewer's yeast, pea, wheat) types of subunits of about 60 kDa, depending on the organism the enzyme was isolated from (Sieber *et al.*, 1983; Bringer-Meyer *et al.*, 1986; Zehender *et al.*, 1987; Mücke *et al.*, 1995; Killenberg-Jabs *et al.*, 1996).

The formation of acylolins (carboligation) as a side reaction of pyruvate decarboxylase has been assumed since Dirscherl's work (1931). Unequivocal proof for PDC as key enzyme of this biotransformation was obtained in several studies using partially purified PDC from different organisms to generate acylolins (Chen and Jordan, 1984; Bringer-Meyer and Sahm, 1988; Crout *et al.*, 1991). Apart from examinations with pyruvate and/or acetaldehyde leading to the production of acetoin, mainly benzaldehyde and substituted analogues thereof were successfully converted to the corresponding acylolins after incubation with PDC and pyruvate or acetaldehyde. The mechanisms of decarboxylation and carboligation have been studied intensively over years, serving as model reactions for thiamin-dependent enzymes (for reviews see Pohl, 1997; Schellenberger, 1998). The production of (*R*)-1-hydroxy-1-phenyl-propan-2-one (PAC, phenylacetyl carbinol) from benzaldehyde by yeast as a chiral precursor for the synthesis of L-ephedrine was one of the first industrially applied biotransformations and is a matter of continuing research (Pohl, 1997).

The evident differences in formation of the homologous phenylbutanoic acylolins by *Z. bisporus* compared to the majority of yeast strains tested (Table 1) raised the question whether distinct structural features of PDC, resulting in modified substrate specificities, might be a reason for the observed results. A purification method was developed to characterise pyruvate decarboxylase from *Z. bisporus* and to confirm its participation in the proposed



**Fig. 2** SDS-PAGE of PDC from *Zygosaccharomyces bisporus* after Different Purification Steps.

Electrophoresis was carried out according to the method of Laemmli (1970). The gel (12.5% T) was stained with Coomassie Brilliant Blue R250. Lane 1: crude extract; lane 2: after  $(\text{NH}_4)_2\text{SO}_4$  precipitation; lane 3: after phenyl Sepharose; lane 4: after Sephacryl S-300; lane 5: after Q Sepharose; lane 6: protein markers.

biosynthesis of the acylolins (1) and (2). Yeast cells were effectively disrupted by grinding in an agitator bead mill after a growth period of 48 h at 24 °C (medium containing 5% glucose). Ammonium sulphate precipitation as a first separation step was followed by three chromatographic procedures, including hydrophobic interaction, gel filtration and anion exchange. The enzyme was purified to homogeneity (Figure 2) with a specific activity of 59 U  $\text{mg}^{-1}$  (Table 2). After concentration (approx. 0.5 mg protein  $\text{ml}^{-1}$ ) PDC was stored at -20 °C in a buffered solution containing its cofactors ThDP and  $\text{MgSO}_4$  without considerable loss of activity for several months.

SDS-PAGE of the isolated pyruvate decarboxylase showed a single protein band of about 61 kDa (Figure 2). In analytical gel filtration on Bio-Silect Sec 400 (Bio-Rad) a single peak equivalent to a molecular mass of 244 kDa was

**Table 2** Purification of PDC from *Zygosaccharomyces bisporus* CBS 702.

Step	Total protein (mg) <sup>a</sup>	Activity (U) <sup>b</sup>	Specific activity (U/mg)	Yield (%)	Purification factor
Crude extract	1135	1624	1.43	100	–
Ammonium sulphate precipitation	462	1283	2.78	79	1.9
Phenyl Sepharose <sup>®</sup> 6 Fast Flow	89.6	1039	11.6	64	8.1
HiPrep <sup>®</sup> Sephacryl S-300	13.5	568	42.2	35	22
HiLoad <sup>®</sup> Q Sepharose	6.59	390	59.2	24	41

The purification procedures were started with 2 l of culture broth, corresponding to approx. 40 g of wet cell weight. Given are average values of 10 preparations.

<sup>a</sup> Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as standard.

<sup>b</sup> Enzymatic activity was measured at 25 °C and 340 nm in the coupled optical test with NADH/ADH (Ullrich, 1970). The test assay contained 0.1 M sodium citrate, pH 6.0, and 20 mM sodium pyruvate.

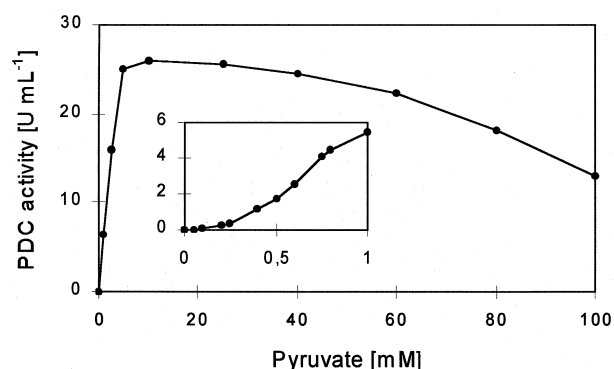
Cell disruption was carried out at 4 °C in 0.1 M potassium phosphate, pH 6.0, containing 1 mM TDP, 5 mM MgSO<sub>4</sub> and 2 mM EDTA (DYNO-Mill KDL, Bachofen AG, 10 min at 3200 rpm, glass beads 0.5 mm). The protein was precipitated with an ammonium sulphate concentration of 60% saturation. Buffers and columns: phenyl Sepharose 6 Fast Flow (2.5 × 50 cm, Pharmacia, flow rate: 3.8 ml min<sup>-1</sup>; equilibration buffer: 0.1 M potassium phosphate, pH 6.0, containing 1 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 5 mM MgSO<sub>4</sub> and 2 mM EDTA, elution with linear decreasing gradient of ammonium sulphate); HiPrep Sephacryl S-300 (2.6 cm × 60 cm, Pharmacia, flow rate: 1.0 ml min<sup>-1</sup>, elution buffer: 0.1 M potassium phosphate, pH 6.0, containing 0.1 M NaCl, 5 mM MgSO<sub>4</sub> and 2 mM EDTA); HiLoad Q Sepharose (1.6 × 10 cm, Pharmacia, flow rate: 3.4 ml min<sup>-1</sup>, equilibration buffer: 20 mM MES/KOH, pH 6.5, containing 1 M NaCl, 5 mM MgSO<sub>4</sub> and 2 mM EDTA, elution with linear decreasing gradient of sodium chloride).

observed. These data are consistent with a tetrameric structure of pyruvate decarboxylase. In contrast to the enzyme of wild-type *S. cerevisiae* strains (WT *S.c.*), PDC of *Z. bisporus* was found to have four subunits of equal size ( $\alpha_4$ -tetramer).

Pyruvate decarboxylase from *Z. bisporus* showed the same sigmoidal dependence of the reaction rate on pyruvate concentration as the enzymes from plants and various yeasts (Figure 3). The  $S_{0.5}$  value of 1.73 mM at pH 6.0 was calculated using a modified Lineweaver-Burk plot ( $1/v$  versus  $1/S^2$ ) and is almost twice as high as that from brewer's yeast (0.9 mM; Kuo *et al.*, 1986). Substrate inhibition is observed at pyruvate concentrations above 25 mM. Decarboxylation of phenylpyruvate proceeded at much

lower rates and was only detectable as aldehyde formation by means of GC (< 1% of the rate observed with pyruvate). In addition, phenylpyruvate proved to be a strong inhibitor of PDC activity (81% inhibition at 10 mM phenylpyruvate).

Up to now various PDC genes have been cloned and sequenced from yeast, bacteria and plant species (Pohl, 1997). The *S. cerevisiae* haploid genome contains three structural genes (*PDC1*, *PDC5*, *PDC6*) coding for pyruvate decarboxylase (Hohmann, 1997). Since other yeast genes for PDC had proven to be similar to *ScPDC1* we reasoned to identify the mRNA coding for PDC from *Z. bisporus* by a PCR-based approach, applying primers deduced from the *ScPDC1* nucleotide sequence. A cDNA library from *Z. bisporus* total RNA was constructed in the  $\lambda$ TriplEx2 vector (Clontech). The PCR screening was performed with primer sets designed according to the vector sequence of the  $\lambda$ TriplEx2 multiple cloning site (upstream primer) and the highly conserved thiamine (ThDP) binding structural motif of *ScPDC1* (downstream primer) (Hawkins *et al.*, 1989), respectively. A 1856 bp cDNA was sequenced by means of primer walking that showed great overall similarity to other yeast PDC genes in the EMBL FASTA data base (Pearson and Lipman, 1988). More than 80% identity at the amino acid level was found for PDCs from *S. cerevisiae* isoenzyme 1 (*ScPDC1*; 81%), from *Kluyveromyces lactis* (KIPDC; 82%), and from *K. marxianus* (KMPDC; 84% identity) (Figure 4). The sequence obtained contains an open reading frame (ORF) of 1689 nucleotides encoding a polypeptide of 563 amino acids corresponding to a calcu-



**Fig. 3** Dependence of the PDC Reaction Rate on the Substrate Concentration.

lated molecular mass of 61.6 kDa, which is in good agreement with the results of SDS-PAGE (61 kDa for the monomeric unit) and gel filtration analysis (244 kDa for the tetrameric enzyme). The deduced ZbPDC amino acid sequence contains only three cysteine residues per enzyme subunit instead of four in KIPDC, KmPDC and ScPDC1. Cysteine 69, which is inaccessible to SH group titration in WT *S.c.* (Baburina *et al.*, 1998), is substituted by an alanine residue in ZbPDC, indicating possible differences in the three-dimensional protein structure. With regard to residues of known catalytical importance (Pohl, 1997), no characteristic differences between the aligned predicted protein sequences were observed.

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60
KmPDC MSETLGLRYLPERLKQVEVQTI FGLPGDFNLSLLDKIYEVPGMRWAGNANELNAAAYADG
K1PDC MSETLGLRYLPERLKQVEVQTI FGLPGDFNLSLLDKIYEVPGMRWAGNANELNAAAYADG
ScPDC1 MSETLGLRYLPERLKQVEVQTI FGLPGDFNLSLLDKIYEVPGMRWAGNANELNAAAYADG
ZbPDC MSETLGLRYLPERLKQVEVQTI FGLPGDFNLSLLDKIYEVPGMRWAGNANELNAAAYADG
*****:*****:*****:*****:*****:*****:*****:*****:*****:*****

120
KmPDC YARLKGMACVITTFVGVGELSNALNGIAGSYAEHVGVLHVGVVPSISSQAKQLLHHTLNG
K1PDC YARLKGMSCIITTFVGVGELSNALNGIAGSYAEHVGVLHVGVVPSISSQAKQLLHHTLNG
ScPDC1 YARLKGMSCIITTFVGVGELSNALNGIAGSYAEHVGVLHVGVVPSISSQAKQLLHHTLNG
ZbPDC YARVKGMAALVITTFVGVGELSNALNGIAGSYAEHVGVLHVGVVPSISSQAKQLLHHTLNG
*****:*****:*****:*****:*****:*****:*****:*****:*****:*****

180
KmPDC DFTVFHRMSSNISETTAMITDINSAPSEIDRCIRTTYISQRPVYVYGLPANLVDLKVPSL
K1PDC DFTVFHRMSSNISETTAMITDINTAPAEIDRCIRTTYVSRPVYVYGLPANLVDLKVPSL
ScPDC1 DFTVFHRMSSNISETTAMITDICTAPAEIDRCIRTTYVSRPVYVYGLPANLVDLKVPSL
ZbPDC DFTVFHRMSSNISETTAMITDINTAPAEIDRCIRTYVYGLPANLVDLKVPSL
*****:*****:*****:*****:*****:*****:*****:*****:*****:*****

240
KmPDC LETPIDLSLKPNDPEAEVEVLELLEIKDANKPVIADACSRHNVKAEVKLIDITQF
K1PDC LETPIDLSLKPNDPEAEVEVLELLEIKDANKPVIADACSRHNVKAEVKLIDITQF
ScPDC1 LETPIDLSLKPNDPEAEVEVLELLEIKDANKPVIADACSRHNVKAEVKLIDITQF
ZbPDC LETPIDLSLKPNDPEAEVEVLELLEIKDANKPVIADACSRHNVKAEVKLIDITQF
*****:*****:*****:*****:*****:*****:*****:*****:*****:*****

300
KmPDC PAFVTPMGKGSIDEQHPFRGGVYVGTLSPEVKEAVESADLVLSVGLSDFNTGSFSYS
K1PDC PAFVTPMGKGSIDEKHPFRGGVYVGTLSPEVKEAVESADLVLSVGLSDFNTGSFSYS
ScPDC1 PAFVTPMGKGSIDEQHPFRGGVYVGTLSPEVKEAVESADLVLSVGLSDFNTGSFSYS
ZbPDC PAFVTPMGKGSIDEQHPFRGGVYVGTLSPEVKEAVESADLVLSVGLSDFNTGSFSYS
*****:*****:*****:*****:*****:*****:*****:*****:*****:*****

360
KmPDC YKTKNIVEFHSDYIKVRNATFPFGQMKFVLQKLLTKVKDAAGKYPVFPVPHAPRDNKQVA
K1PDC YKTKNIVEFHSDYIKVRNATFPFGQMKFVLQKLLTKVKDAAGKYPVFPVPHAPRDNKQVA
ScPDC1 YKTKNIVEFHSDHMKIRNATFPFGQMKFVLQKLLTKVKDAAGKYPVFPVPHAPRDNKQVA
ZbPDC YKTKNIVEFHSDHIKIRNATFPFGQMKFVLQKLLTKVKDAAGKYPVFPVPHAPRDNKQVA
*****:*****:*****:*****:*****:*****:*****:*****:*****:*****

420
KmPDC DSTPLKQEWVWTQVGFLEQEGDVVLTETGTSAFGINQTHFPNDTYGISQVLWGSIGFTTG
K1PDC DSTPLKQEWVWTQVGFLEQEGDVVLTETGTSAFGINQTHFPNDTYGISQVLWGSIGFTTG
ScPDC1 ASTPLKQEWVWNLGNFLEQEGDVVIAETGTSAFGINQTHFPNDTYGISQVLWGSIGFTTG
ZbPDC DSTPLKQEWLWRQVGFLEQEGDVITETGTSAFGINQSHFPNDTYGISQVLWGSIGFTTG
*****:*****:*****:*****:*****:*****:*****:*****:*****:*****

480
KmPDC ATLGAAFAAEIDPKKRVILFIDGSLQLTQVEISTMIRWGLKPYLFLVNLNDGYTIERLI
K1PDC ATLGAAFAAEIDPKKRVILFIDGSLQLTQVEISTMIRWGLKPYLFLVNLNDGYTIERLI
ScPDC1 ATLGAAFAAEIDPKKRVILFIDGSLQLTQVEISTMIRWGLKPYLFLVNLNDGYTIERLI
ZbPDC ATLGAAFAAEIDPKKRVILFIDGSLQLTQVEISTMIRWGLKPYLFLVNLNDGYTIERLI
*****:*****:*****:*****:*****:*****:*****:*****:*****:*****

540
KmPDC HGETAQYNCIQSWKHLLELPTFGAKDYEAARVATTGEWNLKTTDKKQFQENSKIRLIEVML
K1PDC HGETAQYNCIQWQHLLELPTFGAKDYEAARVATTGEWNLKTTDEKQDNTRIRLIEVML
ScPDC1 HGPKAQYNCIQWQHLLELPTFGAKDYETHRVATTGEWNLKTTQDKSFDNDSKIRLIEVML
ZbPDC HGEKAQYNDIQWQHLLELHAFGATDYETHRVATTGEWNLKTTQDKSFDNDSKIRLIEVML
*****:*****:*****:*****:*****:*****:*****:*****:*****:*****

KmPDC PVMDAPSNLVKQAQLTASINAKQE 564
K1PDC PTMDAPSNLVKQAQLTAAASNAKN- 563
ScPDC1 PVFDAPQNLVQAKLTAATNAKQ- 562
ZbPDC ETMDAPSSLVAQAQLTAAINAKQ- 563
.:*****:*****:*****:*****:*****:*****:*****:*****:*****

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**Fig. 4** Alignment of the Predicted Protein Sequences of Pyruvate Decarboxylases from Different Yeast Species.

The alignment was performed using the program ClustalW from EMBL (asterisk: identical residues; colon: conserved substitution; dot: semi-conserved substitution). The ThDP-binding motif is underlined. For construction of the *Z. bisporus* cDNA bank, the SMART PCR cDNA Library Construction Kit (Clontech) was used according to manufacturers instructions. Standard molecular biology procedures were performed according to Sambrook *et al.* (1989). The nucleotide sequence of PDC from *Z. bisporus* was submitted to the EMBL database under the accession number AJ250971.

The purified preparations of *Z. bisporus* PDC were used to examine the formation of aromatic  $\alpha$ -hydroxy ketones (*S*)-3-hydroxy-1-phenyl-butan-2-one (**1**) and (*R*)-3-hydroxy-4-phenyl-butan-2-one (**2**). As proposed in Figure 1, incubation of pyruvate decarboxylase with phenylpyruvate and acetaldehyde yielded predominantly compound (**1**) (68%), while incubation with phenylacetaldehyde and pyruvate resulted in the formation of compound (**2**) (91%). The exclusive synthesis of only one of the two isomers is prevented by keto-enol tautomerisation, especially in the presence of acids or at higher temperatures (Häring *et al.*, 1997). Acyloin (**1**) is reported as a yeast fermentation product in our studies for the first time.

The outstanding capabilities of fermenting cells of *Zygosaccharomyces bisporus* to produce  $\alpha$ -hydroxy ketones from *L*-phenylalanine could not be correlated with differences in the primary amino acid sequence of the pyruvate decarboxylase compared with enzymes from other yeast species. cDNA sequence alignments showed exceptionally high homologies with PDCs from *S. cerevisiae*, *K. lactis* and *K. marxianus*. This was in good agreement with kinetic and structural properties of ZbPDC which were very similar to those reported for the *S. cerevisiae* enzyme. Possibly, the observed variations between *Z. bisporus* and 10 selected yeast strains in converting phenylalanine to acyloins can be attributed to differences in metabolic regulation rather than to different characteristics of PDC.

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