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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 α-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 (α_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 / α-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 α-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 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^{2+} 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 Neuberg and Karczag (1911) and has been found in many other fungi, plants, and bac-

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.)	α-Hydroxy ketones (**1**) + (**2**) [mg × l^{-1}]
Zygosaccharomyces bisporus 702	36.0
Zygosaccharomyces bailii 680	5.9
Zygosaccharomyces rouxii 681	1.0
Saccharomyces cerevisiae 457	14.7
Saccharomyces cerevisiae 1250[a]	2.9
Saccharomyces cerevisiae 5155	0.8
Saccharomyces cerevisiae 6203	0.0
Saccharomyces bayanus 3008[a]	0.2
Torulaspora delbrueckii 1090	0.3
Candida etchelsii 5008	0.6
Candida versatilis 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 × l^{-1}).
[a] Wine yeasts.

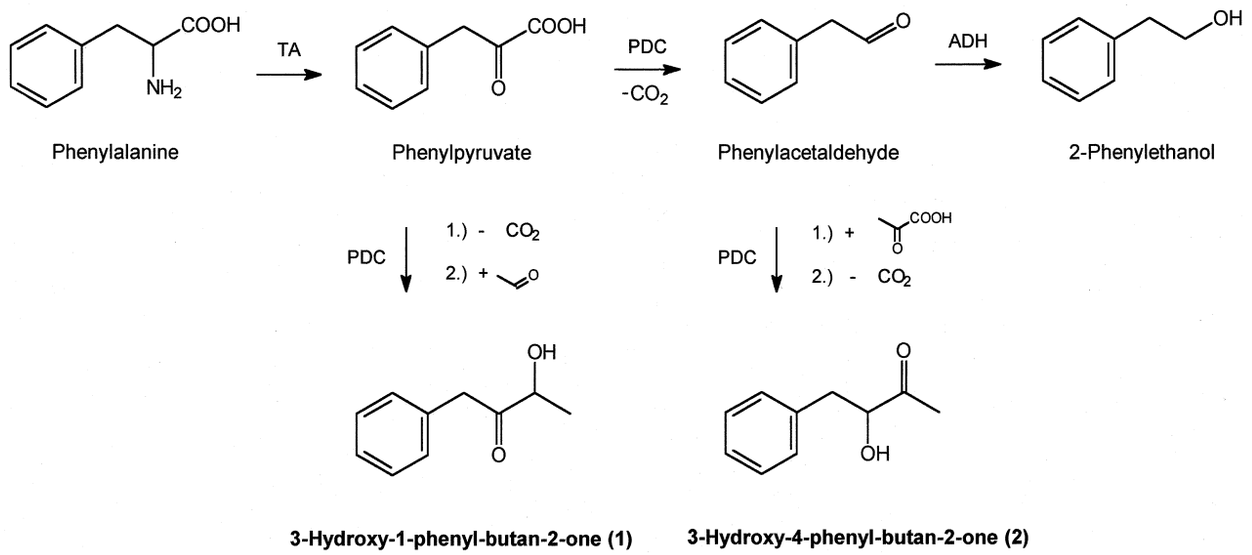

Fig. 1 Proposed Formation of Aromatic α-Hydroxy Ketones from Phenylalanine by Yeasts.
TA, transaminase; PDC, pyruvate decarboxylase; ADH, alcohol dehydrogenase.

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 acyloins (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 acyloins (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 acyloins 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 acyloins 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

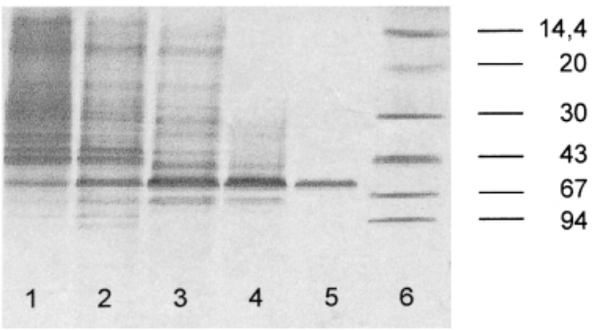

Fig. 2 SDS-PAGE of PDC from *Zygosaccharomyces bisporus* after Different Purification Steps.
Electrophoresis was carried out according to the method of Laemmli (1970). The gel (12.5% T) was stained with Coomassie Brilliant Blue R250. Lane 1: crude extract; lane 2: after $(NH_4)_2SO_4$ precipitation; lane 3: after phenyl Sepharose; lane 4: after Sephacryl S-300; lane 5: after Q Sepharose; lane 6: protein markers.

biosynthesis of the acyloins (**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 mg^{-1} (Table 2). After concentration (approx. 0.5 mg protein ml^{-1}) PDC was stored at –20 °C in a buffered solution containing its cofactors ThDP and $MgSO_4$ 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)[a]	Activity (U)[b]	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® 6 Fast Flow	89.6	1039	11.6	64	8.1
HiPrep® Sephacryl S-300	13.5	568	42.2	35	22
HiLoad® 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.

[a] Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as standard.

[b] 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 $MgSO_4$ 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^{-1}; equilibration buffer: 0.1 M potassium phosphate, pH 6.0, containing 1 M $(NH_4)_2SO_4$, 5 mM $MgSO_4$ 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^{-1}, elution buffer: 0.1 M potassium phosphate, pH 6.0, containing 0.1 M NaCl, 5 mM $MgSO_4$ and 2 mM EDTA); HiLoad Q Sepharose (1.6 × 10 cm, Pharmacia, flow rate: 3.4 ml min^{-1}, equilibration buffer: 20 mM MES/KOH, pH 6.5, containing 1 M NaCl, 5 mM $MgSO_4$ 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 (α_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).

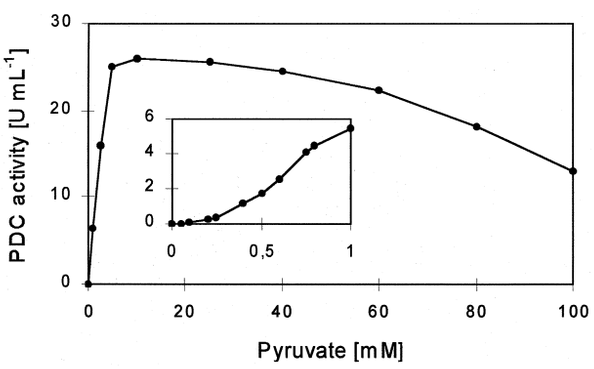

Fig. 3 Dependence of the PDC Reaction Rate on the Substrate Concentration.

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 λTriplEx2 vector (Clontech). The PCR screening was performed with primer sets designed according to the vector sequence of the λTriplEx2 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* (KlPDC; 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-

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 KlPDC, 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  MSEITLGRYLFERLKQVEVQTIFGLPGDFNLSLLDKIYEVPGMRWAGNANELNAAYAADG
KlPDC  MSEITLGRYLFERLKQVEVQTIFGLPGDFNLSLLDNIYEVPGMRWAGNANELNAAYAADG
ScPDC1 -SEITLGKYLFERLKQVNVNTVFGLPGDFNLSLLDKIYEVEGMRWAGNANELNARYAADG
ZbPDC  MSEITLGRYLFERLRQVDTNTIFGVPGDFNLSLLDKIYEVQGLRWAGNANELNAAYAADG
        ******:******:**:.:*:**:**********:**** *:********** *****
                                                                     120
KmPDC  YARLKGMACVITTFGVGELSALNGIAGSYAEHVGVLHVVGVPSISSQAKQLLLHHTLGNG
KlPDC  YARLKGMSCIITTFGVGELSALNGIAGSYAEHVGVLHVVGVPSVSSQAKQLLLHHTLGNG
ScPDC1 YARIKGMSCIITTFGVGELSALNGIAGSYAEHVGVLHVVGVPSISSQAKQLLLHHTLGNG
ZbPDC  YARVKGMAALVTTFGVGELSALNGIAGSYAEHVGVLHIEGVPSISSQAKQLLLHHTLGNG
       ***:***:.::************************: ****:****************
                                                                     180
KmPDC  DFTVFHRMSSNISETTAMITDINSAPSEIDRCIRTTYISQRPVYLGLPANLVDLKVPASL
KlPDC  DFTVFHRMCSNISETTAMITDINTAPAEIDRCIRTTYVSQRPVYLGLPANLVDLTVPASL
ScPDC1 DFTVFHRMSANISETTAMITDICTAPAEIDRCIRTTYVTQRPVYLGLPANLVDLNVPAKL
ZbPDC  DFTVFHRMSANISETTAWLTDITTAPAEIDRCIRVTYLTQRPVYLGLPANLTDQKVPASL
       ********.:******* :*** :**:*******.**::************.* .***.*
                                                                     240
KmPDC  LETPIDLSLKPNDPEAENEVLETVLELIKDAKNPVILADACCSRHNVKAETKKLIDITQF
KlPDC  LDTPIDLSLKPNDPEAEEEVIENVLQLIKEAKNPVILADACCSRHDAKAETKKLIDLTQF
ScPDC1 LQTPIDMSLKPNDAESEKEVIDTILVLAKDAKNPVILADACCSRHDVKAETKKLIDLTQF
ZBbPDC LNTPIDLSLKENDPEAEAEVVETVLELIKEAKNPIILADACCSRXDVKAETKKLIDITQF
       *:****:*** **.*:* **::.:* * *:****:********* :.*********:***
                                                                     300
KmPDC  PAFVTPMGKGSIDEQHPRFGGVYVGTLSSPEVKEAVESADLVLSVGALLSDFNTGSFSYS
KlPDC  PAFVTPMGKGSIDEKHPRFGGVYVGTLSSPAVKEAVESAHLVLSVGALLSDFNTGSFSYS
ScPDC1 PAFVTPMGKGSISEQHPRYGGVYVGTLSKPEVKEAVESADLILSVGALLSDFNTGSFSYS
ZBPDC  PSFVTPMGKGSIDEQNPRFGGVYVGTLSSPAVKKAVESADLVLSVGALLSDFNTGSFSYS
       *:**********.*::**:*********.* **:*****.*:*****************
                                                                     360
KmPDC  YKTKNIVEFHSDYIKVRNATFPGVQMKFVLQKLLTKVKDAAKGYKPVPVPHAPRDNKPVA
KlPDC  YKTKNIVEFHSDYTKIRRPTFPGVQMKFALQKLLTKVADAAKGYKPVPVPSEPEHNEDVA
ScPDC1 YKTKNIVEFHSDHMKIRNATFPGVQMKFVLQKLLTNIADAAKGYKPVAVPARTPANAAVP
ZbPDC  YKTKNVVEFHSDHIKIRNATFPGVQMKFVLEDLLKKVPAAVKGYNPGPVPPAPSPNAEVA
       *****:******: *:*..*********.*:.**.::  *.***:* .**  .  *  *.
                                                                     420
KmPDC  DSTPLKQEWVWTQVGKFLQEGDVVLTETGTSAFGINQTHFPNDTYGISQVLWGSIGFTGG
KlPDC  DSTPLKQEWVWTQVGEFLREGDVVITETGTSAFGINQTHFPNNTYGISQVLWGSIGFTTG
ScPDC1 ASTPLKQEWMWNQLGNFLQEGDVVIAETGTSAFGINQTTFPNNTYGISQVLWGSIGFTTG
ZbPDC  ASTPLKQEWLWRQVGKFLQEGDIVITETGTSAFGINQSHFPNRTYGISQVLWGSIGYTTG
        ********:* *:*:**:***:*::***********: *** *************:* *
                                                                     480
KmPDC  ATLGAAFAAEEIDPKKRVILFIGDGSLQLTVQEISTMIRWGLKPYLFVLNNDGYTIERLI
KlPDC  ATLGAAFAAEEIDPKKRVILFIGDGSLQLTVQEISTMIRWGLKPYLFVLNNDGYTIERLI
ScPDC1 ATLGAAFAAEEIDPKKRVILFIGDGSLQLTVQEISTMIRWGLKPYLFVLNNDGYTIEKLI
ZbPDC  STLGAAFAAEEIDPKKRVVLFIGDGSLQLTVQEISTMVRWGLKPYLFVLNNDGYTIERLI
       :*****************:*****************:*****************:**
                                                                     540
KmPDC  HGETAQYNCIQSWKHLDLLPTFGAKDYEAVRVATTGEWNKLTTDKKFQENSKIRLIEVML
KlPDC  HGETAQYNCIQNWQHLELLPTFGAKDYEAVRVSTTGEWNKLTTDEKFQDNTRIRLIEVML
ScPDC1 HGPKAQYNEIQGWDHLSLLPTFGAKDYETHRVATTGEWDKLTQDKSFNDNSKIRMIEVML
ZbPDC  HGEKAQYNDIQPWKNLELLHAFGATDYETHRVATVGDWDKLTTDSKFNENSRIRMIEVML
       ** .**** ** *.:*.** :***.***: **:*.*:*:*** *..*::*:::**:*****

KmPDC  PVMDAPSNLVKQAQLTASINAKQE 564
KlPDC  PTMDAPSNLVKQAQLTAASNAKN- 563
ScPDC1 PVFDAPQNLVEQAKLTAATNAKQ- 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 α-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 α-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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