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Screening of Yeasts for Cell-Free Production of (*R*)-Phenylacetylcarbinol in a Shake Flask Condition

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Abstract: 10 yeast strains from 4 genera and 10 species were evaluated for cell-free production of (*R*)-phenylacetylcarbinol (PAC), the chiral precursor in the manufacture of the pharmaceuticals ephedrine and pseudoephedrine. Carboligase activity of pyruvate decarboxylase (PDC), forming PAC from benzaldehyde and pyruvate, was found in extracts of 10 strains. PAC was not formed from benzaldehyde and acetaldehyde, an activity of yeast PDCs from *Hansenula polymorpha* and *Hansenula anomola*. Two interesting groups of candidates were identified in the yeast screening: carboligase activities of *Schizosaccharomyces pombe* PDCs were very low but showed best resistance to pre-incubation with acetaldehyde and benzaldehyde; and highest carboligase activities combined with medium resistance were found in strains of *Candida utilis*, *C. tropicalis* and *C. albicans*.

Key words: Biotransformation · Enzyme · *(R)*-phenylacetylcarbinol · Screening · Yeast

INTRODUCTION

(R)-Phenylacetylcarbinol (PAC) is the precursor for the commercial production of ephedrine and pseudoephedrine, which are used primarily as bronchial dilators and nasal decongestants. PAC is produced through a biotransformation catalyzed by the pyruvate decarboxylase (PDC) with thiamine pyrophosphate (TPP) and Mg^{2+} as cofactors (Fig. 1). In its natural function in ethanol fermentation, PDC decarboxylates TPP-bound pyruvate to 'active acetaldehyde'. Subsequently acetaldehyde is released and reduced to ethanol by alcohol dehydrogenase. As a side reaction, PDC can ligate TPP-bound 'active acetaldehyde' to added benzaldehyde resulting in PAC (Figure 1). This carboligase activity was found in various yeasts [1-3], filamentous fungi [4] and bacteria [5]. The ability to form PAC from benzaldehyde and added acetaldehyde has been reported only for the bacterium *Zymomonas mobilis* [6]. As indicated in Figure 1 this activity requires uptake of free acetaldehyde into the 'active acetaldehyde' form. Acetaldehyde strongly inactivates PDC from *Zymomonas mobilis* [7] and to reversibly inhibit yeast PDC activity [8]. Also the substrate, benzaldehyde, is strongly inactivating [9], while no detrimental effects of pyruvate on PDC have been reported.

The current commercial process is based on the biotransformation of benzaldehyde by PDC in whole cells of sugar fermenting baker's yeast with significant benzaldehyde converted to benzyl alcohol due to oxidoreductase activities (e.g. alcohol dehydrogenase). An enzymatic process based on extracted PDC overcame benzyl alcohol formation and has the potential for increased productivities [10,11].While yeast strains have been screened for fermentative PAC production and some ethanol producing filamentous fungi have been screened for cell-free PAC formation [12], no screening data are available for cell-free PAC-formation by yeast PDC. The present study evaluates carboligase activities with benzaldehyde and pyruvate or alternatively benzaldehyde and acetaldehyde in extracts of various yeast strains. Also resistance of carboligase activity towards inactivation by benzaldehyde and towards inactivation and/or inhibition by the potential substrate or by-product acetaldehyde is screened.

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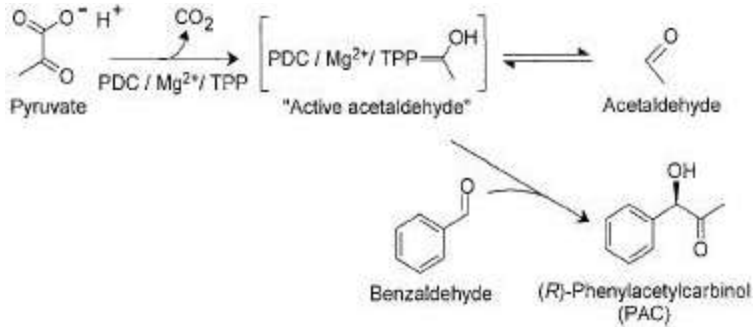

Fig. 1: Acetaldehyde and PAC formation by pyruvate decarboxylase (PDC) with cofactors Mg2+ and thiamine pyrophosphate (TPP).TPP-bound 'active acetaldehyde' is an intermediate and can be formed by decarboxylation of pyruvate or in the case of *Zymomonas mobilis* PDC from acetaldehyde.mg-1. We suggest that the ligation of benzaldehyde and acetaldehyde might be a characteristic feature of bacterial PDCs. However, the detection limit for PAC was 0.3 mM and other bacterial PDCs e.g. from *Acetobacter pasteurianus* [15] or *Sarcina ventriculi* [16] have not been tested yet for PAC formation.

MATERIALS AND METHODS

Enzyme Preparation: The yeast strains as listed in Figure 2 were provided by MTCC Chandigarh, India. These strains were grown at 30°C in 20 ml media (10 g/l yeast extract, 20 g/l peptone, 90 g/l glucose, initial pH 6) in 250 ml Erlenmeyer flasks with cotton stoppers on an orbital shaker at 230 rpm. As a reference for growth, the turbidity of diluted cell suspensions was measured at 600 nm. Because of differences in growth rates, an attempt was made to standardize the procedure by harvesting the cells in the stationary phase. Five ml cell suspension was harvested by centrifugation and washed twice in buffer (50 mM MES, 20 mM $MgSO_4$, adjusted to pH 7 with KOH). They were resuspended in 1.25 ml breakage buffer (50 mM MES/KOH, 20 mM $MgSO_4$, 1 mM thiamine pyrophosphate, 1 tablet 'Complete protease inhibitor cocktail' from Boehringer Mannheim/25 ml, pH 7). 1.25 ml glass beads (diam. 0.5 mm) were added and cells were broken by vortexing 5 times for 15 s at maximum speed with cooling on ice between cycles. After settling of beads, the crude extract was clarified by centrifugation and stored at -70°C. *Zymobacter palmae* was grown in 10 g yeast extract l, 20 g glucose l, 2 g KH2PO4 l, initial pH 6. After 31 h incubation at 30°C, the turbidity at 600 nm was 1.9 and cells were harvested by centrifugation, washed once in water and stored at -70°C. Cells were broken as above but protease inhibitor was omitted and vortexing with glass beads (diam. 0.1 mm) was extended to 10 × 1 min. Residual pyruvate was removed from the crude extract by gel filtration.

Screening for Carboligase Activity and Activity Loss: The screening procedure was designed to provide high specificity for detecting PAC formation while keeping the procedure as simple as possible. PDC carboligase activity was measured as formation of PAC in 30 min at 25°C from 40 mM benzaldehyde and 100 mM pyruvate in carboligase buffer (50 mM MES/KOH, 20 mMMgSO4, 1 mMTPP, 1.5Methanol, pH 7). The reaction was stopped by the addition of trichloroacetic acid (10% w/v) and proteins were removed by centrifugation. PAC was quantified by HPLC as described previously (Rosche *et al.* 2002a). Specific carboligase activities (mM PAC min-1 mg-1) were based on protein concentrations in the crude extract estimated by the Bradford method. Expressing the activities relative to protein concentrations standardized the results regardless of strain differences regarding biomass concentration and cell breakage. For simultaneously testing PDC stability in the presence of benzaldehyde and acetaldehyde and the possible inhibitory effect of acetaldehyde, crude extracts were incubated in carboligase buffer with 40 mM benzaldehyde and 30 mM acetaldehyde for 1 h at 25°C in 1.5 ml closed Eppendorf tubes. An aliquot of the mixture was analyzed for putative PAC, which could have been formed from benzaldehyde and acetaldehyde. To the remaining sample, 100mMpyruvate was added and PAC formation after 30 min at 25°C was measured as above. The % activity loss was calculated based on the difference between this activity and the activity of the untreated crude extract divided by the original activity of the untreated crude extract expressed as a percentage.

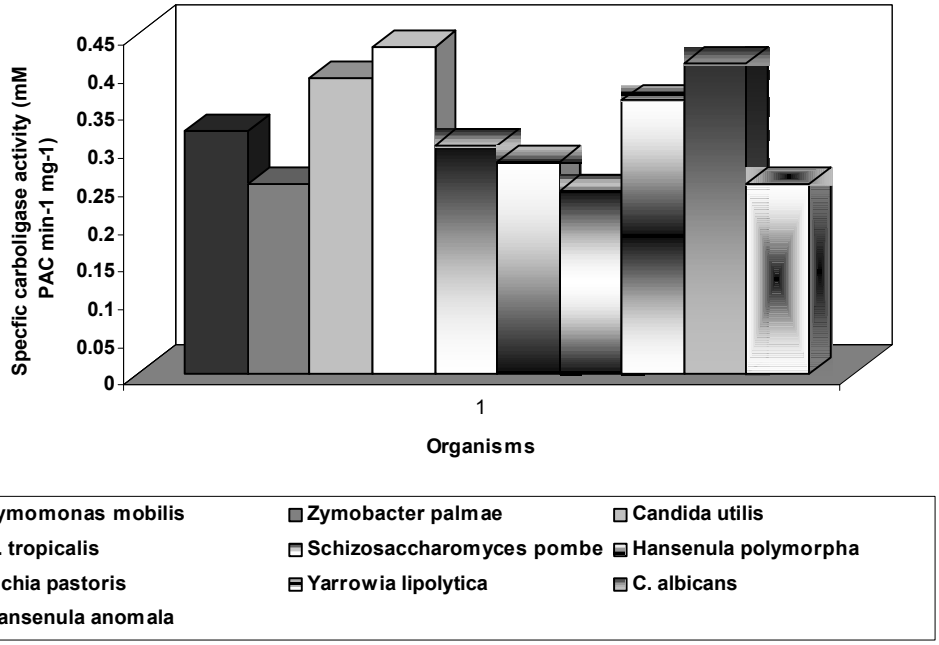

Fig. 2: Carboligase activity in extracts of 10 yeast strains and activity loss after exposure to 40 mM benzaldehyde and 30 mM acetaldehyde for 1 h at 25°C. Strains are sorted by descending activity. Two groups of interesting results are highlighted: squares for less than 30% activity

RESULTS AND DISCUSSION

Ac Formation from Benzaldehyde and Acetaldehyde: PAC formation from benzaldehyde and acetaldehyde was not detected in any of the 10 tested yeast extracts. This activity was not found either in any extract of 14 strains of filamentous fungi (unpublished data), which formed PAC from benzaldehyde and pyruvate [13]. In contrast, it has been reported for PDC from the bacterium *Zymomonas mobilis* [14]. A *pdc* gene from *Zymobacter palmae* has been sequenced recently [15] but no data is available regarding PAC formation. Table 1 establishes that extracts of *Zymobacter palmae* produced PAC from benzaldehyde and pyruvate (0.014mMPAC min-1 mg-1) and to a lesser extend from benzaldehyde and acetaldehyde (0.002mMPAC min-1 mg-1). In comparison, pyruvate decarboxylation in the absence of benzaldehyde was 103 mM pyruvate min-1.

PAC Formation from Benzaldehyde and Pyruvate: Figure 2 presents the screening results for PAC formation from benzaldehyde and pyruvate by extracts of 10 yeast strains from 5 genera and 10 species. All except seven extracts formed PAC. Results are presented with strains sorted by descending specific carboligase activity. The reproducibility of the results was checked with triplicate experiments carried out on some of the yeasts. Variations of ±10% were normally observed. However, occasional variations of up to ±25% were found, possibly due to differences in inoculation conditions and harvest times, although attempts were made to standardize procedures as much as possible.

Most of the species tested here which were listed by [17] as capable of glucose fermentation also showed carboligase activity. This confirmed the strategy of strain selection for this screening based on the rationale that glucose fermentation will involve PDC activity. Five of the seven species that did not form PAC (Figure 2) are listed by [18] as not fermenting glucose or showing more than 7 days' delayed responses. However, *Torulopsis bombicola* and *Saccharomyces exigus* are reported to ferment glucose but no carboligase activity was detected in this study. Furthermore carboligase activity was detected in extracts of *Candida rugosa*, which is described as a species that does not ferment glucose [19]. It is possible that the growth conditions used in this study allowed fermentation of glucose for this yeast or that the enzyme activity was related to a different metabolic function.

Remaining Activity after Incubation with Benzaldehyde and Acetaldehyde: Incubation of the crude extracts in the presence of 40 mM benzaldehyde and 30 mM acetaldehyde for 1 h at 25°C prior to pyruvate addition resulted for nearly all strains (93 of 98) in an activity loss of 32–96% (Figure 2). Despite the low boiling point of acetaldehyde (20°C), no acetaldehyde loss was detected in a control vial during the 1 h incubation at 25°C. Since acetaldehyde was not removed prior to pyruvate addition, the calculated activity loss includes [20]. loss and circles for activity losses less than 50% combined with specific activities higher than 0.3 mM PAC min-1 mg-1.possible reversible inhibition by acetaldehyde in addition to irreversible inactivation[21]. Therefore low activity loss is interpreted as 'carboligase resistance towards pre-incubation with acetaldehyde and benzaldehyde' rather than to stability of carboligase activity. Besides the effect of the aldehydes, a putative inactivation by proteases not inhibited by the 'Complete protease inhibitor cocktail' should be considered also. Figure 2 indicates that the activity losses did not correlate to the specific activities. Least affected by the pre incubation were the carboligase activities in the five strains of *Schizosaccharomyces pombe*.

CONCLUSION

Two interesting groups of candidates were identified in this screening and are highlighted in Fig. 2. The extracts of the strain of *Schizosaccharomyces pombe* showed best resistance towards pre-incubation with acetaldehyde and benzaldehyde with only 5–27% activity loss but the carboligase activities were very low (0.03–0.06 U mg-1 protein) and the strains grew slowly. Highest carboligase activities (0.31–0.49 U mg-1 protein) combined with medium resistance (32–49% activity loss) were observed for strains of *Candida tropicalis*, *Candida utilis* and *Candida albicans* (highlighted by circles). However, *Candida* strains were also distributed over the whole spectrum. Since *Candida albicans* and *Candida tropicalis* can act as opportunistic pathogens the most interesting candidate is *Candida utilis* which has been used as a fodder supplement for animals and has minimal nutritional requirements.

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