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Biotransformation of benzaldehyde into (R)-phenylacetyl carbinol by *Brettanomyces lambicus* or their extracts

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ABSTRACT

Extracts of 7 yeast were examined regarding their potential for production of (R)-phenylacetylcarbinol [(R)-PAC], which is the chiral precursor in the manufacture of the pharmaceuticals ephedrine and pseudoephedrine. Benzaldehyde and pyruvate were transformed at a scale of 1.2 ml into PAC by cell-free extracts of all selected strains, covering the broad taxonomic spectrum of Zygomycota and Basidiomycota. Highest final PAC concentrations were obtained with the extracts of Brettanomyces lambicus. [78-84 mM (11.7-12.6 g/l) PAC within 20 h from initial substrate concentrations of 100 mM benzaldehyde and 150 mM pyruvate]. (R)-PAC was in about90-93% enantiomeric excess. Brettanomyces lambicus were used as an example in a biotransformation process based on whole cells and benzaldehyde and glucose as substrates. The substrate pyruvate was generated through the fungal fermentation of glucose. Only 19 mM PAC (2.9 g/l) was produced within8 h from 80 mM benzaldehyde, with evidence of significant benzyl alcohol production.

Key words: *Brettanomyces lambicus*, Biotransformation, PAC, Ethanol production.

INTRODUCTION

(R)-phenylacetylcarbinol [*(R)*-PAC or *(R)*-1-hydroxy-1-phenyl-propan-2-one] is a chiral intermediate int he production of the pharmaceutical compounds ephedrine and pseudo ephedrine and is currently produced industrially via a biotransformation of benzaldehyde by fermenting yeast cultures. The designation of the *(R)*-PAC enantiomeric refers to the R-/S-system and is identical to (-) PAC.

It is also identical to L-PAC in the D-/L-system, which uses the Fisher projection with the phenyl group at the top. The biotransformation is catalyzed by the enzyme pyruvate decarboxylase (Fig. 1). After pyruvate is decarboxylated, the remaining enzyme-bound-intermediate "active acetaldehyde" can either take up a proton and be released, as in its natural function in ethanol fermentation, or it can react as a nucleophile with added benzaldehyde to form *(R)*-PAC. This carboligation can be conducted using either whole microorganisms (for example, *Saccharomyces cerevisiae, Candida utilis Hansenulla polymorpha and B. lambicus*) or cell-free extracts of microorganisms (for example, *Saccharomyces cerevisiae, Candida utilis Zymomonas mobilis*) (Pohl 1997; Rogers at al. 1997; Oliver *et al.,* 1999). *(R)*-PAC production with whole microorganism has the advantage of pyruvate generation from glucose, while in cell-free reactions pyruvate has to be supplied. On the other hand, biotransformation with whole cells has the disadvantage of substrate loss due to benzaldehyde reduction to the side product benzylalcohol as a consequence of oxidoreductase

activities (e.g. alcohol dehydrogenase). These enzyme activities do not interfere in cell-free extracts due to the lack of electron donors (e.g. NADH), thus allowing higher efficiency.

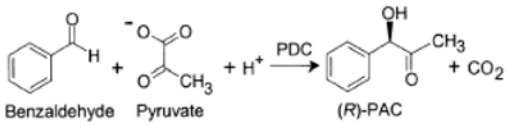

Fig. 1: Biotransformation of benzaldehyde and pyruvate into (R)-PAC by pyruvate decarboxylase (PDC)

For this study, 7 strains of yeast were selected based on their ability to produce ethanol (Singl*et al.,* 1992; Skory *et al.,* 1997), on the assumption that they could be a source of pyruvate decarboxylases with potentially improved characteristics for *(R)*-PAC production. Some information about pyruvate decarboxylases *(R)*-PAC synthesis had not been described. Under oxygen-limited conditions, some yeast produce ethanol from sugars (Singh *et al.,* 1992; Skory *et al.,* 1997), indicating pyruvate decarboxylase activity.

In the literature, the following strains of yeast are reported to conduct acyloin condensations. In a fermentation of benzaldehyde by *Aspergillus niger* a diol was detected after treatment with $NaBH_4$ (Cardillo *et al.,* 1991). *Mucor circinelloides* was reported to carry out acyloin condensations with acyclic unsaturated aldehydes but benzaldehyde conversion was not tested (Stumpf and Kieslich 1991). Strains from the genera *Neurospora, Aspergillus, and Mucor* were included in our screening for *(R)*-PAC formation.

MATERIAL AND METHODS

Sugar cane juice was added into 100 ml conical flasks containing sterile water and incubated at room temperature for two to three days. After incubation one loop full from each flask was added aseptically to separate Petri plates containing YPS medium. Antibiotics like streptomycin and griseofulvin were added YPS medium to prevent the growth of bacteria and fungi respectively. After inoculation, the plates were incubated fro two to three days at room temperature and yeast growth was observed. The obtained yeast colonies were further purified by streaking on pertriplates containing the same medium. The strain was mentioned as SCY.

The strains were motioned as SCY throughout the work until their identification and were used for different studies. These strains were identified at the Institute on Microbial Technology, Chandigarh, basing on sequencing of D1/D2 domain of 26 S rRNA gene these strains were identified as *B. anomalus, B. bruxellensis, B. custersianus, B. lambicus B. naardenensis, B. nanus and Hansunella polymorpha*.

Preparation of inoculum

Cultures of B. anomalus, B. bruxellensis, B. custersianus, B. lambicus, B. naardenesis, B. nanus, B. lambicus and Hansunella polymorpha were inoculated with pieces of culture grown at 30°C on YPS agar slants (Soluble starch 5g/1000ml, Yeast extracts 5g/l, K_2hpo_4 1.20g/l, $Mgso_4$ 0.5g/l, Agar 20g/l pH 3-3.5). The spores were washed from the culture with 15% (w/v) sterile glycerol. This suspension was adjusted to 108 spores/ml and was stored at 70°C.

The flasks were incubated in two phase. An initial rapid shaking (230 rpm) provided oxygen for fast biomass production. This was followed by a phase in which the oxygen supply was reduced (60 rpm, flasks covered with Parafilm) to induce the production of the enzyme pyruvate decarboxylase. *B. lambicus and B. naardenensis.* Were incubated at 23 °C without shaking for the entire time in order to achieve better growth.

Samples of culture supernatant were stored at -20°C for the analysis of glucose and ethanol concentrations. The culture were harvested by filtration in a Buchner funnel, washed twice with cold buffer [50 mM 2-(N-morpholino] ethane sulfonic acid (MES)/KOH, pH 7.0, 20 mM MgSO4] and the excess liquid was drained off. The wet culture were weighed and frozen at -70°C. The frozen culture were ground to a powder in a chilled mortar (-70°C) with an equal weight of chilled glass beads as grinding agent. Cold breakage buffer (50 mM MES/KOH, pH 7.0, 20 mM

MgSO4, 1 mM thiamine pyrophosphate, 1 tablet Complete Protease Inhibitor Cocktail EDTA-free/25 ml) was added and the extracts were clarified by centrifugation (19, 000 g) and adjusted to 5 ml. The resulting crude extracts were about four-fold concentration in relation to the culture volume. They were stored as aliquots at -70°C.

Growth and extraction of yeast

B. lambicus and Hansunella polymorpha were grown in the same medium as the yeast at 30°C and 230 rpm for 17.5 h. Washed cells were disrupted in breakage buffer by vortexing with glass beads at 4°C. After clarification by centrifugation (19, 00o g), the crude extracts were adjusted to a protein concentration of 6 mg/ml.

Biotransformation of benzaldehyde by crude extracts

Biotransformations were carried out at a scale of 1.2 ml in 2-mlscrewed-glass vials with Teflon seals. Benzaldehyde (12.3 µl, final concentration 100 mM) was injected with a glass syringe under the surface of 180 µl of 1 M sodium pyruvate (final concentration 150 mM) in breakage buffer and 47 µl breakage buffer. After vortexing the emulsion, 960 µl crude extract were added and the sample was vortexed again for 10 s. The vials were rotated vertically at 35 rmp at room temperature (23°C). After 20 min and after 20 h, samples of 300 µl were taken and added to 30 µl 100% (w/v) trichloroacetic acid. After removal of protein by centrifugation, the supernatants were analyzed for PAC by HPLC.

Biotransformation of benzaldehyde by fermenting culture

Hansenulla polymorpha was grown in YEMA medium (90 g glucose/l, 10 g yeast extract/ l, 10 g (NH_4) $2SO_4$/l, 3 gKH_2PO_4/l, 2 g Na_2HPO_4.l2H2O/l, l g $MgSO_4.7H_2O$/l. 0.05 g$CaCl_2.2H_2O$/l, pH 6) at 30°C for 20 h. The fermenting culture were harvested by filtration through a Buchner funnel (Whatmanfilter paper no. 1) and resuspended in YEMA medium to a concentration of approximately 7 g dry biomass/l. The flasks were then incubated on a shaker at 30°C and 150 rpm for l h. After wares, the cultures were sub-divided into 25-ml sample in 50-ml Erlenmeyer flasks, covered with Parafilm, and shaken at the same conditions. Flasks 1-3 were fed with 10 mM benzaldehyde every 2 h. Flasks 4-6 were fed with the addition of 15 mM acetaldehyde after 2 h, 4 h,

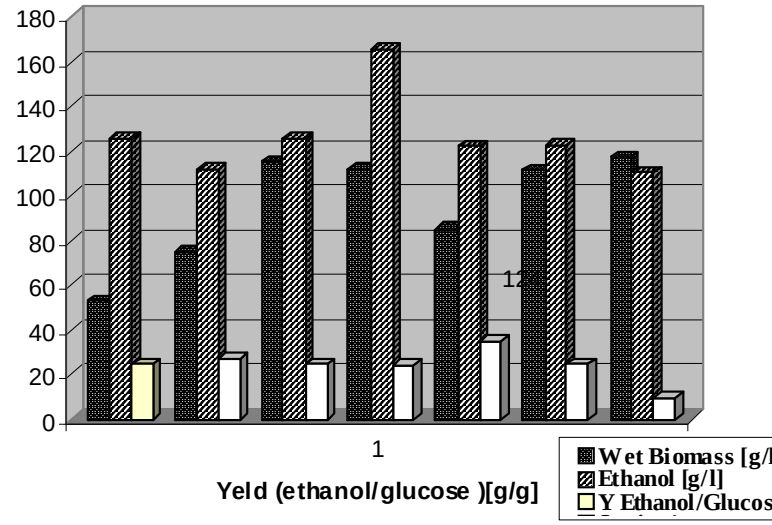

Fig. 2: Biomass and thanol production by filamentous fungi in shake flasks. The cultures were grown at 23° or 30°C with initial rapid shaking followed by a phse in which oxygen supply was reduced. The initial pH was 6.0. The times of harvest are given in parentheses

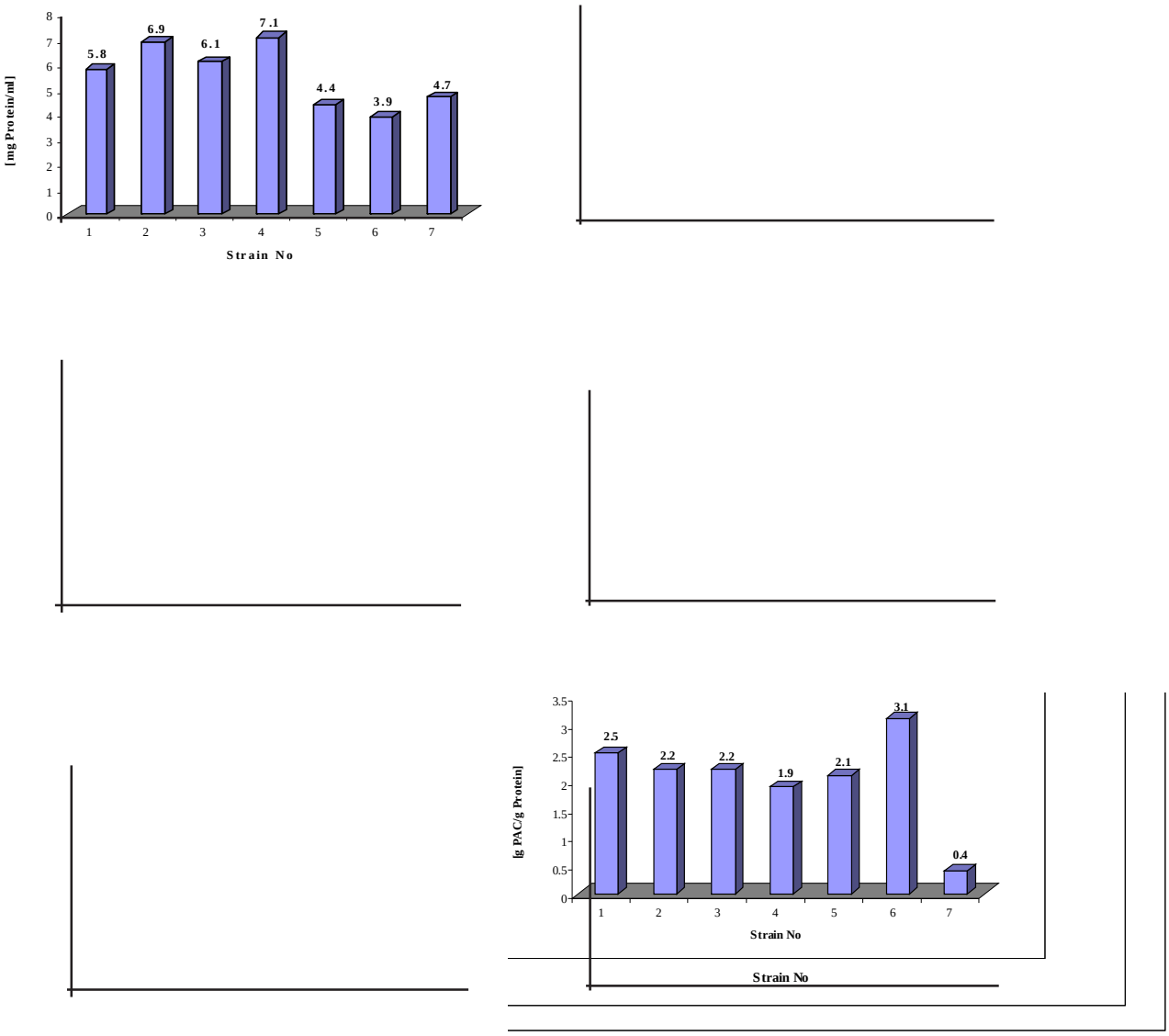

Fig. 3a-f Protein concentrations and activities in crude extracts of yeast. Strain Nos. 1. *B. anomalus*, 2. *B. Bruxellensis,* 3. *B. custersianus,* 4. *Hansunella polymorpha*, 5. *B. naardenensis*, 6. *B. lambicus* and 7. B*. nanus* a Protein concentrations in crude extracts; b pyruvate decarboxylase activities in crude extracts, measured spectrophotometrically by the alcohol-dehydrogenase-coupled assay at pH 6 and 25°C (no data available for strain 5); c initial productivity for PAC by crude extracts (80% v/v). Substrate concentrations were 100 mM benzaldehyde and 150 mM pyruvate at pH 7 and 23°C in 50 mM MES/KOH, 20 mM MgSo4, 1 mM thiamine pyrophosphate, 1 tablet EDTA-free Complete Protease Inhibitor/25 ml. d Final PAC concentrations after 20 h of incubation and theoretical yields based on initial benzaldehyde concentrations; conditions were as in c. e Initial productivity, as in c, related to the protein concentration. f Final PAC concentration, as in d, related to the protein concentration.

analyzed by HPLC for PAC, benzaldehyde, and benzylalcohol.

Analytical methods

The level of glucose was measured by and enzyme-electrode-based glucose analyser. Ethanol concentrations were estimated by gas chromatography using a 1 mx6.3 mm Porapak Q column and a flame ionisation detector (column 180°C, injector and detector 200°C, carrier gas N2 30 ml/min).

PAC, benzaldehyde and benzylalcohol were quantified by HPLC with UV-detection at 283 nm (PAC and benzaldehyde) and 263 nm (benzylalcohol). Concentrations were determined based on peak areas with reference to standards. An C8 column (5 µm, 250 x 4.6 mm) with 32% (v/v) acetonitrile and 0.5% (v/v) acetic acid in water as the mobile phase (1 ml/min). The *(R)*- and (S)-enantiomers of PAC were separated by chiral HPLC with a Chiracel OD column (10 µm, 50 x 4.6 mm) from Sigma-Aldrich in 95% (v/v) *n*-hexane, 4.9% (v/v) isopropanol, and 0.1% (v/v) formic acid (0.8 ml/min). The enatiomeric excess was calculated based on peak areas.

Pyruvate decarboxylase activity was measured spectrophotometrically by the alcohol dehydrogenase-coupled assay as specified by Sigma-Aldrich. Protein concentrations were estimated according to the method of Bradford (1976) with the Coomassie Plus protein assay reagent (Pierce).

RESULTS

Growth of fungi and ethanol production

The growth times needed for the production of a reasonable amount of biomass are given in Fig. 2. The fasted biomass production times were observed for (strains 1-7). All strains produced ethanol. Final ethanol concentrations were up to 36 g/l with yields (ethanol/glucose utilized) of up to 0.43 g/g.

Pyruvate decarboxylase activities in cultural extracts

Extracting the yeast culture by grinding and suspending in buffer mainly yielded protein concentrations between 2.5 and 7 mg/ml (Fig. 3a). The pyruvate decarboxylase activities as estimates since the extracts might also have contained other oxidoreductase activities, for example lactate dehydrogenase, which could have interfered with the spectrophotometric assay.

Biotransformation of benzaldehyde by crude extracts

PAC was produced from benzaldehyde and pyruvate by all extracts of the 7 strains of yeast. The best initial productivities of 3.8 – 6.5 g PAC/l in 20 min were obtained with crude extracts from *all stains* (Fig. 3c). This carboligation followed a similar trend as observe in the decarboxylase activities shown in Fig. 3b. strains produced the highest final PAC concentrations, 78-84 mM (11.7 – 12.6 g/l, Figure 3d) after 20 h of incubation. This was 78-84% of the theoretical yield based on the initial benzaldehyde concentration.

Figure 3e, f relates initial productivities (Fig. 3c) and final PAC concentrations (Fig. 3d) to the protein concentration in the biotransformation mixtures. These relative values were highest for *B. lambicus.* All results were obtained without any optimization of the experimental conditions. The biomass extracts did not produce the byproduct benzylalcohol. PAC was not formed in the absence of biomass extracts. The enantiomeric excess of *(R)*-PAC from the final biotransformation samples (Table 1) was between 90 and 98% respectively.

Biotransformation of benzaldehyde by culture of *Brettanomyces lambicus*

As an example of PAC formation with fermenting culture, shows the benzaldehyde biotransformation by cultures of *Brettanomyces lambicus* With a 10 mM stepwise feed of 40 mM benzaldehyde, 10 mM PAC (1.5 g/l) was produced in 8 h. The benzaldehyde concentration decreased to zero before each new feed and the PAC increase at sampling times was linear over time. The by-product benzylalcohol was produced at approximately double the rate of PAC production, reaching a final concentration of 25 mM.

Eighty mM benzaldehyde (in 20 mM portions) with addition of 45 mM acetaldehyde (in 15 mM portions) yielded 19 mM PAC (2.9 g/l) in

Table 1: Enantiomeric excess of *(R)*-PAC from 100 mM benzaldehyde and 150 mM pyruvate after 20 h at 23°C

No.	Strain	Enantiomeric excess of *(R)*-PAC (%)
1.	*B. anomalus*	93
2.	*B. bruxellensis*	94
3.	*B. custersianus*	93
4.	*Hansenulla polymorpha*	98
5.	*B. nanus*	96
6.	*B. naardenensis*	92
7.	*B. lambicus*	93

mM portions) yielded 19 mM PAC (2.9 g/l) in 8 h. The PAC increase at sampling time was linear at the beginning and slowed down after 4 h. This was accompanied by the build-up of benzaldehyde concentrations in the medium and morphological changes in the culture. In the first 2 h, the formation of the by-product benzylalcohol was as fast PAC production. It slowed down after the first addition of acetaldehyde, which competes with benzaldehyde for the dehydrogenase enzymes, and reached a final concentration of 15 mM. Thus the ration of PAC to benzylalcohol (19 mM/ 15 mM) was three times higher that in the first experiment (10 mM / 5 mM). The yield of PAC with respect to total added benzaldehyde in both experiments as 5% of the theoretical yield.

DISCUSSION

Several yeast species and their pyruvate decarboxylases as well as pyruvate decarboxylases from the bacterium *Zymomonas mobilis* have been well investigated with regard to their *(R)*-PAC-producing potential (Bringer-Meyer and Sahm 1988; Pohl 1997; Rogers *et al.,* 1997; Oliver *et al.,* 1999). This study examines for the first time the potential of using filamentous fungi for *(R)*-PAC production. The screening medium and conditions allowed growth as well as ethanol production for all 14 strains of filamentous fungi. Eleven stains yielded 0.34 – 0.43 g ethanol/g consumed glucose (68-86% theoretical yield), indicating a strongly fermentative metabolisms and thus high activity of the fermentative enzyme pyruvate decarboxylase.

After 2 days *Brettanomyces lambicus and Hansunella polymorpha* accumulate 27, 32, 6 and 33 g ethanol/l, respectively. This compares well to 20, 33, 32 g ethanol/l with the same strains grown for 3 day in 100 g glucose/l by Skory *et al.,* (1997). The lowest ethanol production and the slowest growth were obtained with the basidiomycete *Polyporus eualyptorum*. Crude extracts from all 7 strains of yeast catalyzed the formation of *(R)*-PAC from benzaldehyde and pyruvate, even though not all activities were measurable by the ADH-coupled assay for pyruvate decarboxylase. The strains cover the broad taxonomic spectrum of Ascomycota and Basidiomycota.

The highest final PAC concentrations (11.7 – 12.6 g/l) were obtained with extracts of *B. lambicus and Hansunella polymorpha* can be compared with the 11.3 g PAC/l produced by crude extracts of the other yeast. Partially purified pyruvate decarboxylase of *Candida utilis* has been reported to yield 14.5 g PAC/l from the same substrate concentration under optimised conditions (Shin and Rogers 1996). Evaluating the two fungi that produced the highest PAC levels, *Hansenulla polymorpha* had the advantage of much faster growth than B. lambicus. The best initial productivities of 3.8 – 6.5 g PAC/l in 20 min, achieved with extracts that the 2.6 – 3.2 g PAC/l in 20 min produced by extracts of the yeasts B. lambicus. These initial activities are very high with respect top the final PAC concentrations, indicating the potential for a rapid biotransformation process. However, comparison of Fig. 3c, d demonstrates that a high initial productivity (e. g. strain 4) does not necessarily correspond to the highest final PAC yield. This is consistent with the finding that in fermentative PAC production with yeast, high initial rates did not inevitably result in high final yields (Netrval and Vojtisek 1982).

Only low protein connections (0.8 and 0.2 mg/ml) were recovered from *Polyporus eucalyptorum and Paecilomyces lilacinus*. Nevertheless these extracts yielded 11 and 4 mM PAC, respectively. The low pyruvate decarboxylase activities (0.0 – 1.6 U/ml) and low PAC formation (9- 11 mM) in crude extracts of ethanol were produced during fermentation, indicating active

pyruvate decarboxylase, and the protein recovery from the culture was also good. It is possible that proteases degraded the pyruvate decarboxylase.

Screening of the PAC-producing capacities of the yeast extracts was experimentally based on a defined culture volume. Comparisons of strains therefore fully take into account differences regarding produced biomass; ease of cell breakage, pyruvate decarboxylase concentration, and pyruvate decarboxylase chrematistics and final PAC yields to protein concentrations (Fig. 3e, f, respectively) possibly indicates differences in the relative pyruvate decarboxylase quantity and/or its quality. However, substrate concentrations might have been limiting. Since pyruvate decarboxylase was not purified, it is beyond the scope of this screening to determine qualitative differences between pyruvate decarboxylases of various strains.

For whole-cell process using various yeas strains, concentrations of 10- 22 g PAC/l have been reported (Rogers *et al.,* 1997). Fermenting culture of *Hansenulla polymorpha* yielded only 2.9 PAC/l. This could possibly be improved by the use of higher biomass concentrations and by adapted substrate feeding. However, the organism foes not appear suited for fermentative biotransformation due to the rapid reduction of benzaldehyde to the by-product benzylalcohol. Fungi are well known for their potential to catalyse redox reactions. A list of various fungi used for biotransformations (Kieslich 1997) records 19 to the 48 listed Rhizopus strains as catalyzing keto-reductions and two for the reverse reaction. The only biotransformation recorded fro *Hansenulla polymorpha* in this list is a glycosyl transfer. However, the problem of benzaldehyde loss due to reduction to benzylalcohol is overcome when extracts electron donors (e.g. NADH) are quickly consumed and not regenerated.

CONCLUSION

This study shows that *(R)*-PAC formation is not limited to the use of yeasts. Especially extracts of *Hansenulla polymorpha* are suited fro *(R)*-PAC production with higher initial productivities and slightly higher final yields than obtained with extracts of the yeast *Brettanomyce lambicus*.

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