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Enzymatic (*R*)-phenylacetylcarbinol production in a benzaldehyde emulsion system with *Candida utilis* cells

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Abstract Recent progress in enzymatic (*R*)-phenylacetylcarbinol (PAC) production has established the need for low cost and efficient biocatalyst preparation. Pyruvate decarboxylase (PDC) added in the form of *Candida utilis* cells showed higher stability towards benzaldehyde and temperature in comparison with partially purified preparations. In the presence of 50 mM benzaldehyde and at 4°C, a half-life of 228 h was estimated for PDC added as *C. utilis* cells, in comparison with 24 h for the partially purified preparation. Increasing the temperature from 4 to 21°C for PAC production with *C. utilis* cells resulted in similar final PAC levels of 39 and 43 g l^{-1} (258 and 289 mM), respectively, from initial 300 mM benzaldehyde and 364 mM pyruvate. The overall volumetric productivity was enhanced 2.8-fold, which reflected the 60% shorter reaction time at the higher temperature. Enantiomeric excess values of 98 and 94% for *R*-PAC were obtained at 4 and 21°C, respectively, and benzyl alcohol (a potential by-product from benzaldehyde) was not formed.

Introduction

The enzyme pyruvate decarboxylase (PDC) is a thiamine diphosphate-dependent ketolase with considerable potential for use in industrial biotransformations. (*R*)-phenylacetylcarbinol (PAC), a pharmaceutical intermediate for ephedrine and pseudoephedrine, is synthesized by PDC through the non-oxidative decarboxylation of pyruvate followed by carboligation with benzaldehyde (Fig. 1).

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Values of 10–12 g PAC l^{-1} have been reported for fermentative biotransformations with bakers' yeast (Rogers et al. 1997) and up to 50 g l^{-1} for enzymatic processes with partially purified PDC from *Candida utilis* or *Rhizopus javanicus* based on added pyruvate and a benzaldehyde emulsion (Rosche et al. 2002a,b, 2003). Such enzymatic processes overcome the problems in the traditional yeast-based fermentations of limited availability of substrate pyruvate and the substantial loss of benzaldehyde to benzyl alcohol due to yeast oxidoreductases (Rosche et al. 2002a,b; Shin and Rogers 1996). However, the enzyme processes based on cell-free PDC can result in appreciable PDC deactivation by substrate benzaldehyde (Leksawasdi et al. 2003), although deactivation is less severe when the substrate pyruvate is present (Rosche et al. 2005).

The use of whole cells as the biocatalyst in an enzymatic process has the advantage of simple and economical preparation as expenses for cell lysis and/or purification are saved. A further reported advantage is greater biocatalyst stability and protection within the intracellular environment especially in organic media, although some limitations in mass transfer of substrates and products may be associated with whole cell biocatalysis (León et al. 1998). Previous enzymatic processes with whole cells of *Saccharomyces cerevisiae* resulted in by-product benzyl alcohol formation (0.1–0.5 g l^{-1}) with only 1–3 g PAC l^{-1} produced (Nikolova and Ward 1991, 1994). In a further study with cells of *S. cerevisiae*, 5 g benzyl alcohol l^{-1} and 15 g PAC l^{-1} were reported (Mochizuki et al. 1995). The latter authors maintained the biotransformation at pH 6 and 30°C with progressive addition of low concentrations of benzaldehyde. Benzyl alcohol formation has been reported also for PAC production by whole cells immobilized on polymer matrices, alginates or cyclodextrin (Mahmoud et al. 1990; Nikolova and Ward 1994; Rogers et al. 1997).

In former studies of enzymatic PAC production in benzaldehyde emulsion systems, *C. utilis* PDC has been added as a cell free preparation (Shin and Rogers 1996; Rosche et al. 2002b, 2003, 2005). In the present study, PDC is initially added in the form of *C. utilis* cells (whole cell PDC), and the enzymatic production of PAC is evaluated

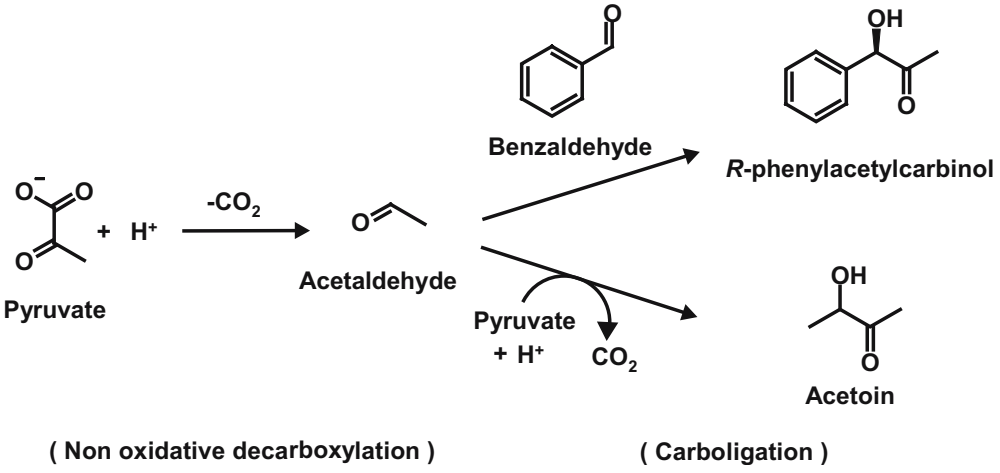

Fig. 1 PDC-catalyzed non-oxidative decarboxylation and carboligation. *R*-PAC production and its associated by-products (acetaldehyde and acetoin) from pyruvate

in a benzaldehyde emulsion at 4 and 21°C. The results are compared with those for crude extract and partially purified PDC preparations to determine whether or not a whole cell PDC process offers any potential advantages.

Materials and methods

Chemicals and analytical methods

All chemicals used were analytical grade. Benzaldehyde of 98% purity was used in this study. Details of the analytical method to determine the concentrations of benzaldehyde, pyruvate, PAC (*R*- and/or *S*-), acetaldehyde, acetoin and benzyl alcohol are specified in Rosche et al. (2001, 2002a). Samples were analysed in duplicate.

PDC preparation

The yeast *C. utilis* UNSW 70940 was obtained from the University of New South Wales Culture Collection, Sydney, Australia; World Federation of Culture Collections, No. 248. The PDC enzyme was produced from a 3-l fermentation in a 5-l bioreactor (B. Braun) using defined minimal medium at 30°C (per litre: 100 g glucose, 10 g $(NH_4)_2SO_4$, 1 g KH_2PO_4, 0.5 g $MgSO_4{\cdot}7H_2O$, 20 mg $CaCl_2{\cdot}2H_2O$, 20 mg $FeSO_4{\cdot}7H_2O$, 10.6 mg $ZnSO_4{\cdot}7H_2O$, 2 mg $MnCl_2{\cdot}4H_2O$ and 0.5 mg $CuSO_4{\cdot}5H_2O$). The yeast cells were harvested after 44 h, reaching 7.8 g dry cell mass (DCM) l^{-1} and a specific PDC carboligase activity of 380 U g^{-1} DCM. The wet cell paste was stored at −20°C prior to use as a whole cell PDC preparation. The preparation of crude extract by attrition with glass beads, partial purification of PDC by acetone precipitation, as well as analysis of carboligase activity, were adapted from Rosche et al. (2002a). One unit (U) carboligase activity is defined as the amount of enzyme that produces 1 μmol PAC from pyruvate and benzaldehyde per minute at pH 6.4 and 25°C.

Experimental system for biotransformation

Prior to the start of reaction by PDC addition, the reaction mixture was magnetically stirred as a benzaldehyde emulsion for 1 h. For the study comparing different PDC preparations, 1.8-ml biotransformation mixtures were stirred in 4-ml teflon screw cap glass vials. The reaction volume was increased to 15 ml in 20-ml glass vials for the kinetic study of whole cell biotransformation. All reactions were initiated at pH 6.5 with 2.5 M MOPS (pKa 7.3) as buffer, and the pH rose to a maximum of pH 7.2–7.6 during biotransformation. The reactions were stopped by adding a trichloroacetic acid solution to the samples to a final concentration of 10% (w/v). Precipitated protein was removed by cold centrifugation at 14,000 rpm for 5 min for subsequent analysis.

Determination of residual PDC activity

PDC as partially purified enzyme, crude extract and whole cell preparation was exposed to 50 mM benzaldehyde in stirred 4-ml teflon screw cap glass vials (1.8 ml reaction volume) at 4°C and 21°C. Residual carboligase activities for each condition and their control (without benzaldehyde) were determined over time by withdrawing and diluting samples for the subsequent analysis. From these data, the half-life values of the various PDC preparations were estimated.

Results

In Fig. 2 the profiles of residual PDC activities in the presence of 50 mM benzaldehyde are compared for a partially purified, a crude extract and a whole cell preparation at 4 and 21°C. From the data it is evident that PDC activity was maintained better in the whole cell preparation and that deactivation occurred more rapidly at the higher temperature. The deactivation kinetics revealed not

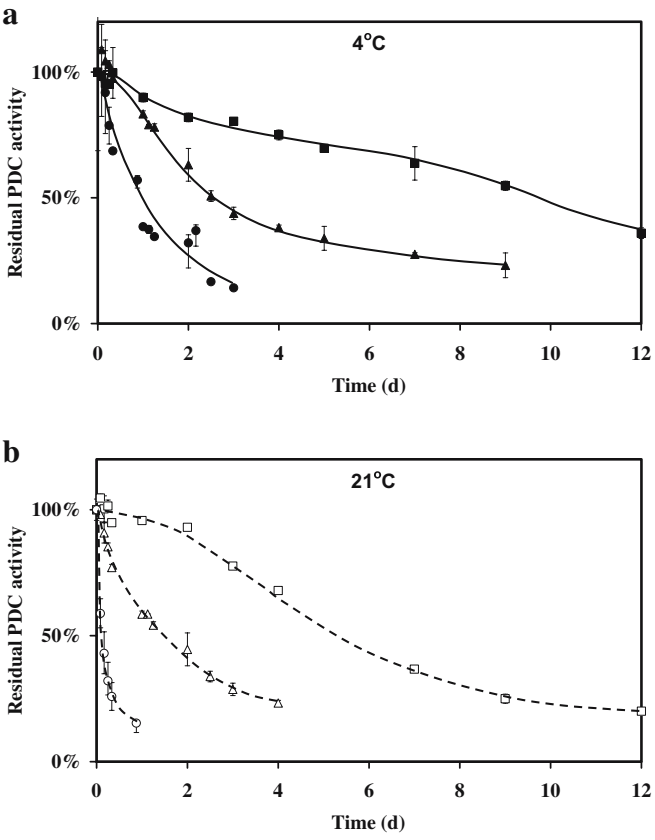

Fig. 2 Profile of residual activity of partially purified (●,○), crude extract (▲, △) and whole cell (■,□) PDC at **a** 4°, *closed symbols* and **b** 21°C, *open symbols*, respectively, in the presence of 50 mM benzaldehyde (initial activity 1.5 U ml^{-1}, 2.5 M MOPS, pH 6.5, 0.5 mM $MgSO_4$, 0.5 mM TPP). *Error bars* show lowest and highest values of duplicates from each of two vials

only differences in stability but also in the curve shapes, as not all deactivation profiles followed an exponential decay model. Therefore, half-life values were estimated graphically from Fig. 2. They are compared with half-life values derived in the absence of benzaldehyde (profiles not shown) in Table 1.

Exposure to benzaldehyde and increasing the temperature from 4 to 21°C reduced PDC stability in all cases, with the greatest impact on the more purified PDC. A half-life of 228 h was estimated for whole cell PDC, compared with 24 h for the partially purified preparation at 4°C in the presence of 50 mM benzaldehyde. When the temperature was increased to 21°C, the half-life for whole cell PDC decreased by 42%, compared with an 85% decrease for the partially purified preparation.

Table 1 The estimated half-lives of different preparations of *C. utilis* PDC in 50 mM benzaldehyde at 4 and 21°C

Benzaldehyde concentration (mM)	Half-life (h) at 4°C		Half-life (h) at 21°C	
	0	50	0	50
Partially purified	168	24	14.4	3.6
Crude extract	480	60	132	48
Cells	600	228	324	132

The values for residual PDC activity in 50 mM benzaldehyde are detailed in Fig. 2

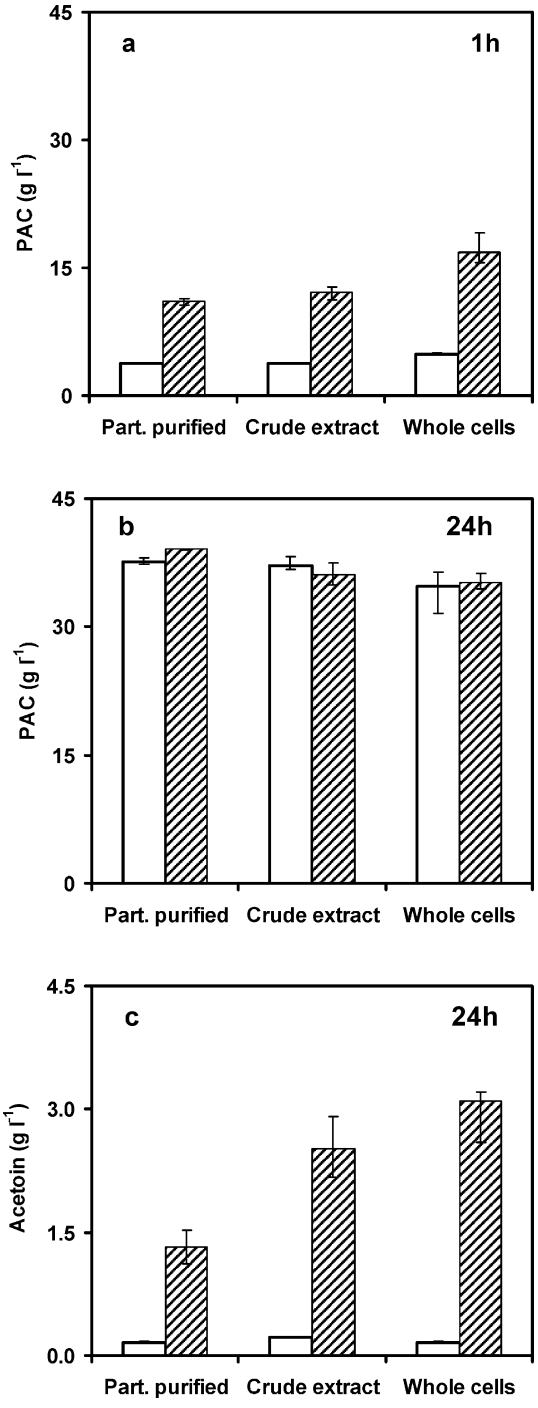

Fig. 3 Comparison of **a** PAC 1 h, **b** PAC 24 h and **c** acetoin 24 h concentrations in the benzaldehyde emulsion system at 4±2 (*blank bars*) and 21±2°C (*shaded bars*) with partially purified, crude extract and whole cell PDC (3 U ml^{-1}, 2.5 M MOPS/KOH, initial pH 6.5, 0.5 mM $MgSO_4$, 0.5 mM TPP, 300 mM benzaldehyde, 360 mM pyruvate). *Error bars* show lowest and highest values of triplicates from two biotransformations

In Fig. 3, the effects of PDC preparation (3 U ml^{-1}) on PAC and by-product acetoin formation at 4 and 21°C are shown. Whole cell PDC at 21°C resulted in increased PAC formation over the initial hour and higher level of acetoin after 24 h than the partially purified PDC preparation. It is also apparent that similar PAC concentrations were achieved after 24 h by all PDC preparations regardless of the temperature (Fig. 3b). The addition of 2 M ethanol or repeated freeze thaw of whole cells to increase cell permeability and/or release intracellular PDC did not result in a higher PAC level (data not shown).

Detailed kinetics for whole cell biotransformations at 4 and 21°C with initial concentrations of 300 mM benzaldehyde and 364 mM pyruvate are shown in Fig. 4a and b, respectively. The profiles demonstrate faster initial PAC production at 21°C than at 4°C as would be expected. At 21°C, a maximum PAC concentration of 43 g l^{-1} (289 mM, ee 94%) was achieved after 12 h, in comparison with

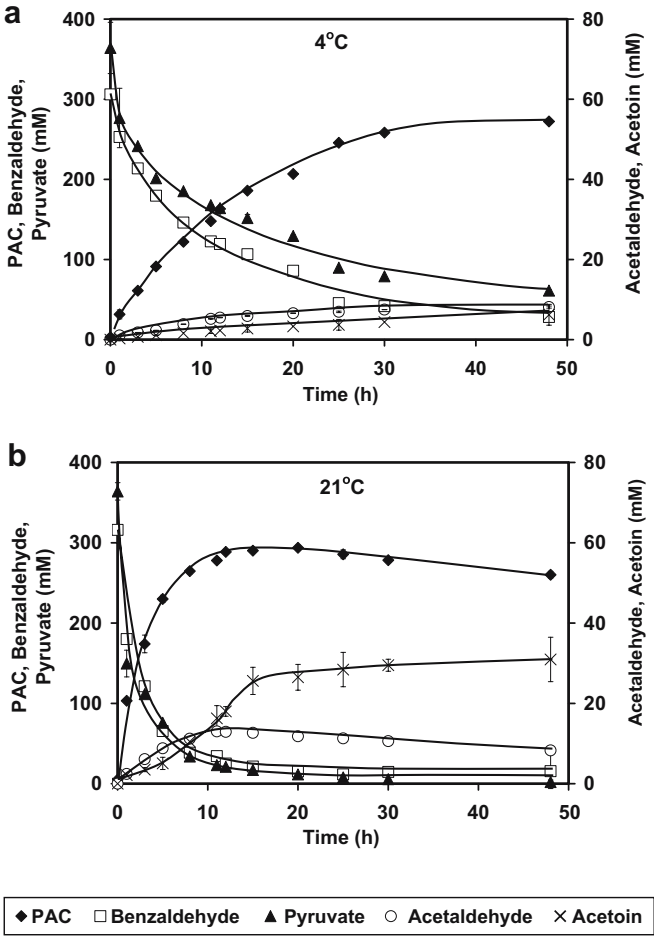

Fig. 4 Profiles of PAC production with PDC added in the form of whole cells in the benzaldehyde emulsion system at **a** 4° and **b** 21°C (initial PDC carboligase activity of 3 U ml^{-1} equivalent to 7.9 g DCM l^{-1}, 2.5 M MOPS/KOH, initial pH 6.5, 0.5 mM $MgSO_4$, 0.5 mM TPP, 300 mM benzaldehyde, 364 mM pyruvate). *Error bars* show lowest and highest values of triplicates from two biotransformations with closely similar values being determined for PAC concentrations

Table 2 Kinetic analysis of biotransformations with PDC added in the form of whole cells (see Fig. 4)

	7.9 g DCM l^{-1} (3.0 U ml^{-1})	
	4°C, 30 h	21°C, 12 h
Initial production rate (g l^{-1} h^{-1})[a]	4.7	15.5
PAC concentration (g l^{-1})	38.7	43.3
Specific PAC production per unit enzyme activity (mg U^{-1})[b]	12.9	14.4
Specific PAC production per biomass (g g^{-1} DCM)[c]	4.9	5.5
Overall volumetric productivity (g l^{-1} h^{-1})	1.3	3.6
Acetoin (g l^{-1})	0.4	1.6
Acetaldehyde (g l^{-1})	0.3	0.6
$Y_{PAC\ per\ pyruvate}$ (%)[d]	91	84
$Y_{PAC\ per\ benzaldehyde}$ (%)[d]	98	99

[a]Based on PAC production in 1 h
[b]Based on initial PDC carboligase activity
[c]Based on the initial amount of biomass added
[d]Molar yield (mol mol^{-1}) based on consumed substrate

39 g l^{-1} (258 mM, ee 98%) after 30 h at 4°C. However, a 4-fold higher acetoin level (1.6 g l^{-1}, 18 mM) was evident at 21°C when compared with the results at 4°C (0.4 g l^{-1}, 4.4 mM) at these times. The remaining time-courses up to 48 h showed a small increase in PAC concentration at 4°C, whereas a gradual PAC loss was noted at 21°C. In the latter case, approximately 13% PAC was lost between 20 h and 48 h with the ee value for *R*-PAC decreasing from 94 to 79%. By comparison, the incubation of a PAC standard at pH 7.5 for 28 h at 21°C showed that the ee value for *R*-PAC decreased from 98 to 82% in the presence of *C. utilis* cells, whereas the ee values with the partially purified PDC or in the absence of PDC under similar conditions remained high at 95 and 97%, respectively.

A comparison of the whole cell biotransformations at 4 and 21°C is given in Table 2. The results show a 12% higher maximum PAC concentration at 21 than at 4°C and a 2.8-fold increase in overall volumetric productivity at the higher temperature. However, increased formation of the by-product acetoin at 21°C lowered the product yield by 7% (based on consumed substrate pyruvate). The molar balances for benzaldehyde and pyruvate were determined for the time points given in Table 2. They closed within 1 and 2%, respectively, based on determination of PAC, acetaldehyde and acetoin together with residual substrate concentrations.

Discussion

In the present study, it was found that the use of PDC in the form of *C. utilis* cells was preferable to cell-free (crude extract or partially purified) PDC preparations for PAC production, as the former preparation achieved similar PAC levels and is a less expensive catalyst.

Comparison of deactivation profiles and half-lives (Fig. 2, Table 1) in the presence of benzaldehyde demonstrated that PDC stability decreased with the greater degree of enzyme purification, especially when the temperature was increased from 4 to 21°C. It appears that the biomass matrix may have provided some support for improved stability or that the cell-free PDCs may have been altered during cell attrition, acetone precipitation or freeze-drying, thereby resulting in a less stable enzyme. Higher stability of trehalosyl-dextrin-forming and trehalose-forming enzymes in whole cell *Escherichia coli* ($t_{1/2}$=30 days) have been reported also in comparison with the purified preparations ($t_{1/2}$=4 days) (di Lernia et al. 2002).

A possible disadvantage of using whole cells in biotransformation is the putative mass transfer limitation of substrates and products across the cell envelope. However, data for PAC production over the initial hour (Fig. 3a) demonstrated higher levels for whole cells than for cell-free preparations, and there was evidence of some PDC activity in the reaction mixture (data not shown) indicating partial cell permeabilization during biotransformation.

The highest published PAC productivity achieved for enzymatic whole cell biotransformation using bakers' yeast was 2.5 g l^{-1} h^{-1} (15 g l^{-1} in 6 h at 30°C, Mochizuki et al. 1995). In this study, the overall productivity of *C. utilis* cells was 3.6 g PAC l^{-1} h^{-1} (43 g l^{-1} in 12 h at 21°C), which was 3-fold higher than at 4°C (Fig. 4, Table 2). In contrast, Shin and Rogers (1996) found that for partially purified *C. utilis* PDC with a different set of conditions (different method for PDC preparation, 70 mM pyruvate, 70 mM benzaldehyde, 40 mM phosphate buffer), a similar temperature increase resulted in an approximately 50% decrease in overall productivity after 6 h. The same study also reported an increase in acetoin formation at higher temperature, which is consistent with the present study.

In comparison with the biotransformation at 4°C, a lower ee value for *R*-PAC was apparent at 21°C following completion of the biotransformation (20–48 h time period). Willeman et al. (2002) have reported a similar trend of decreased ee with increased temperature and exposure time in the biotransformation of benzaldehyde and HCN into *R*-mandelonitrile. They suggested that the competing rates of non-enzymatic racemization and enzymatic reaction for the desired product were the main factors in determining the final ee value for this reaction. In the present study, the decrease in the ee value over time at 21°C occurred only in the presence of whole cells but not in an enzyme-free control or with partially purified PDC, which indicates that cell-associated factors had a greater influence than abiotic factors.

The use of *C. utilis* cells in the present experiments did not result in any benzyl alcohol formation; the same was true when either the crude extract or the partially purified PDC was used. This is in contrast to the results of other enzymatic whole cell studies (Nikolova and Ward 1991, 1994; Mochizuki et al. 1995) in which appreciable benzyl alcohol production occurred. It is possible in the present investigation that the relatively high initial benzaldehyde (300 mM) used and the PAC concentration (33–127 mM) formed within the first hour (Fig. 4a,b) may have inhibited the formation of benzyl alcohol. Mochizuki et al. (1995) indicated that PAC can act as an inhibitor of alcohol dehydrogenase activity and that the formation of benzyl alcohol ceased at approximately 33 mM PAC (5 g l^{-1}) in their study.

In conclusion, the potential of using *C. utilis* cells for PAC production is evident from the present data. PDC added in the form of whole cells showed higher stability particularly at 21°C when compared with the crude extract or partially purified PDC preparations. With no apparent mass transfer limitations, a PAC concentration of 43 g PAC l^{-1} was achieved in 12 h, with 1.6 g by-product acetoin l^{-1} but no benzyl alcohol formation. Having established the potential of the less expensive whole cell PDC preparation, further improvement could be achieved by replacing 2.5 M MOPS with pH control by acid addition for a more economical process. Our results also indicate that, in order to achieve maximum enantiopurity with high productivity at the higher temperature (21°C), the time of PAC harvest is an important parameter.

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