Production of L-Phenylacetylcarbinol (L-PAC) from Benzaldehyde Using Partially Purified Pyruvate Decarboxylase (PDC)

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Received February 23, 1995/Accepted August 1, 1995

Biotransformation of benzaldehyde to L-phenylacetylcarbinol (L-PAC) as a key intermediate for L-ephedrine synthesis has been evaluated using pyruvate decarboxylase (PDC) partially purified from *Candida utilis.* PDC activity was enhanced by controlled fermentative metabolism and pulse feeding of glucose prior to the enzyme purification. With partially purified PDC, several enzymatic reactions occurred simultaneously and gave rise to by-products (acetaldehyde and acetoin) as well as L-PAC production. Optimal reaction conditions were determined for temperature, pH, addition of ethanol, PDC activity, benzaldehyde, and pyruvate:benzaldehyde ratio to maximize L-PAC, and minimize by-products. The highest L-PAC concentration of 28.6 g/L (190.6 m*M*) was achieved at 7 U/mL PDC activity and 200 m*M* benzaldehyde with 2.0 molar ratio of pyruvate to benzaldehyde in 40 m*M* potassium phosphate buffer (pH 7.0) containing 2.0*M* ethanol at 4°C.
Key words: L-phenylacetylcarbinol • biotransformation • benzaldehyde • pyruvate decarboxylase • *Candida utilis*

INTRODUCTION

L-Phenylacetylcarbinol (L-PAC) is an intermediate in the production of L-ephedrine and pseudoephedrine, pharmaceutical compounds used as decongestants and anti-asthmatics. Reports have also indicated its potential use in obesity control.[1,2] It is currently produced via a microbial biotransformation process using different species of yeasts with benzaldehyde as the aromatic substrate. Scheme 1 outlines the biotransformation process which involves the condensation of an "active acetaldehyde" (from pyruvic acid produced by the yeast) and benzaldehyde. The production of the L-PAC is catalyzed by the enzyme pyruvate decarboxylase (PDC) and is associated with by-product formation, viz. benzyl alcohol, due to the activity of an alcohol dehydrogenase (ADH) and/or oxidoreductases.

In previous studies, the kinetics of L-PAC production in fed-batch culture using various strains of yeasts have been reported[9,19,27] as well as a semi-continuous evaluation using immobilized *Saccharomyces cerevisiae*.[20] The role of PDC from both *Zymomonas mobilis* and *S. carlsbergensis* in L-PAC production has been studied also in detail by Bringer-Meyer and Sahm.[6] Other fundamental investigations have indicated that oxidoreductases distinct from ADH may be involved in by-product benzyl alcohol production.[17,21]

In the current investigation, an assessment is made of the potential for developing a process with high L-PAC concentrations and yields using partially purified PDC. This is a significant extension of previous studies which have used only relatively low levels of substrates benzaldehyde and pyruvate. The enzyme itself has been described as a holoenzyme having a tetramer structure with molecular weight of 230–250 kDa for yeasts and *Z. mobilis*[7] or 275 kDa for wheat germ.[29] In both yeast and wheat germ, it consists of two dimeric subunits ($\alpha_2\beta_2$) of slightly different chain lengths; PDC from *Z. mobilis* consists of four identical subunits (α_4). The holoenzyme includes 2–4 molecules of thiamine pyrophosphate (TPP) together with magnesium ions which are obligatory cofactors. The binding strength of thiamine pyrophosphate is pH dependent and differs among the binding sites in the various enzyme molecules.[25]

Following a preliminary evaluation of partially purified PDC from other sources (viz. *S. cerevisiae*, wheat germ), a strain of *Candida utilis* was selected as the source of enzyme. This strain has been shown in our laboratory to produce levels of L-PAC in excess of earlier published values of 10–12 g/L.[9,27] As well as offering the potential for a relatively fast and high productivity L-PAC process, the use of partially purified PDC overcomes the problem of the significant by-product accumulation of benzyl alcohol which occurs with whole cells due to other enzymatic reactions with benzaldehyde.

MATERIALS AND METHODS

Microorganism and Culture Media

Candida utilis was kindly provided by ICI Australia Pty. Ltd. The strain was maintained on culture medium containing (grams per liter) glucose, 20; yeast extract, 3.0; $(NH_4)_2SO_4$, 2.0; KH_2PO_4, 1.0; $MgSO_4 \cdot 7H_2O$, 1.0; agar,

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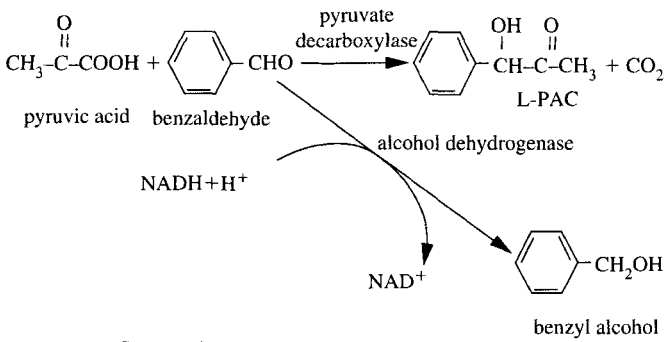
CCC 0006-3592/96/010052-11



Scheme 1. Mechanism of L-PAC formation.

1.0 with initial pH 6.0. For partially purified PDC production, the strain was cultivated in a 100-L fermentor in medium consisting of (grams per liter) glucose, 90; yeast extract, 10; $(NH_4)_2SO_4$, 10; KH_2PO_4, 3.0; $MgSO_4 \cdot 7H_2O$, 1.0; $Na_2HPO_4 \cdot 12H_2O$, 2.0; $CaCl_2$, 0.05; $FeSO_4 \cdot 7H_2O$, 0.05; $MnSO_4 \cdot 4H_2O$, 0.05 at an initial pH 6.0 and temperature of 25°C.

Partially Purified PDC Preparation

In the stationary growth phase at which the PDC activity reached around 0.9 U/mg protein, cells were harvested with a continuous centrifuge (Sharples Centrifuge Co., Ltd., USA) at 15,000 rpm. The cell cake was washed in water twice through a continuous separator (SA 1-01, Westfalia Separator AG., Germany) at 9700 rpm. Washed cells were resuspended in 40 m*M* potassium phosphate buffer (KH_2PO_4/K_2HPO_4, pH 6.0) containing 50 μ*M* TPP and 0.3 m*M* $MgSO_4 \cdot 7H_2O$.

Disruption was effected at 640 kPa with a Manton-Gaulin-APV high pressure homogenizer. The degree of disruption after each pass was estimated microscopically, and the released protein (determined by the Bradford method[5]) and PDC activity were measured. Although higher pressure and multiple passes gave rise to more efficient disruption, the further passage of broken cells resulted in fine debris which would have been difficult to remove further downstream. A 3-cycle homogenization process was selected to disrupt the *C. utilis* cells.

The resulting cell homogenate was diluted with 3 times its volume of 40 m*M* potassium phosphate buffer (pH 6.0), and the cell debris and other solid particles were removed by centrifugation at 3800*g* for 30 min. For further purification, fractionation of the protein mixture by stepwise increase in ammonium sulfate concentration was used. PDC enzyme, which precipitated in the range of 45–55% saturation $(NH_4)_2SO_4$, was harvested and desalted by dialysis in 40 m*M* potassium phosphate buffer (pH 6.0).

Finally, the active PDC enzyme solution was loaded on a Sephacryl S-300 (Pharmacia Chem. Co.) gel chromatography column equilibrated with 40 m*M* potassium phosphate (pH 6.0) containing 50 μ*M* TPP and 0.3 m*M* $MgSO_4 \cdot 7H_2O$. Purification of PDC via chromatography was achieved with 5.6-fold increase in specific activity (4.8 unit/mg protein) compared to homogenized cells (0.85 unit/mg protein) and an overall 54.3% recovery based on cell homogenate.

The results of this partial purification procedure are shown in Table I. The specific activity of the partially purified PDC can be compared with that of a commercial purified preparation from Brewers yeast (Sigma Chem. Co.) which has a specific activity of 5–20 unit/mg protein. Furthermore, the proteolytic activity of the partially purified PDC was very low and measured 0.0043 unit/mg protein at pH 7.0, and 0.0033 unit/mg protein at pH 6.0.

Dry Cell Weight Estimation

After centrifugation of a sample of culture broth and resuspending in isotonic saline, 4 mL of the cell suspension was transferred to a preweighed glass tube and centrifuged at 5000 rpm for 10 min. The samples were dried in an oven at 105°C for 24 h, cooled in a desiccator, and reweighed. The

Table I. Summary of partial purification stages for PDC from *C. utilis*.

Process	Total protein (mg)	Total activity (unit)	Specifc activity (unit/mg protein)	Purification fold	Recovery yield (%)
1. Cell homogenate (640 kPa, 3 cycle)	928.6	789.3	0.85		100
2. $(NH_4)_2SO_4$ fractionation (45–55% saturation)	360	543.6	1.51	1.8	72.5
3. Gel filtration on Sephacryl S-300	85.3	407.6	4.8	5.6	54.3

average values of three measurements for each sample were determined.

Estimation of Glucose Concentration

Glucose concentrations were determined by a YSI glucose analyzer (Yellow Springs Instruments Co., Model 27).

Estimation of Ethanol

Ethanol concentrations were estimated using a gas chromatograph (Packard, USA, Series 427). The relevant column and its operation were as follows: column material, 0.25-in. glass with 1.5 m length; packing material, Porapak Q in mesh range 100–200 μm; carrier gas, nitrogen (30 mL/min); oven temperature, 180°C (isothermal); injector temperature, 220°C; detector temperature, 220°C with a flame ionization detector (FID); injection sample, 3 μL. The ethanol concentration of the sample was determined by comparison with a standard sample.

Estimation of Benzaldehyde and L-PAC Concentrations

Concentrations of both benzaldehyde and L-PAC were determined by gas chromatography. Samples were prepared by extracting with dichloromethane (sample:solvent = 1:5). The biotransformation sample (0.2 mL) was mixed with 1 mL of dichloromethane in a microcentrifuge tube and vortexed for 2 min. A sample from the bottom organic layer was injected into the Packard Series 427 gas chromatograph with the column and its operating conditions as follows: column material, 0.25-in. glass with 1 m length; packing material, Chromosorb W. Hr/SE 30WTX 10 in the mesh range of 80–100 μm; carrier gas, nitrogen (30 mL/min); oven temperature, 115°C (isothermal); injector temperature, 180°C; detector temperature, 180°C with FID; injection sample, 3 μL. The concentrations of benzaldehyde and L-PAC were determined by comparison with standard samples of benzaldehyde and L-PAC, the latter provided by ICI Australia Pty. Ltd.

Pyruvic Acid Determination

Determination of pyruvic acid was carried out by enzymatic analysis (Boehringer–Mannheim Analytical Kit No. 718 882). In the presence of NADH + H^+, lactic dehydrogenase reduces pyruric acid k lactic acid. The amount of NADH + H^+ oxidized to NAD^+ corresponds stoichiometrically to the amount of pyruvic acid. The decrease in NADH + H^+ was determined by difference of its absorbance at 340 nm.

Estimation of Acetaldehyde by Enzymatic Analysis

The determination of acetaldehyde was carried out by enzymatic analysis (Boehringer–Mannheim Analytical Kit, Cat. No. 668 613). Acetaldehyde is oxidized quantitatively at pH 9.0 in the presence of aldehyde dehydrogenase by nicotinamide adenine dinucleotide (NAD^+) to acetic acid. The amount of NADH + H^+ formed is stoichiometrically related to the amount of acetaldehyde. NADH + H^+ was determined by increase of absorbance at 340 nm.

Colorimetric Determination of Acetoin

Determination of acetoin was based on the color reaction with 0.5% creatine and 5% α-naphthol.[28] The color intensity was measured at 540 nm. The colors obtained by this method were reproducible although unstable with extended time. The maximum intensity was reached in 60 min after the addition of the alkaline α-naphthol; fading could not be detected within 75 min. The acetoin concentration of the sample was determined by comparison with standard samples.

Enzyme Recovery

Cells from 1 mL of culture broth were harvested by centrifugation (Eppendorf Centrifuge) at 12,000 rpm for 1 min and washed twice with 30 m*M* Tris-HCl buffer (pH 6.5). Cells were resuspended in the same buffer and the volume adjusted to 0.4 mL. Approximately 1 g of glass beads (size 0.5 mm, B. Braun, Cat. No. 854 170/1) was mixed with 0.4 mL of cell suspension, and vortexed at maximum speed for 2 min. For every 30 s of vortexing, the sample was cooled for 1 min in an ice bath. Cell debris were removed by centrifugation at 12,000 rpm for 3 min. The supernatant was collected for subsequent enzyme assays and protein determination.

Pyruvate Decarboxylase

The activity of pyruvate decarboxylase (PDC) was assayed by coupling the decarboxylation reaction with the ADH mediated reaction and monitoring the oxidation of NADH + H^+ to NAD^+ at 340 nm.[24] The reaction mixture consisted of (microliters): 200 m*M* sodium citrate buffer (pH 6.0), 950; 10 mg/mL NADH (sodium salt), 10; 10 mg/mL alcohol dehydrogenase (Sigma Chem. Co., Prod. No. A-3263), 3; 100 mg/mL sodium pyruvate, 32; enzyme sample, 5. One unit of enzyme activity is defined as that activity which converts 1.0 μmol of pyruvate to acetaldehyde per minute at pH 6.0 and 25°C. The activity of the enzyme was monitored by NAD^+ formation through changes in absorbance at 340 nm.

Alcohol Dehydrogenase

The basic reaction for determination of alcohol dehydrogenase (ADH) activity is oxidation of ethanol to acetaldehyde and monitoring reduction of NAD^+ to NADH + H^+ (modified from Bergmeyer et al.[3]). The reaction mixture consisted of (μL): 35 m*M* Trizma base (pH 8.5), 910; 250 mg/mL semicarbazide hydrochloride, 25; 20 mg/mL

NAD^+, 30; absolute ethanol, 30; enzyme sample, 5. One unit of enzyme activity is defined as that activity which converts 1.0 μmol ethanol to acetaldehyde per minute at pH 8.5 and 25°C. The activity of the enzyme was monitored as NADH + H^+ formation by changes in absorbance at 340 nm.

Protein Determination

Protein determinations of cell free crude extract, PDC, and ADH were carried out by the Bradford method[5] with lyophilized bovine serum albumin as a reference.

RESULTS AND DISCUSSION

Enzyme Profiles of PDC and ADH for *Candida utilis*

In the present study, *C. utilis* was grown on 90 g/L glucose medium in a 100-L fermentor with 0.6 volume of air/volume of liquid/min (vvm) aeration rate at 25°C and pH 6.0 for 8 h; and then its metabolism was shifted toward increased fermentation by the reduction of aeration rate to 0.3 vvm and agitation speed from 300 to 100 rpm (Fig. 1a). The shift in metabolism resulted in further induction of the fermentative enzymes PDC and ADH (Fig. 1b). This sustained a high glucose consumption rate accompanied by ethanol production. Before the initial glucose was completely exhausted, pulse feeding of a supplement containing glucose and yeast extract (approximate concentrations 30 g/L and 5 g/L, respectively) slightly enhanced enzyme activities, while ethanol production increased up to 35 g/L. The highest PDC activity of 0.9 unit/mg protein was achieved after 18-h cultivation. After onset of the stationary phase, there was a slight decrease in the PDC and ADH activities even in the presence of excess glucose.

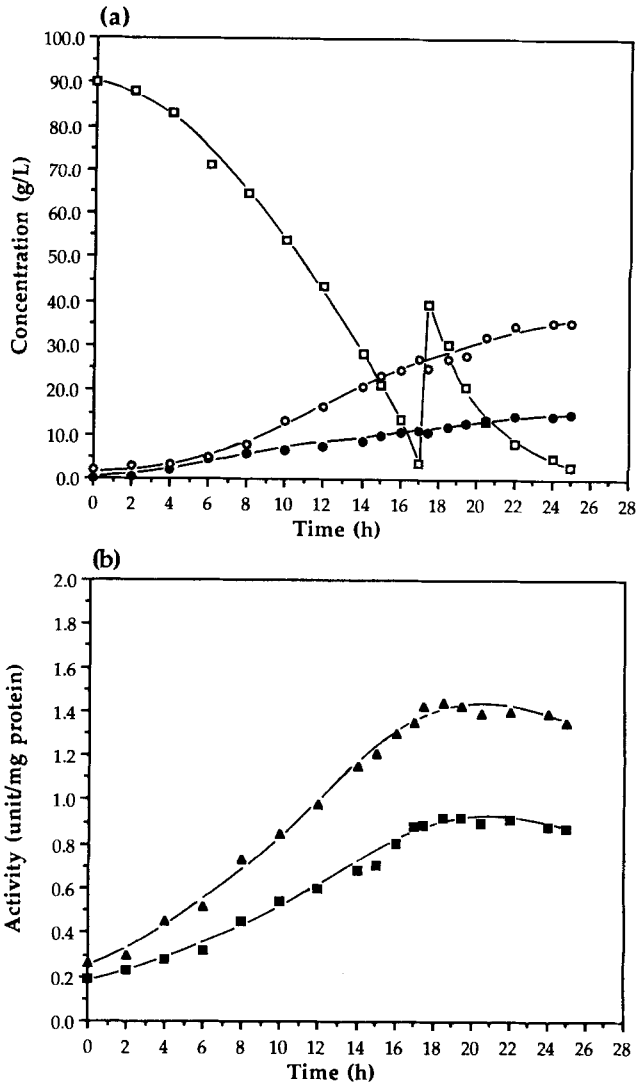


Figure 1. (a) Growth of *Candida utilis* on 90 g/L glucose based medium in 100 L fermentor with pulse feeding 30 g/L glucose and (b) fermentative enzyme profiles: (□) glucose, (●) biomass, (○) ethanol, (▲) ADH, and (■) PDC.

Characteristics of Partially Purified PDC

The stability of the partially purified PDC was evaluated from a time profile of residual activity in various species buffers with and without 30 μ*M* TPP at 25°C and pH 6.0. Although loss of PDC activity occurred in all buffers, it was found that PDC was more stable in potassium phosphate and sodium citrate buffers compared with Tris-HCl buffer (Fig. 2). Furthermore, the stability of PDC was significantly improved by addition of TPP, this being evident for all buffers under test. The loss in activity with time is consistent with a previous study[11] which demonstrated that the dimeric tetramer structure ($\alpha_2\beta_2$) of PDC dissociated in vitro into dimer and monomer subunits with concomitant release of TPP and magnesium ions. This dissociation, predominantly a function of pH,[13] is affected also by the buffer species, and in the present study was greater in Tris-HCl compared with potassium phosphate and sodium citrate buffers.

The loss of activity in potassium phosphate buffer containing TPP was similar to that in sodium citrate buffer with the half-life of PDC being about 28–30 h in both cases; in Tris-HCl buffer, the half-life of PDC was 17 h. It was evident that half-lives without TPP were significantly less than those with TPP. In distilled water for example, PDC retained less than 50% activity after 2 h. This result is strong evidence that TPP is involved in the maintenance of active conformation of PDC holoenzyme. As indicated earlier, the level of contaminating proteolytic enzyme was low in the partially purified PDC and it is unlikely to have a significant influence in the biotransformation experiments with benzaldehyde (see later) which were completed in 5–6 h.

The K_m value of PDC for pyruvate was determined as 2.2 m*M* at 25°C and pH 6.0 (Fig. 3), with saturation at a concentration in excess of 10 m*M* pyruvate. The K_m value is in good agreement with other reported values for yeasts.[4,16,26]

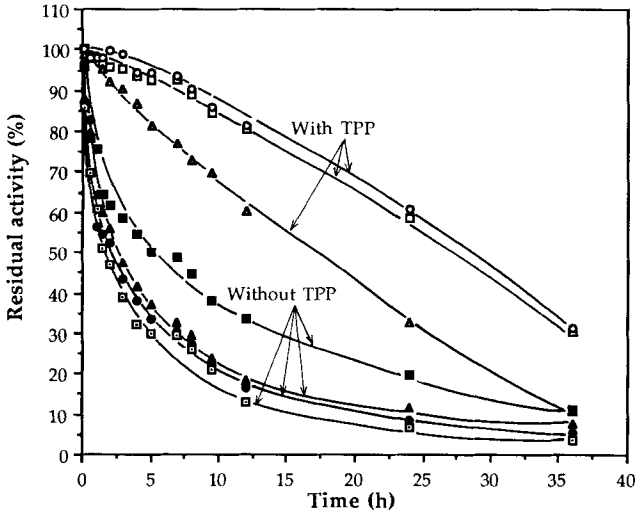


Figure 2. Effect of buffer species (pH 6.0) on the stability of PDC from *C. utilis* in the presence and absence of 30 μ*M* TPP at 25°C: (□) 40 m*M* potassium phosphate, (△) 40 m*M* Tris-HCl, (○) 40 m*M* sodium citrate with TPP; and (■) 40 m*M* potassium phosphate, (▲) 40 m*M* Tris-HCl, (●) 40 m*M* sodium citrate buffer without TPP, and (⊡) water.

When compared with *Z. mobilis*, for which the K_m value is reported as 0.4 m*M*,[7] yeast PDC possesses a lower affinity toward pyruvate.

Biotransformation Kinetics for L-PAC Formation

Effect of Temperature on L-PAC Formation

The dependence of the PDC mediated biotransformation on temperature was evaluated at 4°C, 10°C, and 25°C. A comparison of the kinetic parameters at different temperatures (Table II) shows the interesting result that while the rate of acetaldehyde formation was promoted by increasing temperatures, final L-PAC formation was enhanced at the lower temperatures. Thus, the formation of acetaldehyde was the major reaction at 25°C (on a molar basis), while the formation of L-PAC was predominant at 4°C.

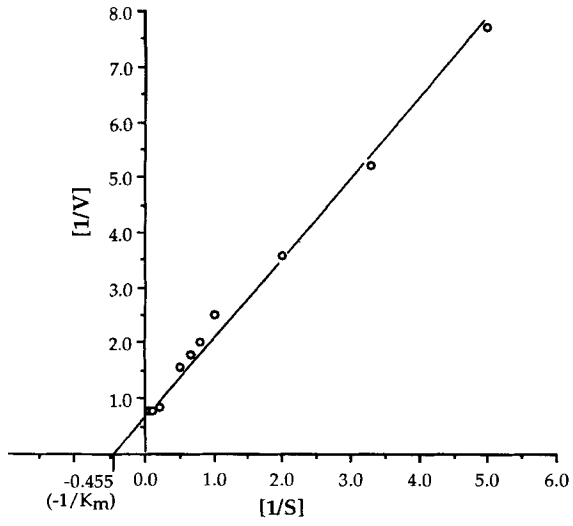


Figure 3. Lineweaver–Burk plot for partially purified pyruvate decarboxylase with pyruvate as a substrate at pH 6.0 and 25°C.

Table II. Comparison of initial kinetic parameters (initial rates determined over first 30 min) and final L-PAC concentration at various temperatures.

Kinetic values (m*M* min^{-1})	Temperature 4°C	10°C	25°C
$\frac{-ds}{dt}$ $AcCOO^-$	0.77	1.05	0.97
$\frac{-ds}{dt}$ PhCHO	0.36	0.45	0.16
$\frac{dp}{dt}$ L-PAC	0.35	0.44	0.14
$\frac{dp}{dt}$ acetoin	0.14	0.19	0.18
$\frac{dp}{dt}$ AcH	0.10	0.22	0.45
$[L\text{-PAC}]_{max}$ (m*M*)	37.7	34.1	22.0

Reactions were carried out with 70 m*M* benzaldehyde, 70 m*M* pyruvate, and 7 U/mL PDC enzyme in 40 m*M* phosphate buffer (pH 6.0) with 30 μ*M* TPP and 0.3 m*M* $MgSO_4 \cdot 7H_2O$. (pyruvate, $AcCOO^-$; benzaldehyde, PhCHO; and acetaldehyde, AcH).

Further comparison of biotransformation kinetics at 4°C and 25°C (Fig. 4) shows that in the first 3–4 h reaction L-PAC formation increased with time. Then due to depletion of pyruvate and possible product inhibition by acetaldehyde and L-PAC, the rate of L-PAC formation declined to zero after several hours.

Effect of pH on L-PAC Formation

The pH dependence of L-PAC and acetaldehyde formation at various pH values in the range of 4.0–8.0 was examined at 4°C. The initial reaction rates were measured with the universal citrate/sodium phosphate buffer (pH adjusted with varying mixture of stock solutions, 0.2 *M* citric acid/0.4 *M* disodium phosphate) over the first 30-min reaction. The results in Figure 5 show that the effect of pH on the rate of L-PAC formation differed from the pH dependence of acetaldehyde formation. The optimum pH for acetaldehyde formation was 6.0; the optimum for L-PAC formation was pH 7.0. It was evident that the PDC had a different pH optimum for the minor condensation reaction, thereby allowing a pH to be selected (viz. pH 7.0) which optimized L-PAC production and reduced acetaldehyde release at 4°C.

Effect of Acetaldehyde Inhibition

To understand more fully the most significant factors influencing the biotransformation process, the effect of acetaldehyde on L-PAC formation was evaluated with various initial concentrations of acetaldehyde. The results shown in Figure 6 indicate that the initial reaction rates for L-PAC

formation were significantly decreased with increasing acetaldehyde concentration. This indicates that the condensation reaction of "enzyme-bound acetaldehyde" with benzaldehyde is greatly affected by free acetaldehyde. Acetaldehyde has been reported by other authors to be a noncompetitive inhibitor of pyruvate decarboxylation.[12] It was found that as acetaldehyde concentration increased, the velocity of the reaction catalyzed by PDC rapidly decreased, even in the presence of an excess of pyruvate. Based on this evidence, Juni[15] suggested a two-site reaction in which pyruvate may be bound and decarboxylated at the first catalytic site and irreversibly transferred to the second site. At the first site, the acetaldehyde formed by decarboxylation and involved in the condensation reaction is described as active acetaldehyde. Acetaldehyde at the second active site can be reversibly released into the medium, and is described as "free acetaldehyde."

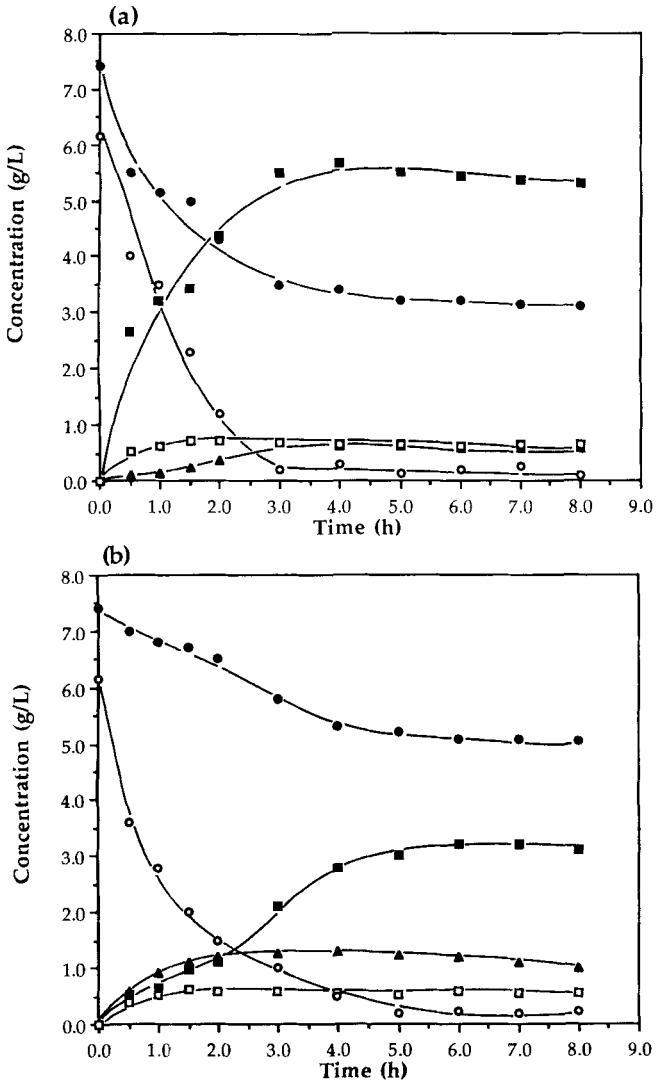


Figure 4. Biotransformation kinetics at (a) 4°C and (b) 25°C. Reaction mixture contained 70 m*M* benzaldehyde, 70 m*M* sodium pyruvate, 7 unit/mL PDC, 30 μ*M* TPP, and 0.5 m*M* $MgSO_4 \cdot 7H_2O$ in 40 m*M* potassium phosphate buffer (pH 6.0): (●) benzaldehyde, (■) L-PAC, (▲) acetaldehyde, (□) acetoin, and (○) pyruvate.

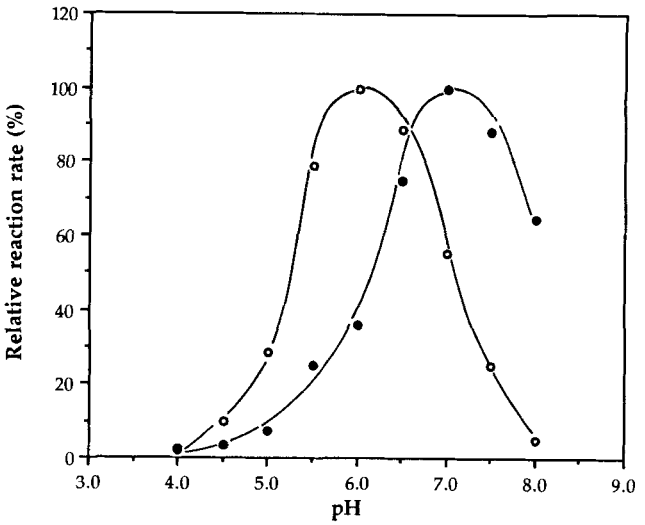


Figure 5. Comparison of effects of pH on relative reaction rates for (○) acetaldehyde and (●) L-PAC formation at 4°C: Reaction mixture contained 70 m*M* benzaldehyde, 70 m*M* sodium pyruvate, 7 unit/mL PDC, 30 μ*M* TPP, and 0.5 m*M* $MgSO_4 \cdot 7H_2O$ in the universal citrate/sodium phosphate buffer at various pH.

Effect of Ethanol on L-PAC Formation

The limited solubility of benzaldehyde (0.3 g/100 mL water) may reduce substrate availability, and it is relevant therefore to evaluate the use of an organic solvent to improve benzaldehyde solubility and the rate of L-PAC production. Other authors[8,14,25] showed that PDC possesses highly hydrophobic substrate binding sites; therefore, the interaction of substrate molecules such as benzaldehyde at these active sites is likely to be important for L-PAC formation. The selection of ethanol as a water miscible organic solvent was based on the following data: PDC has signifi-

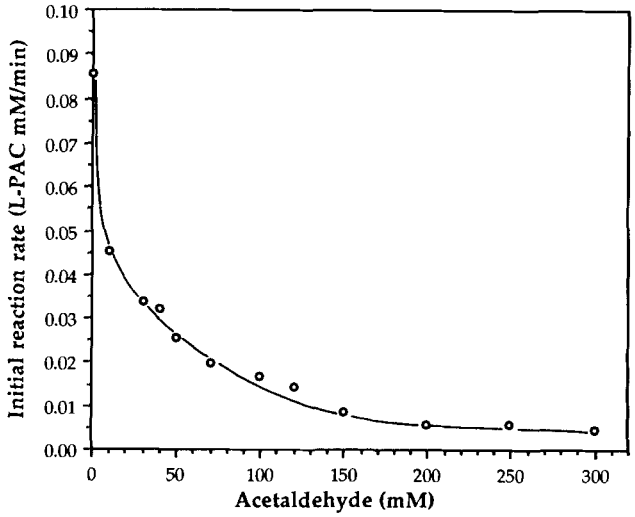


Figure 6. Effect of acetaldehyde on the initial rate for L-PAC formation. Reaction mixture contained 70 m*M* benzaldehyde, 70 m*M* sodium pyruvate, 7 unit/mL PDC, 30 μ*M* TPP, 0.5 m*M* $MgSO_4 \cdot 7H_2O$, and various concentrations of acetaldehyde in 40 m*M* potassium phosphate buffer (pH 7.0) at 4°C.

cant resistance to denaturation by ethanol up to 150 g/L,[23] ethanol is a less hydrophilic solvent than water,[10] and benzaldehyde is reported to have infinite solubility in ethanol.[22]

To evaluate the possible enhancing effect of ethanol, biotransformations were conducted in the presence of 0–6.0 *M* ethanol. This reaction mixture was incubated on a reciprocal shaker at 100 rpm and 4°C. Initial reaction rates were determined for the first 30 min.

As shown in Figure 7, the initial rate of L-PAC formation increased with increasing ethanol concentration up to 2.0–3.0 *M*. At 3.0 *M* ethanol, the initial rate was 1.4 times higher than that in the absence of ethanol. A further increase in ethanol concentration resulted in significantly decreased reaction rates. These results are in agreement with a previous investigation of nonenzymatic decarboxylation with TPP, which established that the rate of decarboxylation was significantly increased in solvents less polar than water, with rates of decarboxylation in ethanol being 10^4 to 10^5-fold higher compared with the rates in water.[8]

Effect of PDC Activity on Biotransformation

The effect of PDC activity on L-PAC formation rates was determined from initial rates for L-PAC formation at various PDC activities from 4.0 to 10.0 U/mL over the first 30-min incubation. The reactions were carried out with various concentrations of benzaldehyde and equimolar ratios of pyruvate in 40 m*M* potassium phosphate buffer (pH 7.0) containing 2.0 *M* ethanol at 4°C.

As shown in Figure 8, the initial L-PAC formation rates were greatly dependent on PDC activity and benzaldehyde concentration. An approximately linear increase in initial rates for L-PAC formation was evident from 30 to 70 m*M* benzaldehyde. The highest L-PAC formation rates with 4.0, 5.0, and 7.0 unit/mL PDC occurred at 70, 100, and 120 m*M* benzaldehyde, respectively. Above 7.0 unit/mL PDC, the L-PAC formation rates were significantly increased with higher levels of benzaldehyde up to 120 m*M*.

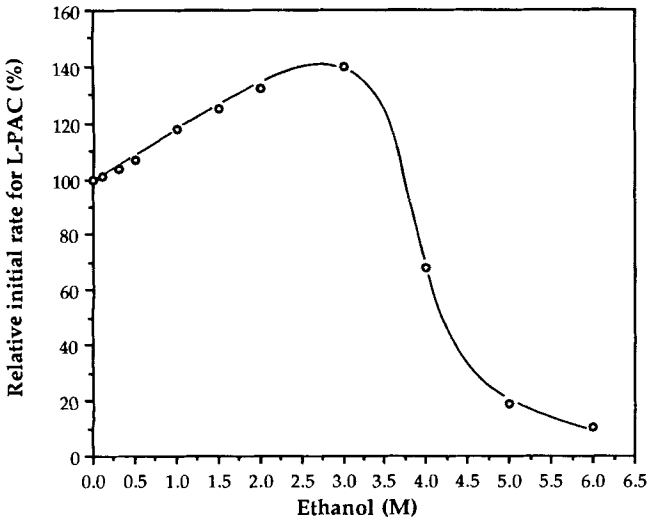


Figure 7. Effect of ethanol on initial rate for L-PAC formation at 4°C. Reaction mixture contained 70 m*M* benzaldehyde, 70 m*M* sodium pyruvate, 7 unit/mL PDC, 30 μ*M* TPP, and 0.5 m*M* $MgSO_4 \cdot 7H_2O$ in 40 m*M* potassium phosphate buffer (pH 7.0).

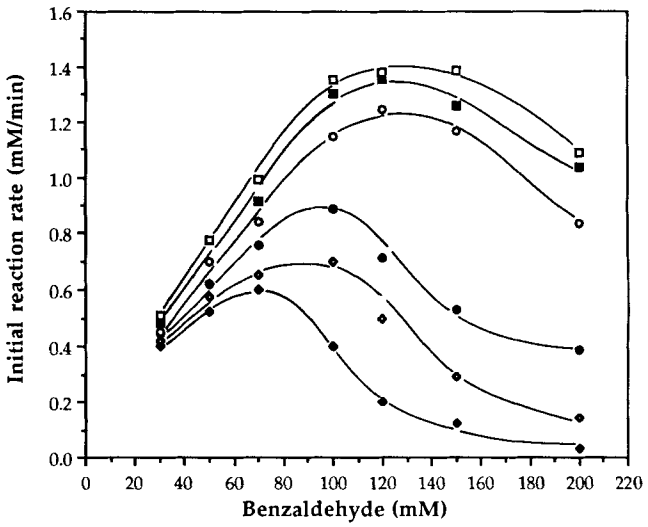


Figure 8. Effect of PDC activity (unit/mL) on initial rate for l-PAC formation in the presence of various concentrations of benzaldehyde and equimolar concentrations of sodium pyruvate in 40 m*M* potassium phosphate buffer (pH 7.0) containing 30 μ*M* TPP, 0.5 m*M* $MgSO_4 \cdot 7H_2O$, and 2.0 *M* ethanol. The initial rates were determined within 30 min at 4°C. The symbols refer to PDC activities of (♦) 4.0, (◇) 5.0, (●) 6.0, (○) 7.0, (■) 8.2, and (□) 10.0 unit/mL.

It is clear that the higher up to 120 m*M* activities of PDC resulted in higher reaction rates for a wide range of benzaldehyde concentrations. This was due presumably to the high initial conversion rates with high PDC activity which resulted in a rapid decrease in the concentration of benzaldehyde and a consequent reduction in its toxic effects. However, after achieving a maximum L-PAC formation rate, the toxic effect of further increasing benzaldehyde was evident. It was dependent also on initial PDC activity.

Increasing PDC activity per unit volume did not correlate directly with increasing final L-PAC formation. Higher PDC activity was accompanied also by more acetaldehyde formation; low PDC activity resulted in incomplete biotransformation at high benzaldehyde concentrations (Table III). A PDC activity of about 7 unit/mL was optimal for efficient biotransformation.

Determination of K_m for Benzaldehyde

As described previously, the rate of L-PAC formation could be significantly affected by various factors, viz. temperature, pH, addition of ethanol, PDC activity, and substrate concentrations. For a typical set of biotransformation conditions, the affinity constant (K_m) for benzaldehyde was determined by measuring the initial rate for L-PAC formation at 4°C over the first 30 min.

As the results show in Figure 9, the initial reaction rate increased with increased benzaldehyde concentration up to

Table III. Final L-PAC and acetaldehyde concentrations with various PDC activities.

PDC activity (unit/mL)	L-PAC (m*M*) [Benzaldehyde] = [Pyruvate] (m*M*) 30	50	100	150
4.0	19.0	34.8	48.2*	3.4*
5.0	20.1	34.8	64.5	30.0*
6.0	17.9	34.2	76.8	94.0
7.0	17.3	33.5	78.3	135.6
8.2	16.2	32.1	71.3	129.3
10.0	15.1	30.5	62.9	125.7

PDC activity (unit/mL)	Acetaldehyde (m*M*) [Benzaldehyde] = [Pyruvate] (m*M*) 30	50	100	150
4.0	6.1	9.5	2.3*	ND*
5.0	6.5	10.2	7.5	ND*
6.0	7.1	10.8	10.5	8.3
7.0	7.5	11.0	11.4	8.4
8.2	8.2	12.0	16.0	9.2
10.0	9.1	13.5	17.8	10.1

Performed in the presence of equimolar concentrations of benzaldehyde and sodium pyruvate at 4°C in 40 m*M* phosphate buffer (pH 7.0) containing 30 μ*M* TPP, 0.3 m*M* $MgSO_4 \cdot 7H_2O$, and 2.0*M* ethanol.

*Reaction did not proceed fully due to toxicity of benzaldehyde at low PDC activity.

ND, not determined.

180 m*M*. The K_m value was determined as 42 m*M* benzaldehyde, which compares well with other results in the literature for PDC from yeasts such as *S. carlsbergensis* (viz. 50 m*M*).[6] It is possible that the enhanced affinity of PDC toward benzaldehyde in the present study may be due to a combination of factors, viz. presence of ethanol, lower reaction temperature, optimal pH, selection of PDC from an efficient yeast for biotransformation, viz. *C. utilis*.

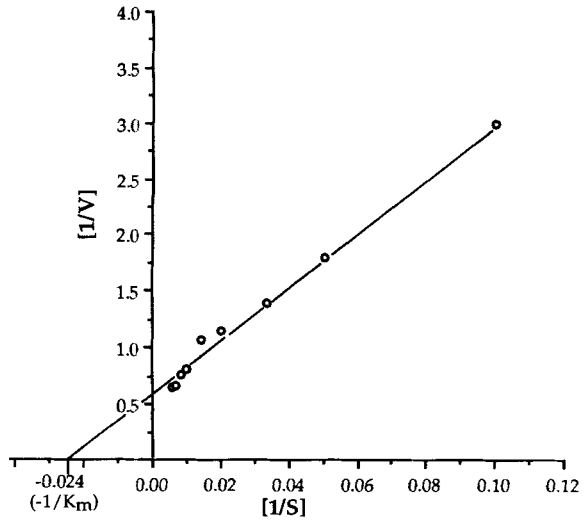


Figure 9. Lineweaver–Burk plot for biotransformation with partial purified PDC at various concentrations of benzaldehyde. Reaction mixture consisted of 40 m*M* phosphate buffer (pH 7.0) containing 7 unit/mL PDC, 200 m*M* sodium pyruvate, 30 μ*M* TPP, 0.5 m*M* $MgSO_4 \cdot 7H_2O$, 2.0 *M* ethanol, and various initial concentrations of benzaldehyde.

Effect of Molar Ratios of Pyruvate to Benzaldehyde on Final L-PAC Concentrations

The final conversion yields of L-PAC as a function of the molar ratios of pyruvate to benzaldehyde were evaluated at 4°C. Biotransformation was considered complete when L-PAC production stopped. The results presented in Figure 10 indicate that an increase in molar ratio was accompanied by an increased final L-PAC yield (based on benzaldehyde). However, the requirement for additional pyruvate to achieve higher conversion yields was dependent on the benzaldehyde concentration used. It was found that higher benz-

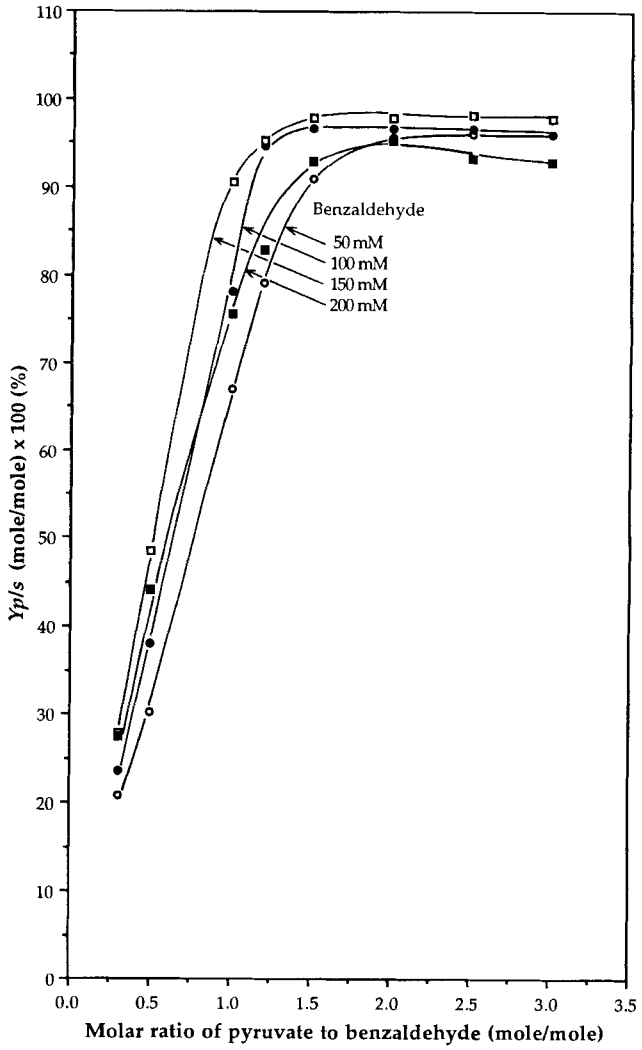


Figure 10. L-PAC yields (mol/mol %) based on benzaldehyde as a function of various molar ratios of pyruvate to benzaldehyde in the presence of various initial concentrations of benzaldehyde: (○) 50 m*M*, (●) 100 m*M*, (□) 150 m*M*, (■) 200 m*M*. Reaction mixture consisted of 7 unit/mL PDC, 30 μ*M* TPP, 0.5 m*M* $MgSO_4 \cdot 7H_2O$, 2.0 *M* ethanol, and various molar ratios of benzaldehyde to sodium pyruvate in 40 m*M* phosphate buffer (pH 7.0) at 4°C.

aldehyde levels required a lower molar ratio to achieve the maximum conversion. For instance, the molar ratio requirements with 50, 100, and 150 m*M* benzaldehyde to achieve over 90% molar yield were 1.5 (i.e., 75 m*M*), 1.2 (i.e., 120 m*M*), and 1.0 (i.e., 150 m*M*), respectively. A likely reason for this is that active acetaldehyde utilization is more effective at higher benzaldehyde concentrations, thereby minimizing release of free acetaldehyde.

Final L-PAC concentrations and yields based on pyruvate and benzaldehyde are summarized in Table IV. Although the highest biotransformation molar yield of 97.8% (based on initial benzaldehyde) was achieved using 150 m*M* benzaldehyde and 1.5 molar ratio, from these results, the highest concentration of 28.6 g/L L-PAC (190.6 m*M*) was obtained using 200 m*M* benzaldehyde and 2.0 molar ratio after 8-h incubation. Molar conversion yields close to 90% (based on initial pyruvate) were achieved only in the range of 150 m*M* to 200 m*M* benzaldehyde when equal or lower molar ratios were used. In other situations, a higher fraction of pyruvate was presumably converted to by-products (free acetaldehyde and acetoin) or remained in excess concentrations in the reaction mixture.

Effect of PDC Activity on L-PAC Formation with Various Molar Ratios of Pyruvate to Benzaldehyde

An attempt was made to achieve higher L-PAC concentrations by evaluating higher PDC activities and benzaldehyde concentrations. The biotransformations were carried out over the ranges of PDC activities of 7.0–20.1 unit/mL, 200–300 m*M* benzaldehyde, and 1.0–1.5 molar ratios of pyruvate to benzaldehyde at 4°C. The results shown in Table V indicate that although the higher levels of PDC activities can function at the higher substrate concentrations, the highest L-PAC formation did not exceed 27.9 g/L.

It was evident that higher benzaldehyde concentrations were required for higher L-PAC formation with increasing PDC activity. However, the higher PDC activities presumably resulted in increased free acetaldehyde which caused product inhibition and/or active acetaldehyde depletion.

Typical Biotransformation Kinetics with Partially Purified PDC

Detailed kinetics of a typical time course for a biotransformation with partially purified PDC shown in Figure 11

Table IV. L-PAC formation with various molar ratios of pyruvate to benzaldehyde at 4°C.

Benzaldehyde (m*M*)	Final Concentration of L-PAC (g/L) and molar conversion yields (%)							
	Molar ratio of pyruvate to benzaldehyde							
	0.3	0.5	1.0	1.2	1.5	2.0	2.5	3.0
50	1.6	2.3	5.0	5.9	6.8	7.2	7.2	7.2[a]
	69.3	60.5	67.1	65.9	60.6	47.8	38.4	32.0[b]
	20.8	30.2	67.1	78.1	90.9	95.7	96.0	96.0[c]
70	2.3	3.8	7.9	9.5	9.9	10.0	10.0	9.9
	72.8	72.0	75.2	75.1	62.8	47.6	38.1	31.4
	21.9	36.0	75.2	90.2	94.3	95.3	95.3	95.3
100	3.5	5.8	11.7	14.2	14.5	14.5	14.5	14.4
	78.4	77.0	78.2	78.3	64.5	48.4	38.6	32.0
	23.3	38.3	78.2	94.6	96.7	96.7	96.7	96.6
120	4.5	7.0	14.6	16.8	17.6	17.6	17.6	17.4
	83.3	77.2	81.1	7.60	65.2	48.9	39.0	32.0
	25.0	38.6	81.1	93.2	97.5	97.5	97.5	96.6
150	6.3	10.9	20.5	21.5	22.0	22.0	22.1	22.0
	92.8	97.0	90.6	79.6	64.9	48.9	39.3	32.5
	27.8	48.6	90.6	95.3	97.8	97.8	98.2	97.8
180	7.4	12.1	24.2	25.1	25.5	25.5	25.0	25.1
	91.8	89.5	90.0	77.5	63.0	47.2	37.0	31.0
	27.2	44.4	90.0	93.0	94.4	94.4	92.6	93.0
200	8.3	13.3	22.7	24.9	27.9	28.6	28.0	28.0
	91.6	88.5	75.6	69.1	62.0	47.6	37.3	31.0
	27.5	44.3	75.6	82.9	93.0	95.3	93.0	93.0

The reaction mixture consisted of 40 m*M* phosphate buffer (pH 7.0) containing 7 U/mL PDC, 30 μ*M* TPP, 0.3 m*M* $MgSO_4 \cdot 7H_2O$, 2.0*M* ethanol, and indicated concentrations of sodium pyruvate and benzaldehyde.

[a]L-PAC (g/L); expressed as g/L for comparison with literature values.

[b]Yp/s (mol L-PAC/mol pyruvate) × 100 (%).

[c]Yp/s (mol L-PAC/mol benzaldehyde) × 100 (%).

Table V. Effect of increased PDC activities and benzaldehyde concentrations on L-PAC formation with various molar ratios of pyruvate to benzaldehyde.

Benzaldehyde (m*M*)	Pyruvate to benzaldehyde (mol/mol)	Final L-PAC concentration (g/L) PDC activity (Unit/mL) 7.0	10.2	15.0	20.1
200	1.0	22.7	18.2	17.6	15.5
	1.2	24.9	20.7	15.9	15.3
	1.5	27.9	23.9	16.2	15.0
230	1.0	20.4	25.3	24.3	22.5
	1.2	25.6	24.8	25.6	23.2
	1.5	21.0	20.3	24.8	19.4
250	1.0	13.7	22.8	23.5	24.8
	1.2	10.7	20.3	20.8	25.6
	1.5	9.1	9.2	20.5	21.2
280	1.0	5.1	6.5	7.9	23.3
	1.2	5.0	6.3	8.1	22.1
	1.5	4.1	6.7	10.5	12.7
300	1.0	1.1	1.2	3.0	4.7
	1.2	1.2	1.3	2.8	4.7
	1.5	1.0	1.7	2.7	4.5

Reaction conditions were specified in Table IV.

demonstrate the time dependence of simultaneous L-PAC, acetaldehyde, and acetoin formation together with biotransformation of pyruvate and benzaldehyde. In the first 2–3 h, L-PAC formation increased rapidly, while further L-PAC formation occurred more slowly and was influenced by substrate depletion and possible product inhibition. After 6-h incubation giving a maximum L-PAC of 147 m*M* (22 g/L), a mass balance on pyruvate indicated that of the original 225 m*M* pyruvate, 147 m*M* pyruvate contributed to L-PAC formation, 25 m*M* pyruvate was converted to acetoin, and 22 m*M* pyruvate was converted to free acetaldehyde. The residual pyruvate was about 30 m*M* indicating a closing mass balance based on pyruvate. The mass balance based on benzaldehyde conversion to L-PAC closed to within 2% with complete utilization of 150 m*M* benzaldehyde. The small mass balance discrepancy is due to some evaporation losses of products and/or minor experimental error. In addition after 6-h incubation, 20–30% of initial PDC activity still remained, indicating the potential for further biotransformation if more benzaldehyde were available.

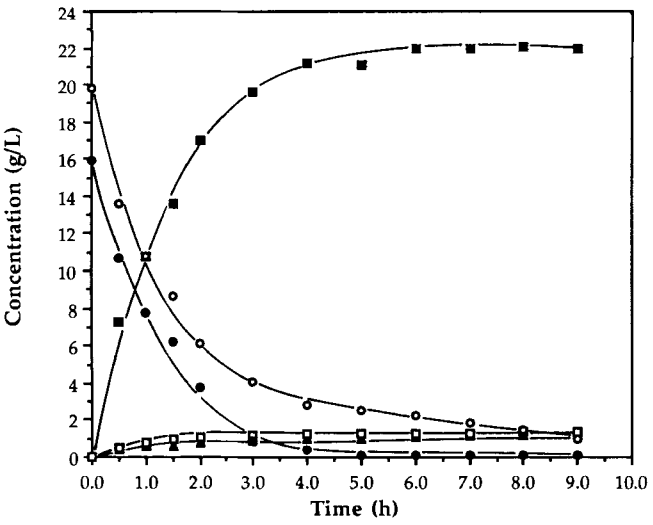


Figure 11. Biotransformation kinetics. Reaction mixture consisted of 40 m*M* potassium phosphate buffer (pH 7.0) containing 150 m*M* benzaldehyde, 225 m*M* sodium pyruvate, 30 μ*M* TPP, 0.5 m*M* $MgSO_4 \cdot 7H_2O$, 2.0 *M* ethanol, and 7 unit/mL PDC at 4°C: (●) benzaldehyde, (■) L-PAC, (▲) acetaldehyde, (□) acetoin, and (○) pyruvate.

DISCUSSION AND CONCLUSIONS

From the present evaluation it is evident that relatively high concentrations and yields of L-PAC can be achieved under optimal conditions using partially purified pyruvate decarboxylase from *C. utilis*. For example at pH 7.0 and 4°C, with an initial concentration of 200 m*M* benzaldehyde (20.8 g/L), a concentration of 190.6 m*M* L-PAC (28.6 g/L) can be achieved with a yield in excess of 95% based on benzaldehyde. Pyruvate excess is required to maximize yields, with a molar ratio (pyruvate:benzaldehyde) of 1.2–1.5 sufficient to meet pyruvate requirements under optimal conditions. Acetoin and acetaldehyde are the by-products resulting from this excess pyruvate utilization.

The results with partially purified PDC compare with previously published data using either free or immobilized yeast cells. In the former case, L-PAC concentrations of 10–12 g/L were reported,[9,27] for the latter, 10 g/L was obtained with immobilized *S. cerevisiae*.[20] The yields based on benzaldehyde were normally in the range 60–70%, with significant diversion of benzaldehyde to benzyl alcohol.

A further interesting point that emerges is that benzaldehyde appears to have a much more significant denaturation effect on the PDC when it is in vivo within the yeast by comparison to its denaturation of the partially purified PDC. For example, the purified PDC can carry out the biotransformation at benzaldehyde concentrations of 20–30 g/L; for both *S. cerevisiae* and *C. utilis* the maximum benzaldehyde concentration for biotransformation is about 5 g/L. Cell growth stops at 1–1.5 g/L.[18] The reason for this lies in the multiple effects which benzaldehyde has on the yeast including altered cell wall permeability and denaturation of enzymes involved in pyruvate synthesis, as well as its direct effect on PDC. In addition, selection of optimal buffer conditions and cofactor concentrations for the partially purified PDC also serves to minimize the denaturation effects.

By comparison with free or immobilized yeasts, the partially purified enzyme system clearly offers advantages in terms of higher L-PAC concentrations and yields, with easier product recovery resulting from the absence of aromatic by-products. Further assessment is necessary of an immobilized PDC system to determine its potential for scale-up and continuous or semicontinuous operation. The disadvantages of the partially purified enzyme system compared to a

yeast biotransformation process lie in the costs of enzyme recovery and purification, together with the additional costs of provision of one of the substrates (viz. pyruvate). With free or immobilized yeast cells, no pyruvate addition is required as sufficient pyruvate is produced by the fermentative metabolism of glucose. Ultimately the selection of one mode of biotransformation process in preference to another will depend on a detailed economic assessment based on the type of data obtained in this evaluation.

Financial support is gratefully acknowledged for one of the authors (Dr. H. S. Shin) under the Korea–Australia Postgraduate Research Scholarship Program.

References

1. Astrup, A., Breum, L., Toubro, S., Hein, P., Quaade, F. 1992. The effect and safety of an ephedrine-caffeine compound compared to ephedrine, caffeine and placebo in obese subjects on an energy restricted diet: A double blind trial. Int. J. Obesity **16**: 269–277.
2. Astrup, A., Buemann, B., Christensen, N. J., et al. 1992. The effect of ephedrine/caffein mixture in energy expenditure and body composition in obese women. Metabolism **41**: 686–688.
3. Bergmeyer, H. U., Grassl, M., Walter, H. E. 1983. Alcohol dehydrogenase from yeast. pp. 139–141 In: H. U. Bergmeyer (ed.), Methods of enzymatic Analysis, 3rd, edition, Vol. II. VCH, Weinheim.
4. Boiteux, A., Hess, B. 1970. Allosteric properties of yeast pyruvate decarboxylase. FEBS Lett. **9**: 293–296.
5. Bradford, M. M. 1970. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing, the principle of protein-dye binding. Anal. Biochem. **72**: 248–254.
6. Bringer–Meyer, S. Sahm, H. 1988. Acetoin and phenylacetylcarbinol formation by the pyruvate decarboxylase of *Zymomonas mobilis* and *Saccharomyces carlsbergensis*. Biocatalysis **1**: 321–331.
7. Bringer–Meyer, S., Schimz, K. L., Sahm, H. 1986. Pyruvate decarboxylase from *Zymomonas mobilis*. Isolation and partial characterization. Arch. Microbiol. **146**: 105–110.
8. Crosby, J., Stone, R., Lienhard, G. E. 1970. Mechanisms of thiamine-catalysed reactions, decarboxylation of 2-(1)-carboxyl-1-hydroxyethyl-3,4-dimethylthiazolium chloride. J. Am. Chem. Soc. **92**: 2891–2900.
9. Čulič, J., Netrval, J., Ulbrecht, S., Souhrada, J., Vojtišek, V., Vodnansky, M. 1984. Method of the cost reduction in the production of D-(−)-1-phenyl-1-hydroxy-2-propanone for the preparation of L-(−)ephedrine. Czech Patent 222,941 (CI. C07C 49/24).
10. Godfrey, N. B. 1972. Solvent selection via miscibility number. Chemtech **2**: 359–363.
11. Gounaris, A. D., Turkenkopf, I., Buckwald, S., Young, A. 1971. Pyruvate decarboxylase: 1. Protein dissociation into subunits under conditions in which thiaminepyrophosphate is released. J. Biol. Chem. **246**: 1302–1309.
12. Gruber, M., Wassenaar, J. S. 1960. Inhibition of yeast carboxylase by acetaldehyde. Biochim. Biophys. Acta **38**: 355–357.
13. Hopmann, R. F. W. 1980. Hydroxyl-ion-induced subunit dissociation of yeast cytoplasmic pyruvate decarboxylase. A circular dichroism study. Eur. J. Biochem. **110**: 311–318.
14. Jencks, W. P. 1975. Binding energies, specificity and enzymatic catalysis: The Circe effect. pp. 250–251 In: A. Meister (ed.), Advances in enzymology and related areas of molecular biology, Vol. 43. Wiley, New York.
15. Juni, E. 1961. Evidence for a two site mechanism for decarboxylation of α-keto acid by α-carboxylase. J. Biol. Chem. **236**: 2302–2308.
16. Lehmann, H., Fischer, G., Hübner, G., Kohnert, K. D., Schellenberger, A. 1973. The influence of steric and electronic parameters on the substrate behaviour of α-oxo acids to yeast pyruvate decarboxylase. Eur. J. Biochem. **32**: 83–87.
17. Long, A., Ward, O. P. 1989. Biotransformation of aromatic aldehydes by *Saccharomyces cerevisiae:* Investigation of reaction rates. J. Ind. Microbiol. **4**: 49–53.
18. Long, A., Ward, O. P. 1989. Biotransformation of benzaldehyde by *Saccharomyces cerevisiae:* Characterization of the fermentation and toxicity effects of substrates and products. Biotechnol. Bioeng. **33**: 933–941.
19. Mahmoud, W. M., El-Sayed, A. M. M., Coughlin, R. W. 1990. Production of L-phenylacetylcarbinol by immobilized yeast cells: I. Batch Fermentation. Biotechnol. Bioeng. **36**: 47–54.
20. Mahmoud, W. M. El-Sayed, A. M. M., Coughlin, R. W. 1990. Production of L-phenylacetylcarbinol by immobilized yeast cells: II. Semicontinuous Fermentation. Biotechnol. Bioeng. **36**: 55–63.
21. Nikolova, P., Ward, O. P. 1991. Production of L-phenylacetylcarbinol by biotransformation: Product and by-product formation and activation of the key enzymes in wild-type and ADH isoenzyme mutants of *Saccharomyces cerevisiae*. Biotechnol. Bioeng. **38**: 493–498.
22. Perry, R. H. 1984. Perry's chemical engineering handbook, 6th ed, McGraw–Hill International Editions, Chemical Engineering Series, pp. 3–27. New York.
23. Scopes, R. K. 1989. Effect of ethanol on glycolytic enzymes. pp. 89–100 In: N. van Unden (ed.), Alcohol toxicity in yeast and bacteria. CRC Press Inc., Boca Raton, FL.
24. Ullrich, J. 1985. Thiamine pyrophosphate. pp. 457–467 In: H. U. Bergmeyer (ed.), Methods of enzymatic analysis, 3rd ed, Vol. VII. VCH, Weinheim.
25. Ullrich, J., Donner, I. 1970. Fluorimetric study of 2-*p*-toluidinonaphthalene-6-sulphonate binding to cytoplasmic yeast pyruvate decarboxylase. Hoppe–Seyler's Z. Physiol. Chem. **351**: 1030–1034.
26. Urk, H., Schipper, D., Breedveld G. J., Paul, R. M., Scheffers, W. A., van Dijken, J. P. 1989. Localization and kinetics of pyruvate-metabolizing enzymes in relation to aerobic alcohol fermentation in *Saccharomyces cerevisiae* CBS 8066 and *Candida utilis* CBS 621. Biochim. Biophys. Acta **992**: 78–86.
27. Vojtisek, V., Netrval, J. 1982. Effect of pyruvate decarboxylase activity and pyruvate concentration on the production of L-hydroxy-1-phenylpropanone in *Saccharomyces carlsbergensis*. Folia Microbiol. **27**: 173–177.
28. Westerfeld, W. W. 1945. A colorimetric determination of blood acetoin. J. Biol. Chem. **161**: 495–502.
29. Zehender, H., Trescher, D., Ullrich, J. 1987. Improved purification of pyruvate decarboxylase from wheat germ. Its partial characterization and comparison with the yeast enzyme. Eur. J. Biochem. **167**: 149–154.