Biotransformation for L-Ephedrine Production

P.L. Rogers, H.S. Shin and B. Wang. Department of Biotechnology, University of New South Wales, Sydney 2052, Australia

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L-ephedrine is widely used in pharmaœutical preparations as a decongestant and anti-asthmatic compound. One of the key intermediates in its production is L-phenylacetylcarbinol (L-PAC) which can be obtained either from plants (*Ephedra sp*.), chemical synthesis involving isolation of a racemic mixture, or by biotransformation of benzaldehyde using various yeasts.

In the present review, recent significant improvements in the microbial biotransformation are assessed for both fed-batch and continuous processes using free and immobilized yeasts. From previous fed-batch culture data, maximal levels of L-PAC of 10-12 g L^{-1}were reported with yields of 55-60% theoretical based on benzaldehyde. However, recently concentrations of more than 22 g L^{-1} have been obtained using a wild-type stain of *Candida utilis*. This has been achieved through optimal control of yeast metabolism (via microprocessor control of the respiratory quotient, RQ) in order to enhance substrate pyruvate production and induce pyruvate decarboxylase (PDC) activity. Processes involving purified PDC have also been evaluated and it has been demonstrated that L-PAC levels up to 28 g L^{-1} can be obtained with yields of 90-95% theoretical based on the benzaldehyde added. In the review the advantages and disadvantages of the various strategies for the microbial and enzymatic production of the L-PAC are compared.

In view of the increasing interest in microbial biotransformations, L-PAC production provides an interesting example of enhancement through on-line control of a process involving both toxic substrate (benzaldehyde) and end-product (L-PAC, benzyl alcohol) inhibition.

1 Introduction

The transformation of organic compounds using biocatalysts - enzymes, cell organelles or whole cells are important processes in organic synthesis, and have been widely used in the production of steroids, antibiotics, vitamins and other high-value products (1). The advantages of such biotransformations are that they are reaction-specific processes (e.g. condensation, hydrogenation, esterification, decarboxylation, etc.), and can involve high degrees of regio- and stereo specificity. In addition, in most cases relatively mild reaction conditions are used (2). As well as these advantages some problem areas have been identified. These include low substrate and product solubilities in the aqueous phase, substrate and product toxicities, together with low yields and low productivities particularly with whole cell biotransformation. As a result there has been considerable recent research on the enhancement of biotransformation kinetics by the use of two phase systems (3,4,5), biotransformation in a 'micro-aqueous' environment in the presence of an organic solvent (6,7,8), the use of reverse micelles (9,10) and the application of supercritical fluid extraction for product removal (11,12).

Microbial biotransformation for the production of biologically active chiral compounds (L-form), which are important building blocks in the pharmaceutical industry, is a field which has grown significantly in recent times. This has been largely due to the toxicological problems which have been encountered with the pharmaceutical use of racemic mixtures (13).

In the present review, various strategies are assessed for a biotransformation process which involves the conversion of benzaldehyde to L-phenylacetylcarbinol (L-PAC), an intermediate in the production of L-ephedrine and related psuedoephedrines. This can be considered as an interesting biotransformation process for evaluation, as it includes both toxic substrate and end-product inhibition.

L-ephedrine is a natural plant alkaloid isolated originally from the dried young branches of Ephedra, a plant with interesting pharmacological activities. Extracts of Ephedra sp., particularly Ephedra sinica, E.equisetina and E.distachya commonly called 'Ma Huang' in China, have been used for several thousand years as folk remedies for inducing sweat, soothing breath and easing excretion of urine. The active ingredient of these extract, L-ephedrine, was first isolated in 1855, and international interest in this drug was stimulated by the classical investigations of Chem & Schmidt in 1930, who reported on its cardiovascular effects and its similarity to epinephrine (14)

Ephedrine, known chemically as 1-methylamino-ethyl-benzyl alcohol or 2-methylamino-l-phenyl-l-propanol, contains two asymmetric carbon atoms, so that there are four optically active forms (Fig. 1), of which the (L-) isomers are used clinically. These four isomers occur naturally in Ephedra plants, and can be extracted with alcohol and benzene. Purified ephedrine is obtained as odourless, colourless crystals or as a white crystalline powder with bitter taste.

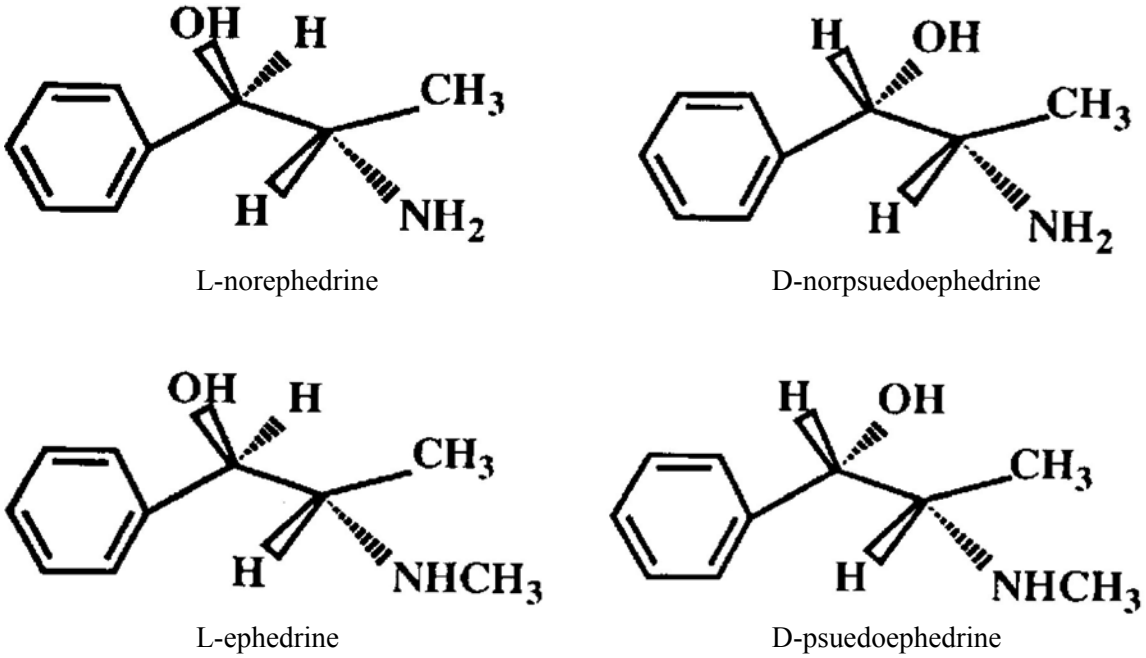

Fig. 1. The Structural formulae for ephedrine and its analogs (15)

Ephedrine and pseudoephedrine are very stable. A solution of ephedrine hydrochloride sealed for six years showed neither oxidation nor loss of activity (15, 16). Its main use pharmacologically is as a decongestant or anti-asthmatic compound, although recent reports have indicated its potential in obesity control (17).

Three production methods have been used for L-ephedrine: traditional extraction from plant species of *Ephedra,* a synthetic chemical process involving resolution of the racemic mixture, and a process which involves the biotransformation of benzaldehyde to L-PAC by various species of yeasts followed by reductive animation (18, 19).

The biotransformation process which involves the condensation of an ‘active benzaldehyde' (from pyruvic acid) and benzaldehyde is shown in Fig. 2. The production of the L-PAC is catalysed by the enzyme pyruvate decarboxylase (PDC), and can be associated with benzyl alcohol by-product formation, due to the activity of alcohol dehydrogenase(s) (ADH) and/or other non-specific oxidoreductases. Some traces of benzoic acid as a by-product have also been reported.

Previous studies have reported concentrations if 10-12 g L^{-1} L-PAC in batch culture with free cells (20, 21) and with immobilized yeast (22, 23). The addition of cyclodextrins (particularly β-cyclodextrin) was shown to enhance L-PAC production with immobilized *Saccharomyces cerevisiae* (24) although again maximal concentrations of 12 g L^{-1} L-PAC were reported. Evaluation of a large number of yeast strains by Netrval and Vojtisek (19) identified strains of *Saccharomyces*, *Candida* and *Hansenula* sp. which were capable of significant L-PAC production in shake-bask cultures (up to a maximum of 6.3 g L^{-1}). Strain improvement studies to develop acetaldehyde and L-ephedrine resistant mutants have been reported by Seely et al. (25) with mutants capable of increased L-PAC levels compared to wild-type strains. However, maximal L-PAC levels did not exceed 10 g L^{-1}.

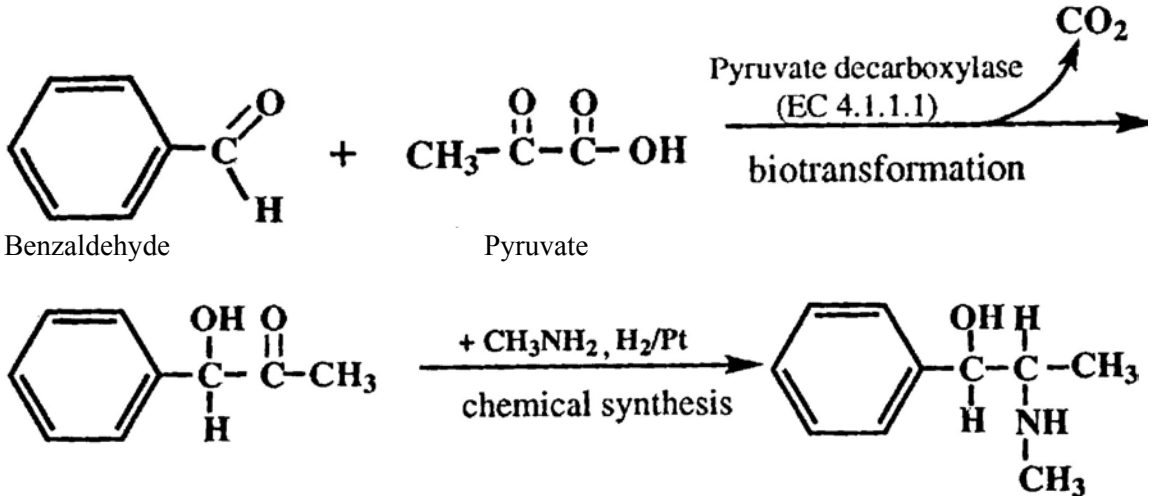

Fig.2. Mechanism of L-PAC and ephedrine production

In further fundamental investigations into the biotransformation of aromatic substrates by yeasts, other authors have indicated that oxidoreductases other than alcohol dehydrogenates may be involved in benzyl alcohol by-product formation (26-31).

Current commercial practice involves a fed-batch process with fermentative yeast growth on sugars to produce biomass, pyruvic acid and induce PDC activity. The growth phase is followed by biotransformation, with the further addition of sugars and the programmed feeding of benzaldehyde to maximise L-PAC production. Cessation of L-PAC formation occurs as a result of the following factors acting either together or independently:

(a) reduction of PDC activity due to benzaldehyde inhibition;
(b) pyruvic acid limitation at end of biotransformation phase;
(c) cell viability loss due to extended exposure to benzaldehyde and/or increasing concentrations of benzyl alcohol and L-PAC.

2 Factors Affecting L-phenylacetylcarbinol (L-PAC) Production

2.1 Pyruvate Decarboxylase (PDC) Activity

Although L-PAC production depends on PDC activity, little research has been published on the induction and deactivation of this enzyme during the biotransformation process. In our research. studies have been carried out or a strain of *Candida utilis* selected for its ability to tolerate benzaldehyde and produce relatively high levels of L-PAC. The yeast has been grown in batch culture under partial fermentation conditions to produce biomass and also induce PDC activity.

As shown in Fig. 3, after 18-20 h the PDC activity reached a level of 0.9 U mg protein $^{-1}$, while the ADH activity was 1.4 U mg protein $^{-1}$. The fermentative nature of the metabolism was illustrated by ethanol accumulation of 35 g L^{-1}. As indicated earlier, the enhanced PDC activity should be conducive to rapid L-PAC production, while the higher ADH activity may result in more
benzyl alcohol.

2.2 Metabolic Status of Yeast

In a study to develop strategies for on-line control of the L-PAC process, the effect of the respiratory quotient (RQ) on the production of L-PAC has been investigated by our group. An RQ value of 1.0 for growth on glucose corresponds to full respiratory growth while RQ values greater than 1.0 indicate fermentative metabolism. Increasing fermentation results in increasing RQ vales. Data using cells of *C.utilis* grown in continuous culture at controlled RQ values of 1.0, 1.9 and 4.4 are shown in Fig. 4. The biotransformations were carried out in shake flasks at 30^0C, pH = 6.0 at various initial concentrations of benzaldehyde (0.5-4.0 g L^{-1}). Initial specific rates L-PAC ($_{qPAC}$) and benzyl alcohol production ($_{qBA}$) were estimated from the kinetic data. From Fig. 4 it is evident that full respiratory growth (RQ = 1.0) resulted in a low specific rate of L-PAC and a high specific rate of benzyl alcohol production. Increasing RQ had a significant effect in improving L-PAC production rates and yields (65-70% theoretical at higher RQ values). For the biotransformation process an RQ value of 4-5 was optimal for the yeast growth phase as this corresponded to an RQ which produced sufficient yeast biomass for rapid biotransformation, while enhancing L-PAC production rates and yields. Through the use of on-line gas analysers for O_2 (paramagnetic) and CO_2 (infrared) measurements, interfaced to a microprocessor for calculation of RQ and subsequent stirrer speed adjustment, an effective feedback control strategy for RQ has been developed.

2.3 Benzaldehyde Concentration

The effect of benzaldehyde on L-PAC production by *S.cerevisiae* has been investigated by Gupta et al. (32) and Agarwal et al. (33). The latter authors reported that once the benzaldehyde concentration increased above 16 mM (1.7 g L^{-1}), the species rate of L-PAC production decreased, and beyond 20 mM (2.1 g L^{-1}) it was inhibited completely. When the residual benzaldehyde declined below 4 mM (0.4 g L^{-1}), the formation of benzyl alcohol was predominant over L-PAC. They estimated that the optimum benzaldehyde concentration range
was 4-16 mM. Further studies (34) cited the optimum concentration for L-PAC
production as 10 mM (1.1 g L^{-1}).

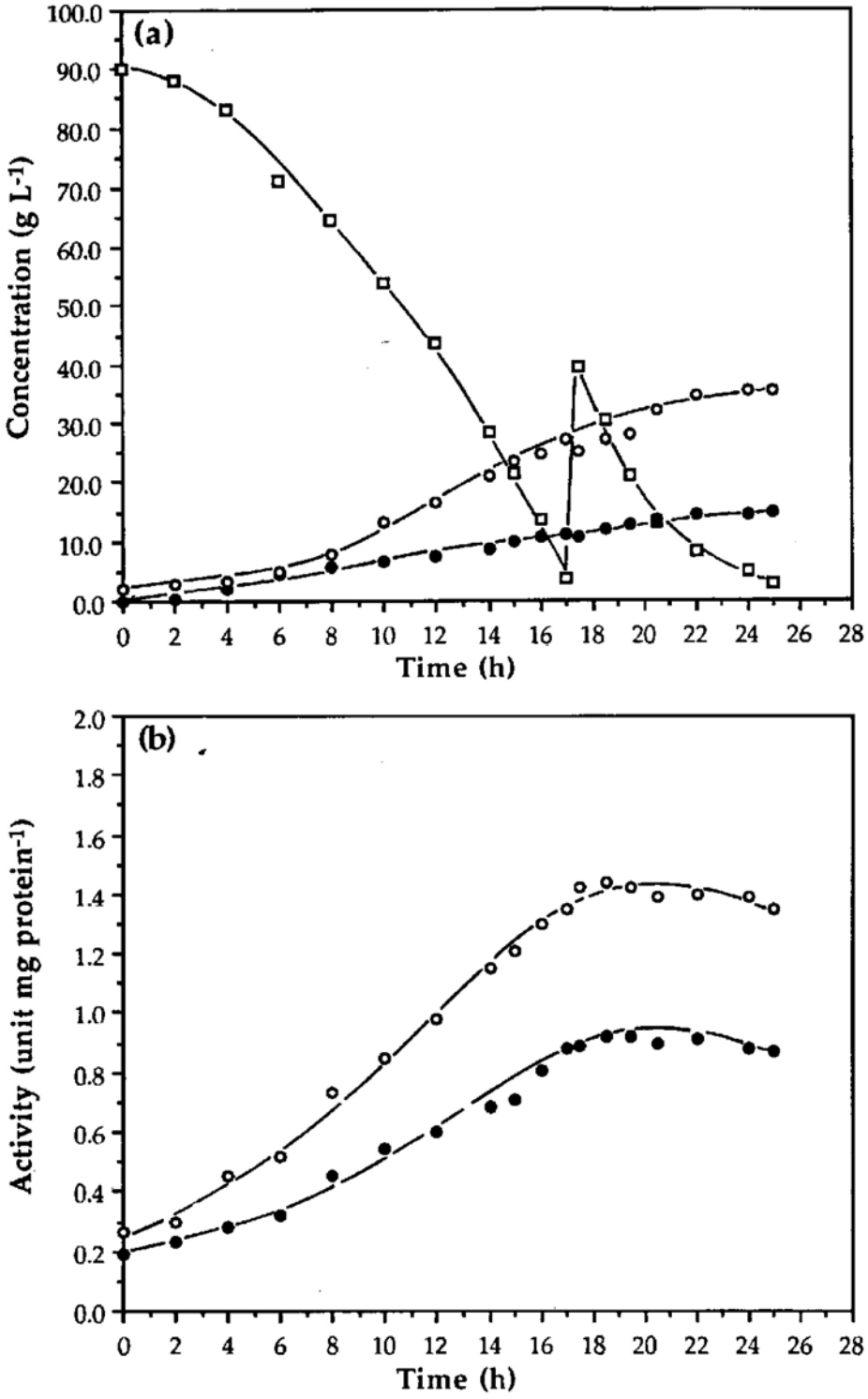

Fig. 3. Kinetics of growth and enzyme profiles of *Candida utilis*. **a** Cell growth: (●) biomass, (O) ethanol, (□) glucose. **b** Enzyme profiles: (●) PDC, (O) ADH

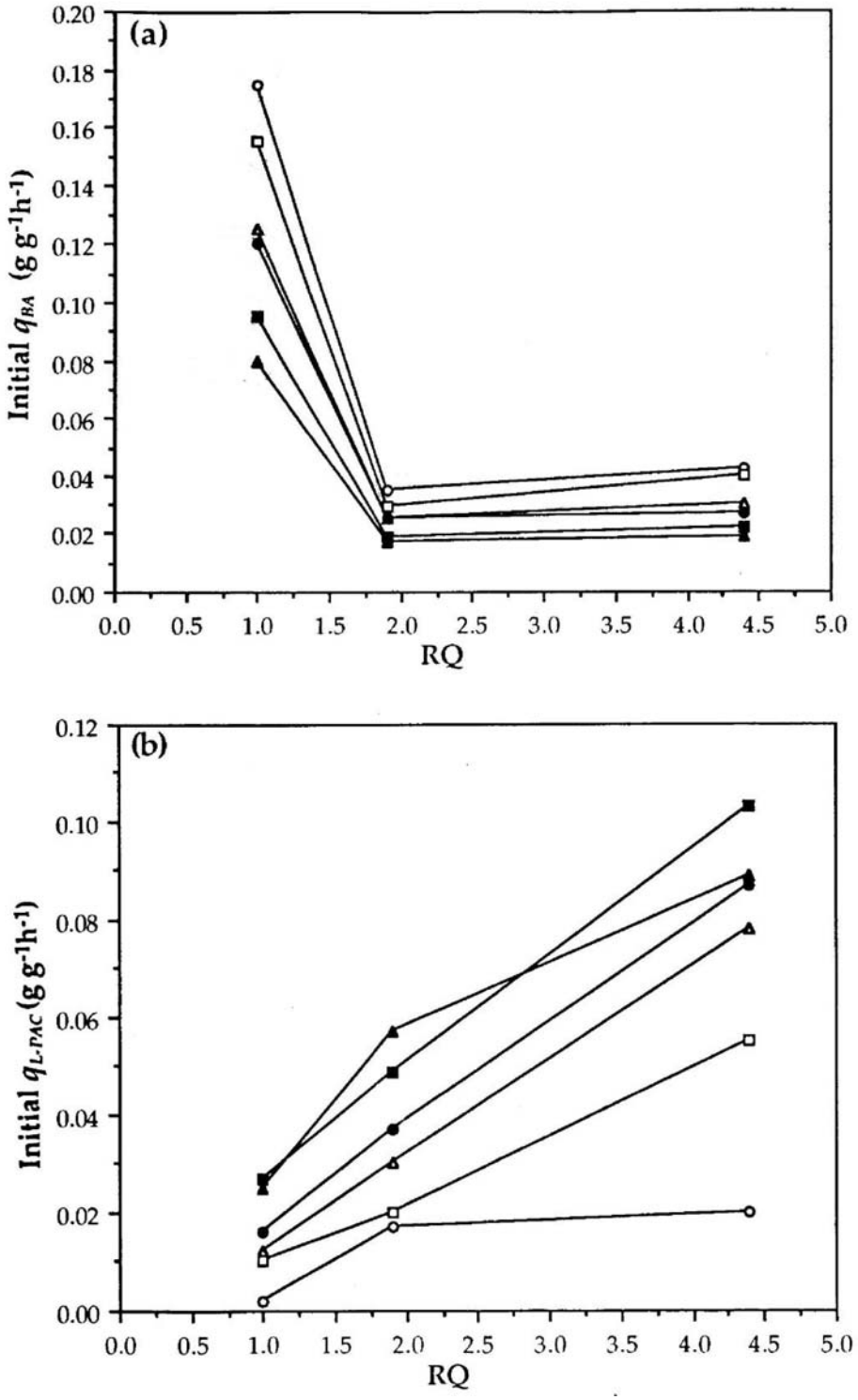

Fig 4a-c. Effect of RQ and benzaldehyde concentrations on **a** initial specific rate of L-PAC ($_{qPAC}$) production, **b** specific rate of benzyl alcohol ($_{qBA}$) productions, and **c** final L-PAC conversion efficiency. Benzaldehyde concentrations: (o) 0.5, (□) 1.0, (△) 1.5, (●) 2.0, (■) 3.0, (▲) 4.0 g L^{-1}

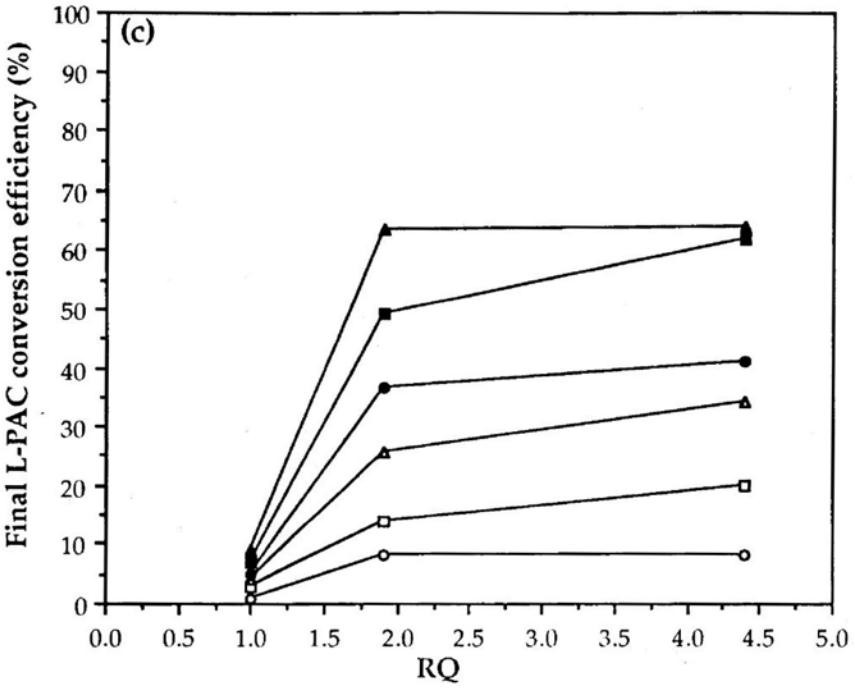

Fig. 4. (Continued)

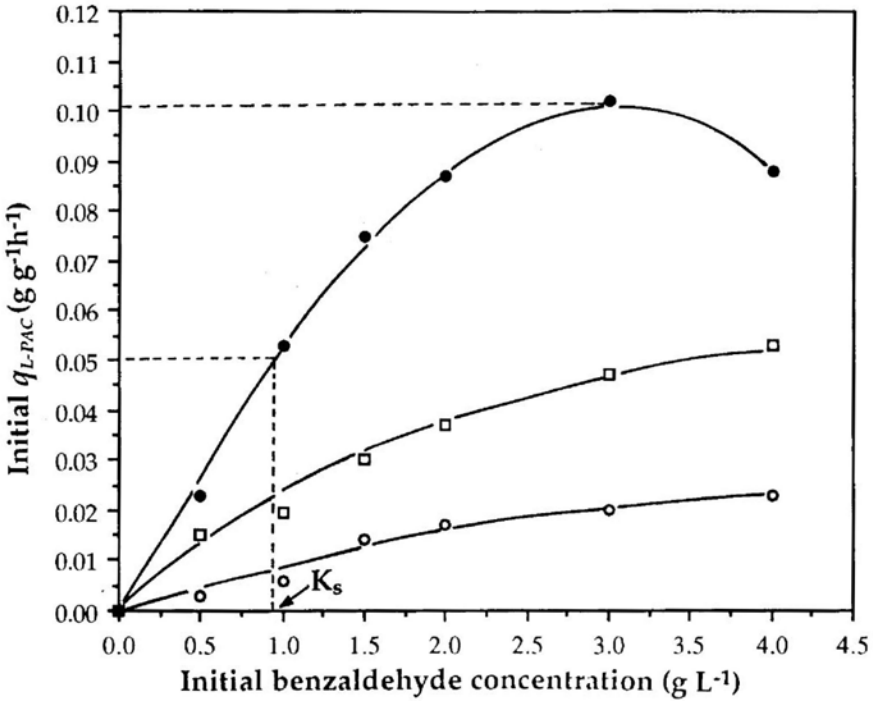

Fig. 5. Effect of benzaldehyde concentration on the specific rate of L-PAC production by *C.utilis.* Cells grown in continuous culture at T = 30^{0}C, pH = 5.2, D = 0.15 h $^{-1}$ and RQ values (o) 1.0, (□) 1.9, (●) 4.4

In recent studies by our group it has been found with *C.utilis* that cessation of growth occurred for benzaldehyde concentrations above 1.0 g L^{-1}. A growth inhibition constant for benzaldehyde was estimated at 0.30 g L^{-1} (unpublished data). Using initial rate analysis, the effect of benzaldehyde on L-PAC production has been investigated. An analysis of the data for a range of initial benzaldehyde concentrations (0.5--4.0 g L^{-1}) is shown in Fig. 5. From the analysis. the maximum specific rate of L-PAC production ($_{qPAC}$) was estimated as 0 10 gg^{-1} h^{-1} for RQ = 4.4, the Ks value (saturation constant) was 1.0 g L^{-1} benzaldehyde and concentrations of benzaldehyde in excess of 3.0 g L^{-1} caused a significant decrease in $_{qPAC}$. In studies of Tripathi ct al. (34) with a strain of *S.cerevisiae*, a maximum $_{qPAC}$ value of 0.07 gg^{-1} h^{-1} was reported.

2.4 End Product Inhibition

The main products which are likely to influence yeast growth and metabolism as well as L-PAC production are: ethanol produced by fermentative metabolism, benzyl alcohol by-product and the L-PAC product itself. It is possible also that acetaldehyde or benzoic acid accumulation might influence L-PAC production, although only very low levels were detected during biotransformation with free cells of *C.utilis*.

In this context, Seely et at. (25) have mutagenized strains of *S.cerevisiae* and *C.flareri* using classical methods, eg. N-methyl-N'-nitro-N-nitrosoguanidine (NTG), ultra-violet light (UV) and gamma rays and have selected mutants with increased resistance to acetaldehyde and L-ephedrine. Strains were isolated capable of producing L-PAC to higher levels than parent cultures, and final concentrations to a maximum of L-PAC of 9,9 g L^{-1} were reported.

Studies in our laboratory on a strain of *C.utilis* have indicated the potential for significant end-product and by-product inhibition of growth. Product inhibition constants for growth (Kp) have been estimated as 4.1 g L^{-1} L-PAC, 5.0 g L^{-1} benzyl alcohol and 39 g L^{-1} ethanol (unpublished data). The effect of these products on the specific rate of L-PAC production ($_{qPAC}$) for the free cells are yet to be determined.

3 Biotransformation Using Yeasts- Free Cells

3.1 Fed-Batch Process

A fed-batch process for L-PAC production can be subdivided into three basic phrases:

(1) growth of the yeast under partial fermentation conditions to produce sufficient biomass for the biotransformation process, and also to facilitate the accumulation of pyruvate for subsequent L-PAC production;

(2) optimal induction of PDC for biotransformation - however PDC induction in an increasingly fermentative environment results in conversion of pyruvate to ethanol via acetaldehyde, and an increase in alcohol dehydrogenases which may enhance benzyl alcohol formation;
(3) programmed feeding of benzaldehyde to maintain concentrations of 1-2 g L^{-1} and the subsequent production of L-PAC.

As discussed earlier, L-PAC concentrations of 10-12 g L^{-1} have been reported in the literature using various species of yeasts. However, it is only recently that higher levels have been found. Presumably pyruvate depletion occurred in many of the earlier studies and insufficient attention was paid to initial pyruvate accumulation, PDC activity and the metabolic status of the yeast. Some studies (20,28) evaluated the addition of pyruvate and acetaldehyde to achieve higher L-PAC levels; however the effects were variable and in many cases no enhancement was found.

In a recent study by our group, L-PAC levels up to 22 g L^{-1} have been achieved through the optimal control of metabolism of a strain of *C.utilis* (viz. RQ = 4-5) and the sustained feeding of benzaldehyde while maintaining a concentration of 1-2 g L^{-1} in the bioreactor (35). The results of a typical experiment in a controlled bioreactor (T = 20^0C, pH = 6.0) using a fully-defined medium are shown in Fig. 6. Prior to PDC activation, pyruvic acid levels were typically 10-15 g L^{-1} with PDC activity low (0.2 U mg $protein^{-1}$). After cell growth and pyruvate accumulation (total 22 h) with glucose addition to maintain active metabolism, PDC activity was enhanced (to greater than 1.0 U mg $protein^{-1}$). Benzaldehyde feeding was initiated and resulted in subsequent L-PAC production. As shown in Fig. 6, accumulation of benzyl alcohol occurred to 4 g L^{-1} and L-PAC reached 22 g L^{-1}. Biotransformation finally ceased due to pyruvate depletion accompanied by declining PDC activity. Cell viability studies after 14 h biotransformation indicated 100% viability loss. The productivity for the biotransformation phase was 1.6 g L^{-1} h^{-1} with a yield of 65% theoretical based on the benzaldehyde utilized. No benzoic acid was detected.

3.2 Continuous Process

Continuous promises are often cited as having the advantages of higher productivities, easier process control and compatible operation with subsequent downstream product recovery. However, there are relatively few reports on the application of free cells in continuous culture to biotransformation processes, although Rohner et al. (36, 37) have reported on the successful application of chemostat cultures of *S.cerevisiae* for the stereospecific reduction of acetoacetic acid esters to the 3-(5)-hydroxy-butanoic acid esters. A continuous single-stage steady-state production system was found to be superior to the pulse-batch and fed-batch systems in terms of product optical purity, biomass concentration and production rates. It was established also in this process that the reduced product

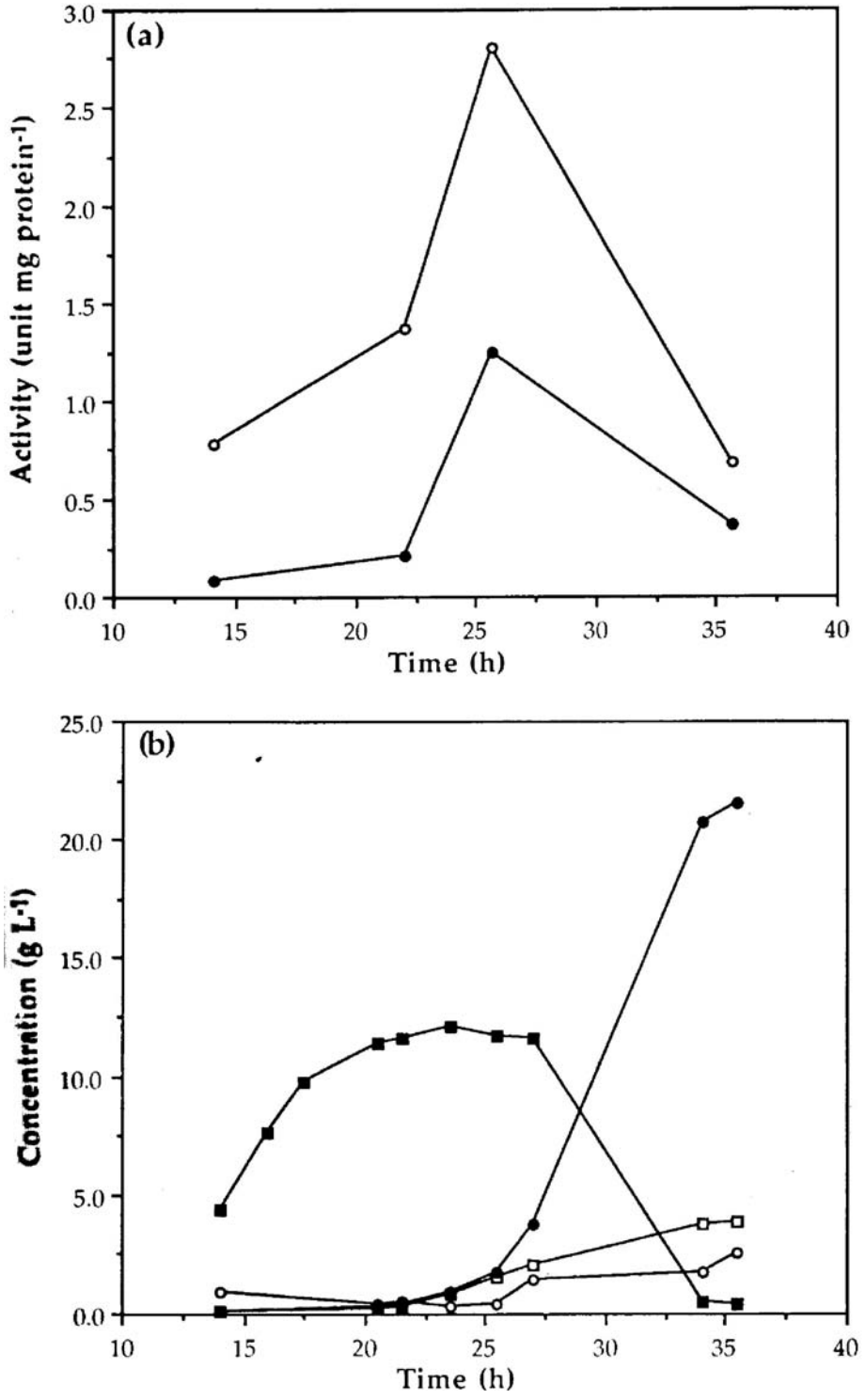

Fig. 6a, b. Fed-batch biotransformation of benzaldehyde to L-PAC by C.utilis. **a** Enzyme profiles: (●) PDC, (O) ADH, **b** Biotransformation kinetics: (O) benaldehyde, (□) benzyl alcohol, (■) pyruvate, (●) L-PAC

(3-(5)-hydroxybutanoic acid ester) did not inhibit the cells (36) although the starting material (acetoacetic acid ester) influenced both the respiration and cell physiology of *S.cerevisiae*.

With L-PAC production, both the substrate (benzaldehyde) and the product (L-PAC) have significant inhibitory effects on yeast metabolism and PDC activity. These factors necessitate the design of a more complex continuous culture process, and suggest one involving multistage operation and later stage benzaldehyde feeding in order to sustain steady-state conditions. Other workers have reported on the use of a single-stage continuous culture for the production of active yeast cells for L-PAC production (34), however the biotransformation of benzaldehyde was carried out in batch culture.

In recent studies in our laboratories a detailed evaluation of various continuous culture systems has been carried out, and the results of this research can be summarised as follows:

(1) A single-stage continuous culture operating as a glucose-limited chemostat with continuous feeding of benzaldehyde was not suitable for the biotransformation process due to the strongly inhibitory effects of benzaldehyde on cell growth.

(2) For a two-stage system, partially fermentative conditions can be established in the first stage to provide for pyruvic acid accumulation and PDC induction, while benzaldehyde can be added to the second stage to facilitate the biotransformation. However, it was found that the biomass yield under the partially fermentative conditions in the first stage was too low to produce sufficient yeast cells for rapid L-PAC production in the second stage.

(3) To overcome the disadvantages of the single- and two-stage processes, a three-stage system was designed as follows:

Stage 1. A fully aerobic stage (RQ = 1) designed to give yields to *C.utilis* of Yx/s = 0.450.50 gg^{-1} glucose, close to the theoretical maximum biomass yield.

Stage 2. A partially fermentative stage (RQ = 4-5) designed to increase PDC activity as well as providing for some accumulation of pyruvic acid. A supplementary feed of glucose was supplied to this second stage.

Stage 3. A continuous biotransformation stage was established with benzaldehyde addition at various feed rates. Low level glucose feeding was also supplied to this stage to provide substrate for continuing yeast metabolic activity.

A diagram of the three-stage process together with a typical set of operating conditions is shown in Fig. 7.

This system was evaluated with a range of benzaldehyde and supplementary glucose feed rates using defined medium containing 60 g L^{-1} glucose to the first stage. Under fully aerobic conditions (DO > 20% air saturation), it was established that close to 30 g L^{-1} biomass was produced in the first stage (RQ = 1). PDC induction up to 0.50 U mg protein $^{-1}$ and pyruvate accumulation to 2-3

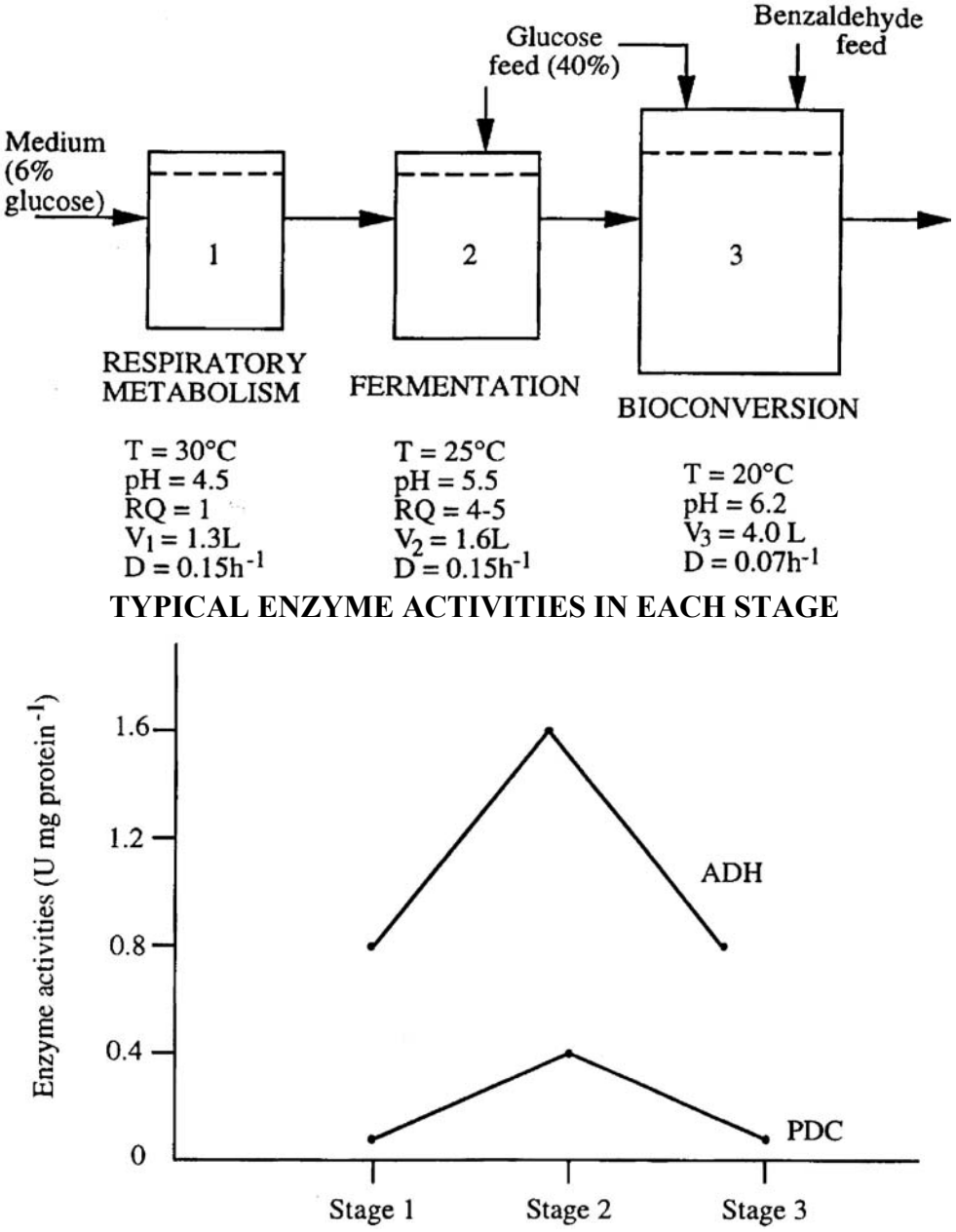

Fig. 7. Operating conditions for a three-stage continuous biotransformation process, showing typical enzyme activities for each stage

g L^{-1} occurred during Stage 2, as well as the formation of 45-50 g L^{-1} ethanol. The ethanol has been shown to be beneficial for enhancing the solubility of benzaldehyde during Stage 3; however it is represented a significant consumption of glucose. As shown in Fig. 8, when the benzaldehyde feeding rate was increased

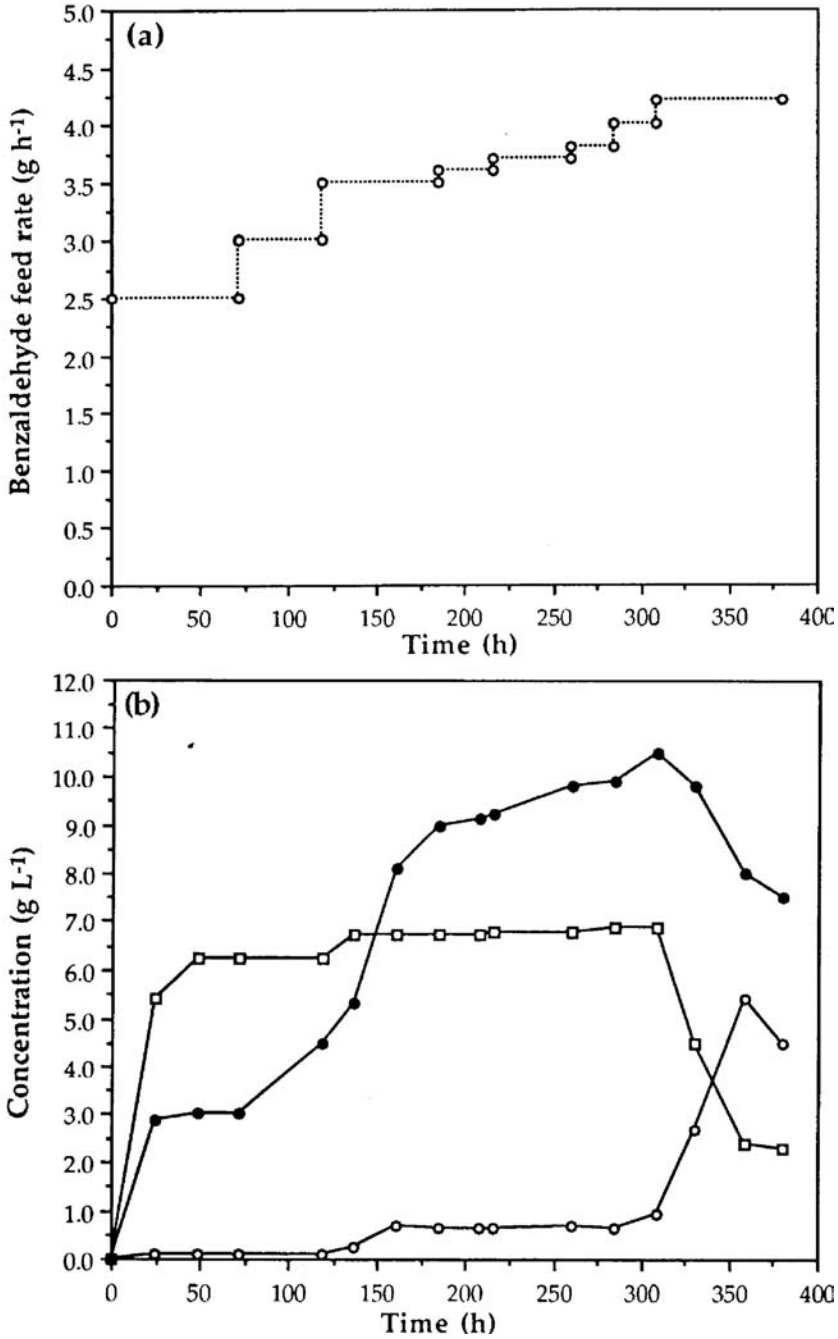

Fig. 8a, b. Effect of increased benzaldehyde feeding on L-PAC production in a three-stage process, **a** Benzaldehyde feed rate; **b** concentrations: (o) benzaldehyde, (□) benzyl alcohol, (●) L-PAC

Incrementally during 300 h, the L-PAC concentration increased to 10.6 g L^{-1} to a controlled fermentor of 4 L working volume during Stage 3 resulted in a decline in L-PAC levels and the accumulation of benzaldehyde. Under these conditions, the PDC activity declined to less than 0.05 U mg protein $^{-1}$.

At the maximal steady-state L-PAC concentration of
10.6 g L^{-1}, the productivity, based on the total volume of the three fermentors, was 0.44 g L^{-1} h^{-1} with a yield of 0.80 gg^{-1} (56% theoretical) based on the benzaldehyde used.

4 Biotransformations Using Yeasts - Immobilized Cells

It has been reported that cell immobilization of yeasts is able to reduce the toxic effects of benzaldehyde by virtue of divisional limitations and the toxic substrate gradients that are established within the immobilizing matrix (22, 23, 38).

Recent studies by our group using cells of C.utilis immobilized within calcium alginate beads of 2-3 mm diameter have confirmed this enhanced resistance to benzaldehyde (39), with the immobilized cells producing higher L-PAC levels than free cells in shake bask experiments. In experiments with the programmed feeding of benzaldehyde in a controlled bioreactor (T = 20 DC, pH = 5.0), under similar conditions to those described previously with free cells, the final L-PAC concentration was 15 g L^{-1} (39). The PDC activity of the cells in the beads which was initially at 0.65 U mg $protein^{-1}$ declined to 0.2 U mg $protein^{-1}$, with cessation of L-PAC production resulting from pyruvate depletion. Electron microscope pictures taken at the end of the biotransformation (Fig. 9) indicated that major cell wall damage had occurred within the calcium alginate beads. The reason that the final L-PAC level of 15 g L^{-1} with immobilized cells was significantly less than the 22 g L^{-1} achieved with the free-cell fed-batch system is that better control of yeast metabolism (via RQ) in the latter case provided for greater pyruvate accumulation. Levels of 10-15 g L^{-1} were achieved with the free cells, while for the immobilized cell system the maximum concentration was only 5 g L^{-1}.

When this evaluation with immobilized C.utilis was extended to a continuous bioreactor, the steady-state L-PAC levels were low (no more than 4 g L^{-1} in sustained operation). These results were similar to those of Mahmoud et at.(22, 38) who reported on a semicontinuous immobilized cell process. These authors found with immobilized *S.cerevisiae* that L-PAC production in the first and second cycles (with an intervening reactivation period of 24 h) could reach 4.5 g L^{-1}. Attempts to extend the process to three or more cycles resulted in rupturing of the cells/beads in the continuing presence of benzaldehyde.

5 Biotransformation with Purified PDC - Free Enzyme

One of the problems of using yeast whole cells for the biotransformation process is that considerable amounts of benzaldehyde are converted to the unwanted

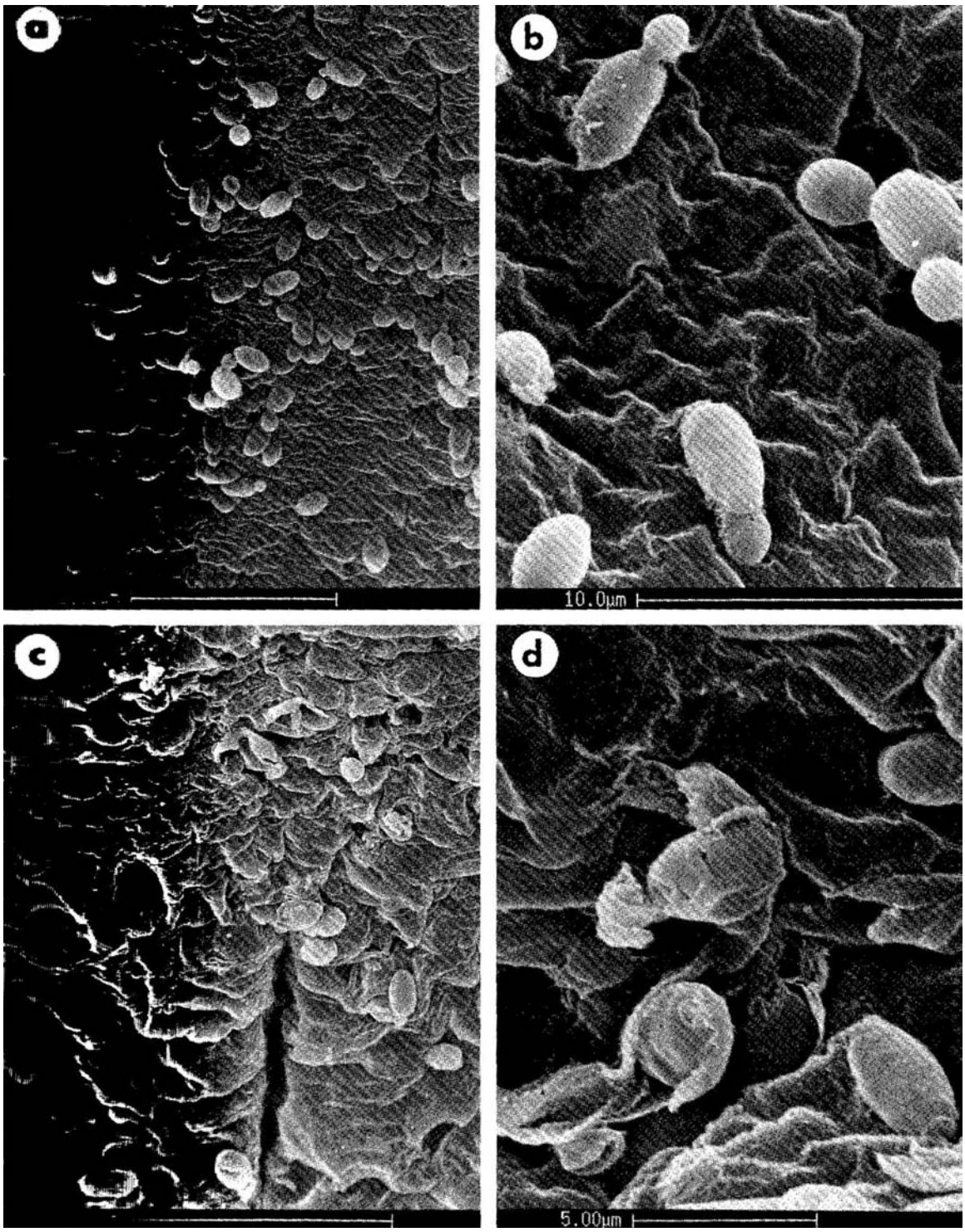

Fig. 9a-d. Comparision of electron microscope pictures of immobilised cells of *C.Utilis* in calcium alginate beads. **a,b** Before exposure to benzaldehyde, c,d following prolonged exposure to 1-2 g L^{-1} benzaldehyde. The scales are given on the four photographs.

by-product, benzyl alcohol. The use of purified PDC offers the possibility of overcoming this problem. However, in the latter case, pyruvate will have to be supplied as a substrate and there is the further likelihood that the pyruvate added will be removed via decarboxylation to acetaldehyde and condensation to acetoin.

Bringer-Meyer and Sahm (40) have investigated the use of purified PDC from *Zymomonas mobilia* and *S.carlsbergensis* for L-PAC production, and demonstrated that the PDC from *Z.mobilis* is unsuitable for the biotransformation due to its low affinity for benzaldehyde and because of significant substrate inhibition effects. However, the substrate levels and consequent L-PAC concentrations achieved with *S.carlsbergensis* were relatively low in this investigation.

To provide an effective comparison between the yeast biotransformation process and one which involves the purified enzyme, it is necessary to evaluate similar operating conditions and substrate concentrations. Details of our recent study (41) are as follows.

5.1 Characteristics of Purified PDC

Purified PDC was prepared from cells of *C.utilis* growing under partially fermentative conditions in a 100-1 fermented at a constant temperature of 25^0C and a pH of 6.0. When the PDC activity had reached 0.9 U mg protein^{-1}, the cells were harvested, disrupted in a high pressure homogenizer and the PDC was purified by means of $(NH_4)_2SO_4$. precipitation and gel chromatography. Previous studies with PDC have demonstrated that the enzyme has a dimeric tetramer structure ($\alpha_2\beta_2$) which dissociates in vitro into dimer and monomer subunits with the concomitant release of the cofactors thiamine pyrophosphate (TPP) and magnesium ions (42). This dissociation, predominantly a function of pH, is affected also by the buyer species. The dissociation was found to be greater in Tris-Cl compared with phosphate and citrate buffers. As a result, in the reported study (41), the reaction mixtures contained various PDC activities and pyruvate : benzaldehyde ratios with 30 mM TPP, 0.5 mM $MgSO_4.7H_2O$ in 40mM phosphate buffer (pH 6.0) in order to achieve maximal enzyme stability.

As shown in Fig. 10, the Km value of PDC for pyruvate was determined to be 2.2 mM at 25^0C and pH 6.0, with saturation at concentrations in excess of 10mM pyruvate. The Km value is in good agreement with other reported values for PDC from yeasts (43-45).

5.2 Factors Influencing Biotransformation Kinetics

The effects of various factors on L-PAC formation with purified PDC have been reported in detail (41) and can be summarised as follows

(a) Higher yields of L-PAC were achieved at lower temperatures due to reduced acetaldehyde production. For this reason, a temperature of 4_0C was selected for the enzyme biotransformations.

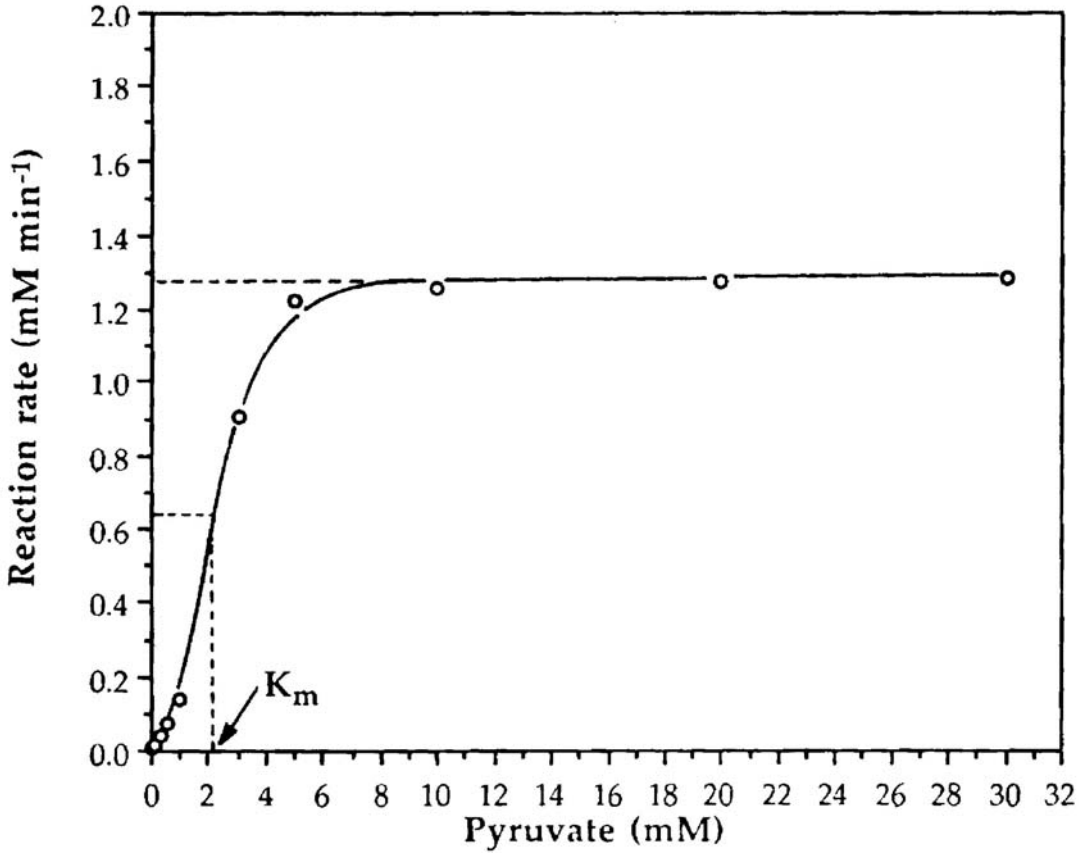

Fig. 10. Estimation of the Km value for pyruvate for PDC from *C.utilis* at T = 25^0C, pH = 6.0

(b) The optimum pH for L-PAC formation was 7.0 while that for acetaldehyde production was 6.0.
(c) As acetaldehyde is an inhibiting by-product, its role in L-PAC formation was investigated. Initial rate studies established that the inhibition constant (Kp) for acetaldehyde was of the order of 20 mM (0.9 g L^{-1}) indicating that it could play a significant role in reducing the L-PAC formation rate.
(d) The influence of an organic solvent such as ethanol in enhancing the benzaldehyde solubility and increasing the L-PAC production rate was evaluated. The selection of ethanol as a water miscible organic solvent was based on the following information: PDC has significant resistance to denaturation by ethanol up to a concentration of 3M (46), PDC has been reported to have highly hydrophobic substrate binding sites and the presence of ethanol may assist enzyme/substrate interactions (47). Furthermore, benzaldehyde is reported to have infinite solubility in ethanol (48) compared to its very limited solubility in water (0.3 g 100 mL^{-1}). Initial rate studies showed that an increase of 30-40% in the rate could be achieved by addition of 2.0-3.0M ethanol.

Based on the above optimal conditions, the substrate saturation constant (Km) for benzaldehyde was determined (Fig. 11), and it is interesting to note that substrate inhibition (benzaldehyde toxicity) was evident only above concentrations of 180 my (19.1 g 1- 1) for the free enzyme. This compares with data for the

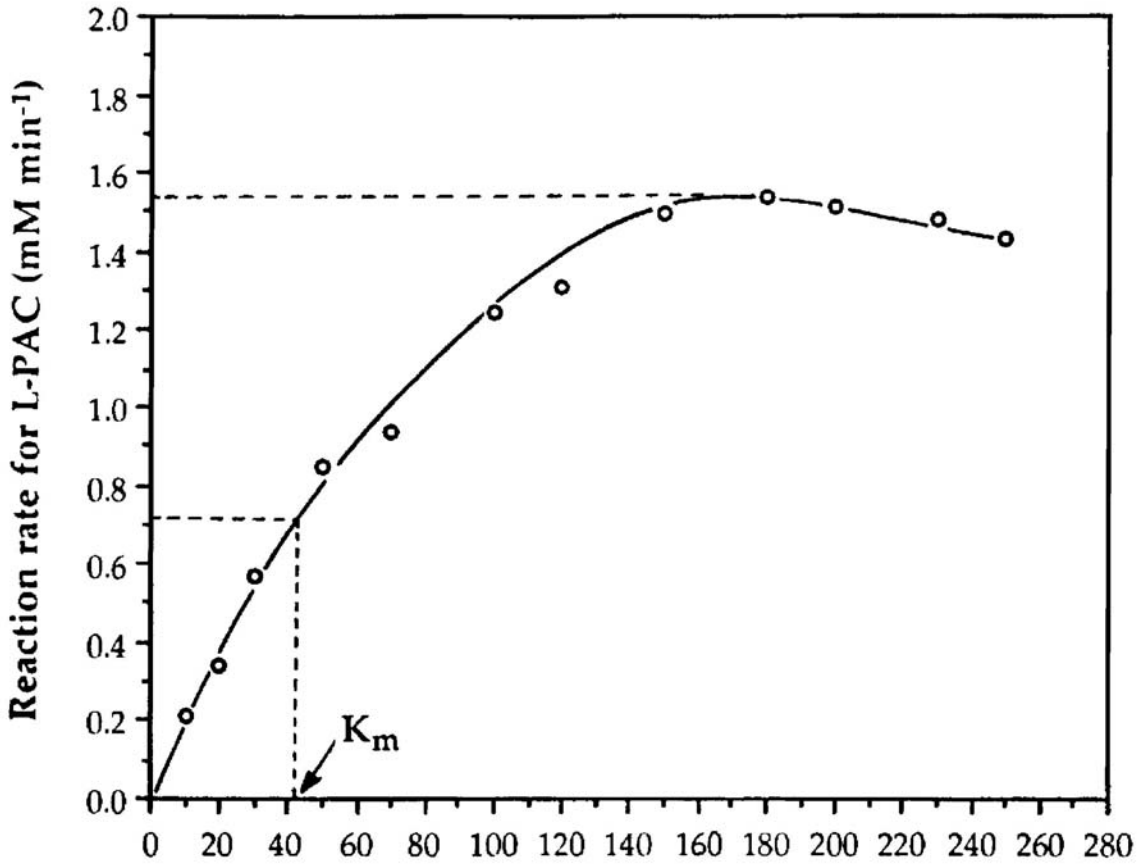

Fig. 11. Estimation of Km value for benzaldehyde for PDC from *C.utilis* at T = 4 ^{0}C, pH = 7.0

influence of benzaldehyde on the growth of *C.utilis* where concentrations above 10 mM were found to inhibit growth completely. The Km value for benzaldehyde substrate limitation for the free enzyme was determined to be 42 mM which compares well with values for Km of 50 mM for PDC from *S.carlsbergen*sis in the literature (40)..

As well as pyruvate and benzaldehyde concentrations, it has been established that initial L-PAC formation rates are influenced significantly by PDC activity. As evident from Fig. 12, higher PDC activities resulted in higher initial rates over a wide range of benzaldehyde concentrations (up to 200 mM). Benzaldehyde toxicity was greater for the lower PDC activities. However, although the initial rates were strongly affected by PDC activity, the final L-PAC concentrations were not influenced to the same extent. There was a trend also towards increased acetaldehyde production at the higher PDC activity levels (Table 1).

The influence of the molar ratio of pyruvate:benzaldehyde is shown in Table 2. From the data, while the highest molar biotransformation yield of 97.8% (based on initial benzaldehyde) was achieved using 150 mM benzaldehyde and a 1.5 molar ratio, the highest concentration of 191 mM (28.6 g L^{-1}) L-PAC was obtained using 200 mM benzaldehyde and a 2.0 molar ratio after an 8 h biotransformation. Molar conversion yields close to 90% (based on initial pyruvate) were achieved only in the range of 150-200 mM benzaldehyde when molar ratios of 1.0 or 0.5 were used. In other situations, a higher proportion of pyruvate was converted to by-products (free acetaldehyde and acetoin) or

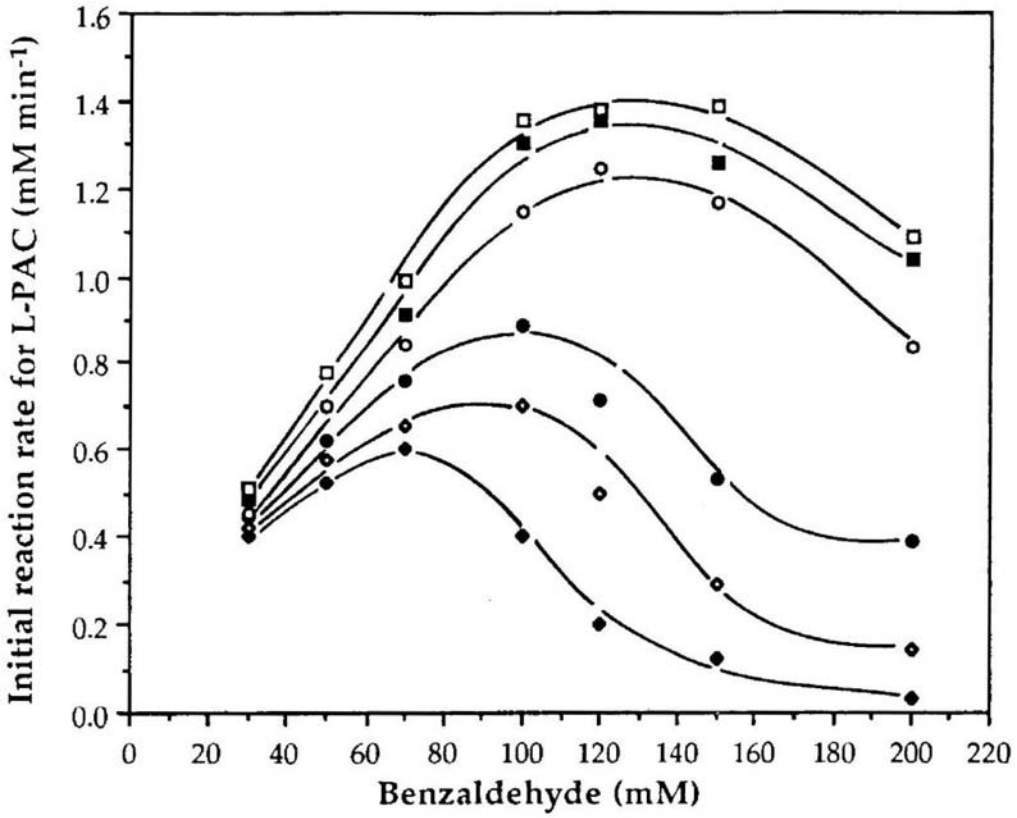

Fig. 12. Effect of PDC activity on initial rate of L-PAC formation in the present of various concentrations of benzaldehyde and equimolar concentrations of sodium pyruvate in 40 mM phosphate buffer (pH = 7.0) containing 30 μM TPP, 0.5 mM $MgSO_4.7H_2O$ and 2.0 M ethanol. The initial rates were determined within 30 min at 4^0C. The symbols refer to PDC activities of (■) 4.0, (□) 5.0, (●) 6,0, (O) 7.0, (■) 8.2 and (□) 10.0 unit.ml^{-1}

remained in excess concentrations in the reaction mixture. It is interesting to note also that increases in initial benzaldehyde (200-300 mM) and PDC activity (7.0-20 U mL^{-1}) did not further raise the final L-PAC concentrations, presumably because of substrate toxicity effects.

5.3 Kinetic Analysis

Detailed kinetics of a typical time course for the biotransformation of 150 mM benzaldehyde with purified PDC are shown in Fig. 13; they 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 probable product inhibition. After a 6 h incubation period, giving a maximum L-PAC concentration of 147 mM (22 gL^{-1}), a mass balance based on pyruvate indicated that of the original 225 mM pyruvate, 147 mM had contributed to L-PAC formation, 25 mM pyruvate had been converted to acetous and 22 mM had been converted to free acetaldehyde. The residual pyruvate was 30 mM, indicating

Table 1. Final L-PAC and acetaldehyde concentrations (mM) with various PDC activities in the presence of equimolar benzaldehyde and sodium pyruvate at 4^0C and pH 7.0

PDC activity (Unit mL^{-1})	L-PAC (mM) Benzaldehyde (mM) 30	50	100	150
4.0	19.0	34.8	48.2[a]	3.4[a]
5.0	20.1	34.8	64.5	30.0[a]
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^{-1})	Acetaldehyde (mM) Benzaldehyde (mM) 30	50	100	150
4.0	6.1	9.5	2.3[a]	ND[a]
5.0	6.5	10.2	7.5	ND[a]
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

[a] Reaction did not proceed fully due to toxicity of benzaldehyde at low PDC activity

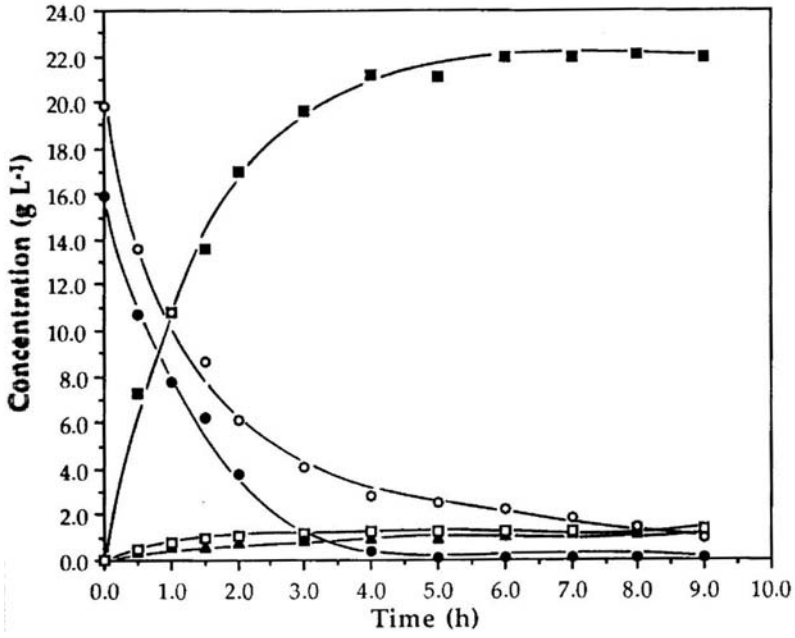

Fig. 13. Typical biotransformation kinetics with purified PDC from *C.utilis.* T = 4^0C, pH = 7.0. Concentrations (●) benzaldehyde, (O) pyruvic acid, (▲) acetaldehyde, (□) acetoin, (■) L-PAC

Table 2. L-PAC formation with various molar ratios of pyruvate to benzaldehyde at 4^0C. The reaction mixture consisted of 40mM phosphate buffer (pH 7.0) containing 7 U mL^{-1} PDC, 2.0 M ethanol and various concentrations of sodium pyruvate and benzaldehyde.

Final concentration of L-PAC (g L^{-1}) and molar conversion yeilds (%)					
Molar ratio of pyruvate to benzaldehyde					
BZ (mM)	0.5	1.0	1.2	1.5	2.0
100	5.8	11.7	14.2	14.5	14.5[a]
	77.0	78.2	78.3	64.5	48.4[b]
	38.3	78.2	94.6	96.7	96.7[c]
120	7.0	14.6	16.8	17.6	17.6
	77.2	81.1	7.6	65.2	48.9
	38.6	81.8	93.2	97.5	97.5
150	10.9	20.5	21.5	22.0	22.0
	97.0	90.6	79.6	64.9	48.9
	48.6	90.6	95.3	97.8	97.8
180	12.1	24.2	25.1	25.5	25.5
	89.5	90.0	77.5	63.0	47.2
	44.4	90.0	93.0	94.4	94.4
200	13.3	22.7	24.9	27.9	28.6
	88.5	75.6	69.1	62.0	47.6
	44.2	75.6	82.9	93.0	95.3

[a]L-PAC (g L^{-1}); expressed as g L^{-1} for comparison with literature values.
[b]$Y_{p/s}$ (mole L-PAC,mole added pyruvate^{-1}) x 100 (%).
[c]$Y_{p/s}$ (mole L-PAC,mole added benzaldehyde^{-1}) x 100 (%).

a closing mass balance based on pyruvate. With the complete utilization of 150 mM benzaldehyde, the mass balance based on benzaldehyde conversion to L-PAC closed to within 2%. The small mass balance discrepancy was probably due to evaporative losses of benzaldehyde and/or minor experimental errors. 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.

6 Biotransformation using Periled PDC - Immobilized Enzyme

Previous studies have reported that cell and enzyme immobilization in suitable gels and matrices could minimise substrate inhibition effects by means of dimensional limitation and the substrate gradients which exist within the immobilizing material (22, 23, 38). Furthermore, the development of an immobilised system provides the technology for long-term continuous operation, provided that enzyme stability can be maintained.

6.1 PDC Immobilization

In our recent work, various immobilizing methods have been evaluated for PDC, including adsorption on cationic exchange resins and entrapment in gel matrices (49). It was found that entrapment in calcium polyacrylamide gel provided a higher activity than adsorption on either Amberlite IR-200 or CM-sephadex (activities expressed as U mL^{-1} immobilizing material), and that the addition of 0.2-0.3% glyceraldehyde to the polyacrylamide gel enhanced the PDC binding capacity and increased PDC activity by 40%. The 'apparent' Km for PDC immobilized with respect to pyruvate was determined at 25^0C from initial-rate data, and a value of 3.2 mM estimated from Lineweaver-Burk analysis. This compares with 2.2 mM for the free enzyme - the higher Km for the immobilized enzyme being consistent with mass transfer limitations within the gel.

6.2 Factors Influencing Biotransformation Kinetics

In a similar evaluation to that with free PDC, the optimal conditions for the immobilized PDC were determined. Although higher temperatures gave rise to an initial increase in the L-PAC production rate, the relatively high acetaldehyde and acetoin concentrations at 25^0C resulted in reduced final L-PAC formation. As for the free enzyme, a temperature of 4^0C was selected to maximise the L-PAC yield. The optimum pH for L-PAC with immobilized PDC was shifted slightly to more acidic conditions (pH = 6.5) compared to the free enzyme (pH = 7.0). Ethanol was again found to enhance initial rates (due to improved benzaldehyde solubility) and a concentration of 3-4 M gave a 40% increase in initial rates compared to the control.

For a batch biotransformation process, the immobilized PDC process did not provide any real advantages compared to the free PDC system. The results were much as expected with the immobilized enzyme able to function better at higher benzaldehyde concentrations (up to 300 mM). The maximum final L-PAC concentrations were similar, viz. 27.1 gL^{-1} with 300 mM benzaldehyde and 1.5 molar ratio of pyruvate:benzaldehyde.

6.3 Continuous Biotransformation

The operating parameters for a continuous process are shown in Fig. 14 with increasing molar ratios and residence times (viz. decreasing dilution rates) resulting in the increased formation of L-PAC and by-products, acetaldehyde and acetoin. While the highest concentration of 5.3 g L^{-1} L-PAC was achieved with a 2.0 molar ratio at D = 0.05 h^{-1} higher L-PAC productivities could be achieved at higher dilution rates. In an evaluation of the long-term operation of the process it was found that a gradual decline in PDC activity occurred, with the enzyme half-life estimated to be 32 days.

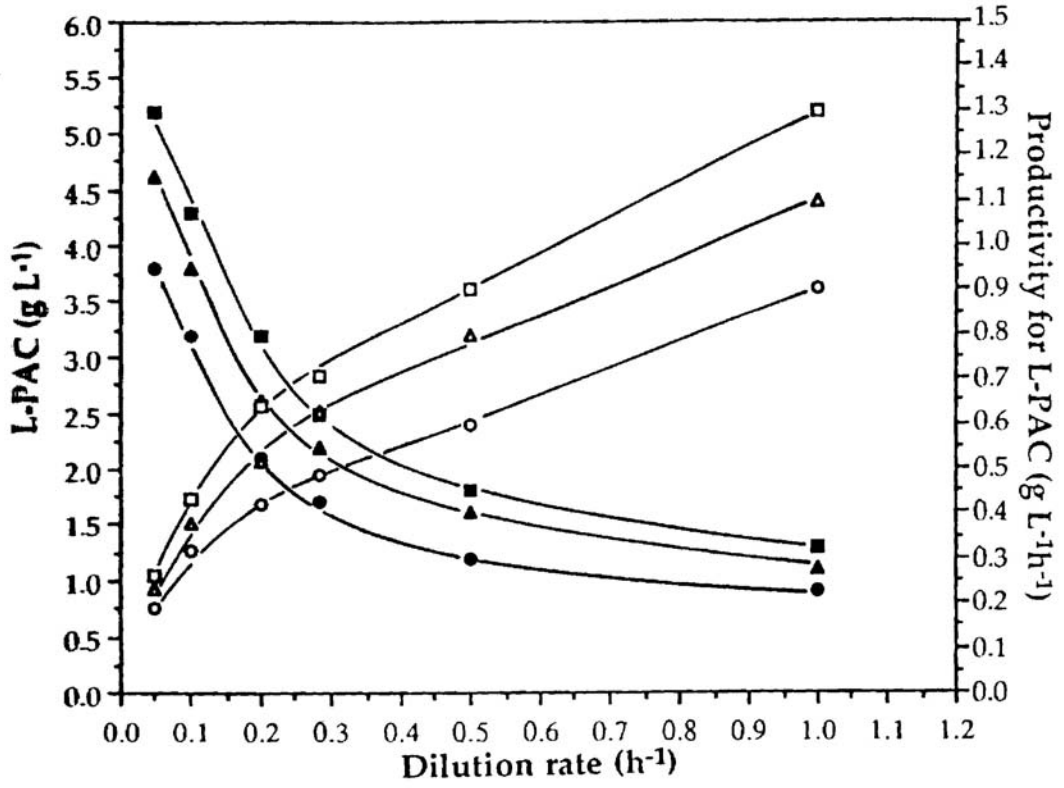

Fig. 14. Effect of dilution rate and molar ratios of pyruvate: benzaldehyde on L-PAC production using immobilised PDC in a packed-bed bioreactor. T = 40c, pH = 7.0. L-PAC concentrations for molar ratios: (●) 1.0, (▲) 1.5, (■) 2.0; productivities for molar ratios: (O) 1.0, (△) 1.5, (□) 2.0

7 Discussion and Conclusions

A comparison of the various biotransformation processes for the conversion of benzaldehyde to L-PAC is given in Table 3. From the data it is evident that for both free and immobilized yeast, the biotransformation is a relatively low efficiency process in which there is significant diversion of benzaldehyde to benzyl alcohol and a loss of up to 30-40% due to the formation of by-products. This diversion has been attributed to the activity of alcohol dehydrogenates in yeast. However, studies by Nikolova and Ward (29) using mutant strains of *S.cervisiae* that lack ADH-I, -11 and -111 demonstrated that these strains were still able to produce ethanol and benzyl alcohol and suggested that other oxidoreductases were catalyzing these reductive biotransformations. Other authors (50, 51) have demonstrated the presence of ADH-IV, activated by Zn, in low amounts in *S.cerevisiae* and it is possible that this enzyme may play some part in ethanol and benzyl alcohol production.

Using yeast strain selection, immobilized cell technology and cyclodextrin addition, previous studies have reported maximal L-PAC concentrations of 10-12 g L^{-1}. Recent studies by our group, however, have demonstrated with the control of yeast activity and metabolism (via control of the respiratory quotient,

Table 3. Comparison of kinetic evaluations for various methods of L-PAC production

Process	L-PAC ($g\ L^{-1}$)	Biotrans. time (h)	Productivity ($g\ L^{-1}h^{-1}$)	Yield theorectical based on benzaldehyde	Ref.
(1) Batch and fed-batch processes					
Free cells	12.4	17	0.73	57	(21)
	22.4	14	1.6	65	(35)
Free cells (cyclodextrins)	12				(24)
Immobilised cells	9.9	3	3.3	60	(23)
	10	24	0.42	59	(22)
	15	22	0.7	58	(39)
Free PDC	28.6	8	3.6	95	(41)
Immobilised PDC	27.1	12	2.3	93	(49)
(2) Continuous processes					
Immobilised cells	4	-	0.6	45	(39)
Immobilised cells (semi-continuous)	4.5	-	0.4-0.8	57	(22)
Three-stage system (free cells)	10.6	-	0.44	56	(53)

RQ), that L-PAC concentrations up to 22 $g\ L^{-1}$ can be achieved in defined media in the normal biotransformation time. This has resulted from accumulation of pyruvic acid, induction of PDC and controlled feeding of benzaldehyde. It is interesting to note that other authors (52) using defined media and a *Candida* strain have also achieved relatively high levels of pyruvic acid by manipulation of PDC activity. Furthermore, the use of defined media also offers the advantages of easier solvent-extraction of L-PAC and, fewer pollution problems compared to the use of industrial substrates such as molasses.

From Table 3 it is evident that continuous biotransformation processes do not offer any advantages when compared to a fed-batch process under optimal control. This results from the highly toxic nature of the substrate benzaldehyde which considerably reduces cell viability and PDC activity in a continuous process. Rohner et al (36, 37) demonstrated that a chemostat culture was the most suitable for a biotransformation which involved the stereo-specific reduction of acetoacetic acid esters; however, in this case the inhibition effects of substrate/products on *S. cerevisiae* were much lower.

The use of purified PDC for the biotransformation demonstrated that the PDC could maintain its activity at benzaldehyde concentrations greater than 2?0 mM (21 $g\ L^{-1}$) and give L-PAC concentrations up to 28 $g\ L^{-1}$. Very high conversion efficiencies were achieved (greater than 95% theoretical based on benzaldehyde added) as no significant aromatic by-products were produced. However, with the purified PDC process it was necessary to add pyruvic acid with a mole ratio of pyruvate : acetaldehyde greater than 1.0, due to formation of the by-products acetaldehyde and acetoin. Mass balances on both the

benzaldehyde and the pyruvate closed to within 2% indicating the accuracy of the analytical procedures and the identification of all major products in the biotransformation process.

In an overall assessment of the L-PAC process, it is clear that significant enhancements can be achieved by either improved process control of the yeast biotransformation or by the development of an enzymatic process based on purified PDC. The choice will ultimately be based on economic criteria; however, it may also be influenced by the development of genetically engineered strains of yeasts with enhanced PDC and reduced oxidoreductase activities, resulting in higher L-PAC yields and productivities.

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