Appl Microbiol Biotechnol (1995) 44:7–14 © Springer-Verlag 1995

ORIGINAL PAPER

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Biotransformation of benzeldehyde to L-phenylacetylcarbinol, an intermediate in L-ephedrine production, by immobilized *Candida utilis*

Received revision: 24 February 1995 / Accepted: 1 March 1995

Abstract Biotransformation of benzaldehyde to L-phenylacetylcarbinol (L-PAC) as a key intermediate for L-ephedrine synthesis has been evaluated using immobilized *Candida utilis*. During biotransformation, the benzaldehyde level and respiratory quotient significantly affected both L-PAC and by-product benzyl alcohol formation. By controlling the benzaldehyde level at 2 g/l, maintaining a respiratory quotient of 5–7 and pulse feeding glucose, a final concentration of 15.2 g/l L-PAC was achieved in a fed-batch process. This compares with previous published results of 10–12 g/l in batch culture and 10 g/l L-PAC in a semicontinuous process with immobilized *Saccharomyces cerevisiae*. In a single stage continuous process with immobilized *C. utilis*, the steady state L-PAC concentration was significantly reduced because of the sustained toxic effects of benzaldehyde.

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 indicated also its potential use in obesity control (Astrup et al. 1992a, b). It is currently produced via a microbial biotransformation process using different species of yeasts with benzaldehyde as the aromatic substrate. The following diagram (Fig. 1) outlines the biotransformation process, which involves the condensation of an "active acetaldehyde" (from pyruvic acid produced by the yeast) with benzaldehyde. The production of the L-PAC is catalysed by the enzyme pyruvate decarboxylase (PDC), and is associated with the formation of benzyl alcohol as a by-product resulting from the activity of an alcohol dehydrogenase (ADH) and/or oxidoreductases.

Previous studies have reported concentrations of 10–12 g/l L-PAC in batch culture (Vojtisek and Netrval 1982; Culic et al. 1984) and 10 g/l for a semicontinuous culture using immobilized *Saccharomyces cerevisiae* (Mahmoud et al. 1990a, b). Strain-improvement studies with acetaldehyde-resistant mutants have been reported by Seely et al. (1989) with similar levels of L-PAC. The role of purified PDC in L-PAC production has been studied by Bringer-Meyer and Sahm (1988) and other fundamental investigations have indicated that oxidoreductases distinct from ADH may be involved in by-product benzyl alcohol formation (Long and Ward 1989; Nikolova and Ward 1991).

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

1. Significant reduction of PDC activity due to benzaldehyde or end-product inhibition
2. Pyruvic acid limitation at the end of the biotransformation phase
3. Cell viability loss due to extended exposure to benzaldehyde and/or increasing concentrations of benzyl alcohol and L-PAC.

In the present study, an immobilized cell system with *Candida utilis* has been investigated. The immobilized cell system was selected for detailed evaluation, as the research by Mahmoud et al. (1990a, b) has suggested that the toxic effects of benzaldehyde may be minimized by the diffusional limitations of immobilizing matrices. The kinetics of both batch and continuous biotransformation processes have been assessed in the current investigation.

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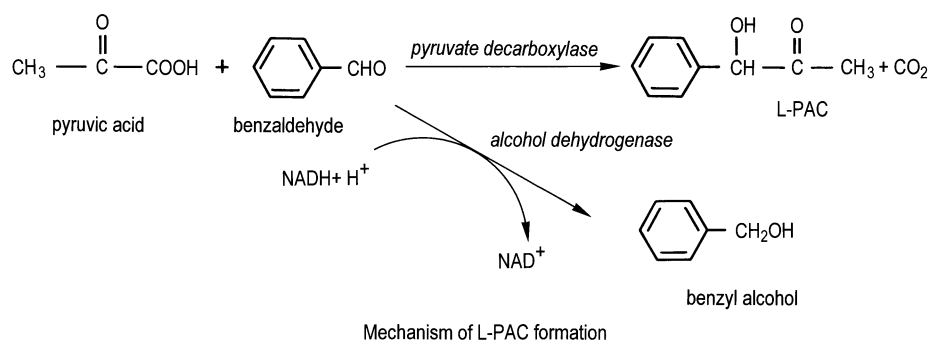


Fig. 1 Mechanism of L-PAC formation

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 (g/l) 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 1.5 with an initial pH of 6.0. For its growth and subsequent immobilization, this strain was cultivated in a fermentation medium consisting of (g/l) glucose 60, yeast extract 10, $(NH_4)_2SO_4$ 10, KH_2PO_4 3.0, $Na_2HPO_4 \cdot 12H_2O$ 2.0, $MgSO_4 \cdot 7H_2O$ 1.0, $CaCl_2$ 0.05, $FeSO_4$ 0.05, Mn $SO_4 \cdot 4H_2O$ 0.05 at an initial pH of 5.0 and temperature of 25° C.

Culture and biotransformation system

The system consisted of several components: fermenter, benzaldehyde-feeding pump, exit-gas analysers and computer-linked on-line respiratory quotient measurement. In this system, the 2-l LH fermenter (working volume 1.5c) was used for cell growth under controlled conditions at 25° C and pH 5.0 and for biotransformation at 20° C and pH 6.0. For the continuous process, the overflow outlet was covered with a stainless-steel sieve (mesh size 1.0 mm) to maintain the immobilized cell beads in the fermenter. The culture medium was fed into the fermenter by means of a peristaltic pump (Gilson Minipul). To feed benzaldehyde into the fermenter, a syringe pump (Perfusor VII, B. Braun) was used with variable feed rates in the range of 0.1–99.0 ml/h by means of a 50-ml disposable syringe.

Prior to analysis for O_2 and CO_2, the exit gas was dehumidified by a cold dehumidifier (Komatsu Electronics Inc. Model DH 1052G) to meet the requirements of the gas analysers. The content of oxygen was measured by the paramagnetic susceptibility of the sample (Servomix type 1400A), while the content of carbon dioxide was measured by an infrared gas analyser with single-beam dual wavelength (Servomix-R type 1410). Output signals (4–20 mA) from both gas analysers were fed into a data interface (Data system FC-4, Real Time Engineering, Australia) linked to an NEC Powermate computer. RQ values were calculated instantaneously by this computer and the results were used for control via aeration and/or agitation.

Immobilization of *C. utilis* cells

When the level of PDC reached a maximum with pulse feeding of glucose, the cells were harvested and resuspended in sodium alginate (3% w/v). Samples of 150 g wet cells (approximately 30 g dry weight)/300 ml solution were prepared for immobilization. This preparation was extruded into 2% $CaCl_2$ solution through a 0.5-mm-diameter needle, and stabilized in fresh 2% $CaCl_2$ solution containing 3% glucose for 1–2 h at 4° C. For further stabilization and prior to biotransformation, the immobilized beads were reintroduced into their own former supernatant with further supplementation of glucose and yeast extract.

Dry cell weight estimation

After centrifugation of a sample of culture broth and resuspension in isotonic saline, 4 ml of the cell suspension was transferred to pre-weighed glass tubes and centrifuged at 5000 rpm for 10 min. The glass tubes containing the cells were dried in an oven at 105° C for 24 h, cooled in a desiccator and reweighed. The average values from three measurements were determined for each sample.

Estimation of glucose concentration

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

Estimation of ethanol concentration

Ethanol concentrations were estimated using a gas chromatograph (Packard, series 427). The relevant column and its operation were as follows: column material, 6.4-mm glass 1.5 m long; packing material, Porapak Q in mesh range 100–200 μm; carrier gas, nitrogen (30 cm^3/min); oven temperature, 180° C (isothermal); injector temperature, 220° C; detector temperature, 220° C with flame ionization detector; injection sample, 3 μl. The ethanol concentrations of the sample were estimated by comparison with standard samples.

Estimation of benzaldehyde, L-PAC and benzyl alcohol concentrations

Concentrations of benzaldehyde, L-PAC and benzyl alcohol were determined by gas chromatography. Samples were prepared by extraction into dichloromethane (sample:solvent = 1:5). The biotransformation sample (0.2 ml) was mixed with 1 ml dichloromethane in a microcentrifuge tube and vortexed for 2 min. A sample from the bottom organic layer was injected into a gas chromatograph with the column and its operating conditions as follows: column material, 6.4-mm glass 1 m long; packing material, Chromosorb W. Hr/SE 30WTX 10 in the mesh range of 80–100 μm; carrier gas, nitrogen (30 cm^3/min); oven temperature, 115° C (isothermal); injector temperature, 180° C; detector temperature, 180° C with flame ionization detector; injection sample, 3 μl. The concentrations

of benzaldehyde and benzyl alcohol were determined by comparison with standard samples (from Aldrich) and L-PAC (from ICI Australia Pty. Ltd).

Pyruvic acid determination

Determination of pyruvic acid was carried out by an enzymatic analysis (Boehringer-Mannheim analytical kit no. 718 882). In the presence of NADH + H^+, lactate dehydrogenase reduces pyruvic acid to lactic acid and 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 in sample absorbance at 340 nm.

Analysis of enzyme activities

Extraction of enzymes from free cells

Cells from 1 ml of culture broth were harvested by centrifugation (Eppendorf Centrifuge) at 12000 rpm for 1 min and washed twice with 30 mM TRIS buffer (pH 6.5). Cells were resuspended in the same buffer and the volume adjusted to 0.4 ml. Approximately 1 g glass beads (size 0.5 mm, B. Braun, catalogue no. 854 170/1) were mixed with 0.4 ml 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 were collected for subsequent enzyme assays and protein determination.

Extraction of enzymes from immobilized cells

To extract enzymes from immobilized cells, 3 ml immobilized beads containing *C. utilis* were put into a ceramic hammer mill and gently extracted with 3 g pretreated fine sand (which was washed three times with 3 M HCl and Reverse Osmosis (RO) water until neutralized). Crushed immobilized beads were suspended in 20 ml water and centrifuged at 1000 rpm for 2–3 min. From the resultant supernatant, yeast cells were harvested and washed with 30 mM TRIS buffer (pH 6.0) by centrifugation at 5000 rpm for 10 min. The harvested cells were resuspended into 3.0 ml 30 mM TRIS buffer, and then 1 ml yeast suspension was centrifuged at 12 000 rpm for 1 min, and the enzymes were extracted by ball milling.

Pyruvate decarboxylase

The activity of 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 (Bergmeyer 1974). The reaction mixture consisted of (µl) 200 mM sodium citrate buffer (pH 6.0) 950, 10 mg/ml NADH (sodium salt) 10, 100 mg/ml sodium pyruvate 32, 10 mg/ml alcohol dehydrogenase (Sigma Chem. Co., Product no. A-3263) 3, enzyme sample 5. One unit of enzyme activity is defined as that activity which converts 1.0 µmol of pyruvate to acetaldehyde/min at pH 6.0 and 25° C. The activity of the enzyme was monitored as NAD^+ formation by changes in absorbance at 340 nm.

Alcohol dehydrogenase for ethanol

The basic reaction for determination of ADH activity is the oxidation of ethanol to acetaldehyde with monitoring of the reduction of NAD^+ to NADH + H^+ (modified from Bergmeyer 1974). The reaction mixture consisted of (µl): 35 mM Trizma base (pH 8.5), 935, 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/min at pH 8.5 and 25° C. The activity of the enzyme was monitored as NADH formation by changes in absorbance at 340 nm.

Protein determination

Protein determinations of cell-free crude extract, following enzyme extraction were carried out by the Bradford method (Bradford 1970) with lyophilized bovine serum albumin as a reference.

Results

Optimal fed batch culture of *C. utilis* and its fermentative enzyme profiles

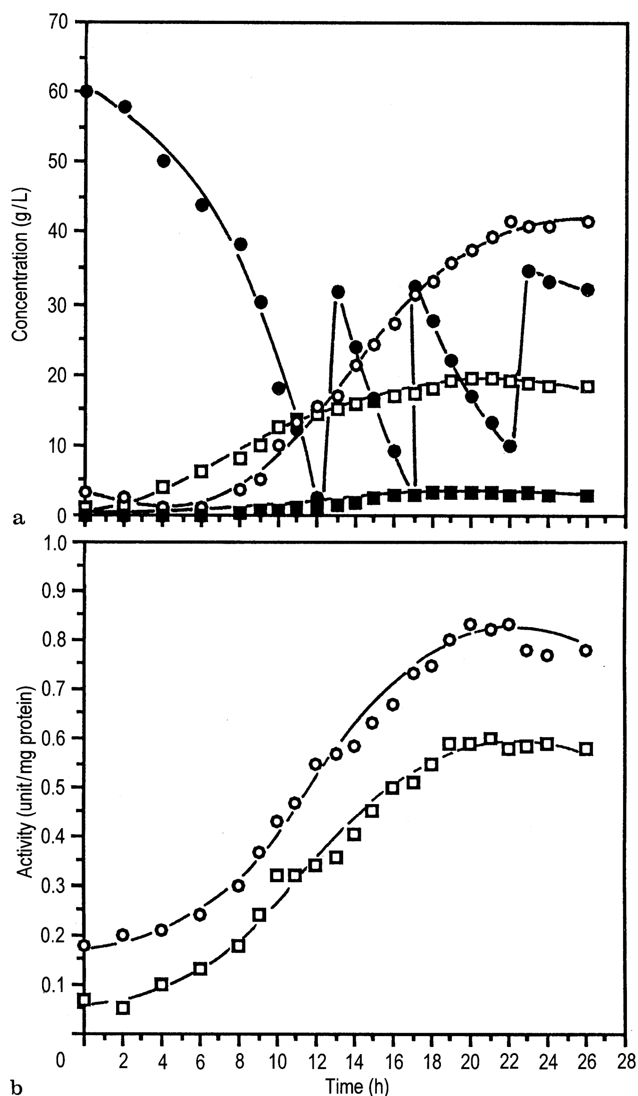
In order to enhance PDC activity prior to cell immobilization, an extended fed-batch culture under partially fermentative conditions was developed. Initially, respiratory metabolism was maintained for the first 8–9 h to obtain a high biomass concentration for immobilization, and then a switch from aerobic respiration to fermentative growth was made by reducing agitation (from 1000 rpm to 500 rpm) and aeration rate (0.6 vvm to 0.3 vvm). 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) was initiated. As shown in Fig. 2, this resulted in enhanced PDC and ADH activities to maximum values of 0.59 unit/mg and 0.83 unit/mg protein respectively.

Immobilization of *C. utilis* cells and associated enzyme profiles

Following cell immobilization (which involved cell harvesting and entrapment in calcium alginate), there was a decline in enzyme activities because of reduced levels of cellular metabolism. As a result, a glucose-feeding protocol was initiated, which resulted in the levels of PDC and ADH in immobilized *C. utilis* increasing as shown in Fig. 3. The highest activities of PDC, ADH were 0.61 unit/mg and 0.93 unit/mg protein respectively, after 12 h incubation. Comparison of enzyme profiles indicated that ADH activity was a little higher in the immobilized cells compared to the free cells.

Comparison of biotransformation by free and immobilized cells with various initial concentrations of benzaldehyde

Biotransformation studies were carried out in shake flasks with addition of benzaldehyde and 30 g/l glucose



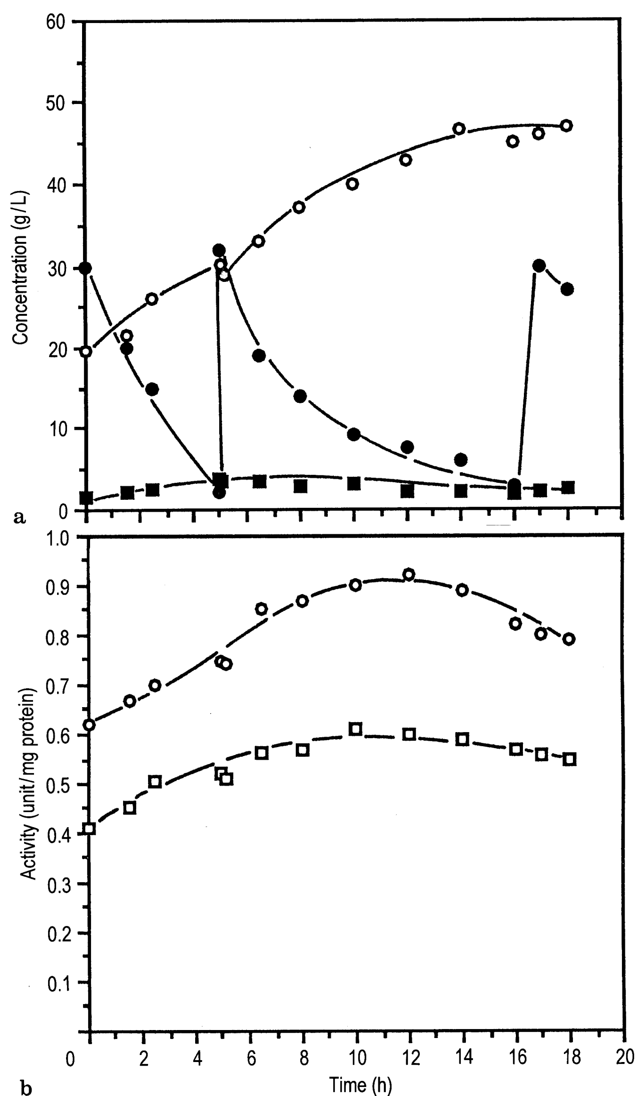


Fig. 2a,b Effect of pulse feeding of glucose on (**a**) kinetics of *Candida utilis* growth: □ biomass, ● glucose,○ ethanol, ■ pyruvate, and (**b**) fermentative enzyme profiles: ○ alcohol dehydrogenase, □ pyruvate decarboxylase

Fig. 3a,b Kinetics of immobilized cells: (**a**) ● glucose consumption, ○ ethanol, ■ pyruvate production; (**b**) fermentative enzyme profiles with pulse feeding of glucose: ○ alcohol dehydrogenase, □ pyruvate decarboxylase

with free and immobilized cells after PDC had been fully induced in both systems. Results for various initial concentrations of benzaldehyde on L-PAC and by-product benzyl alcohol formation are shown in Fig. 4 with data expressed on a millimolar basis to illustrate molar conversion of benzaldehyde to products. No evidence of benzoic acid production was found following biotransformation.

With free and immobilized cells below 30 mM benzaldehyde, benzyl alcohol was preferentially produced instead of L-PAC. However, L-PAC was preferentially formed above 40 mM benzaldehyde in both systems. Higher concentrations of benzaldehyde were accompanied by higher L-PAC formation and by an enhanced molar ratio of L-PAC formation to benzyl alcohol until benzaldehyde inhibition occurred.

It was evident also that higher L-PAC concentrations could be produced with immobilized cells compared to free cells, an observation consistent with the results of Mahmoud et al. (1990a). However, benzyl alcohol production with immobilized cells was higher than for free cells over the range of benzaldehyde concentrations. From these results it is evident that selection of an optimum level of benzaldehyde is necessary to enhance L-PAC formation as well as to minimize benzyl alcohol formation.

Biotransformation kinetics with various sustained concentrations of benzaldehyde

Investigations of the effect of a relatively constant benzaldehyde level on L-PAC formation were carried out

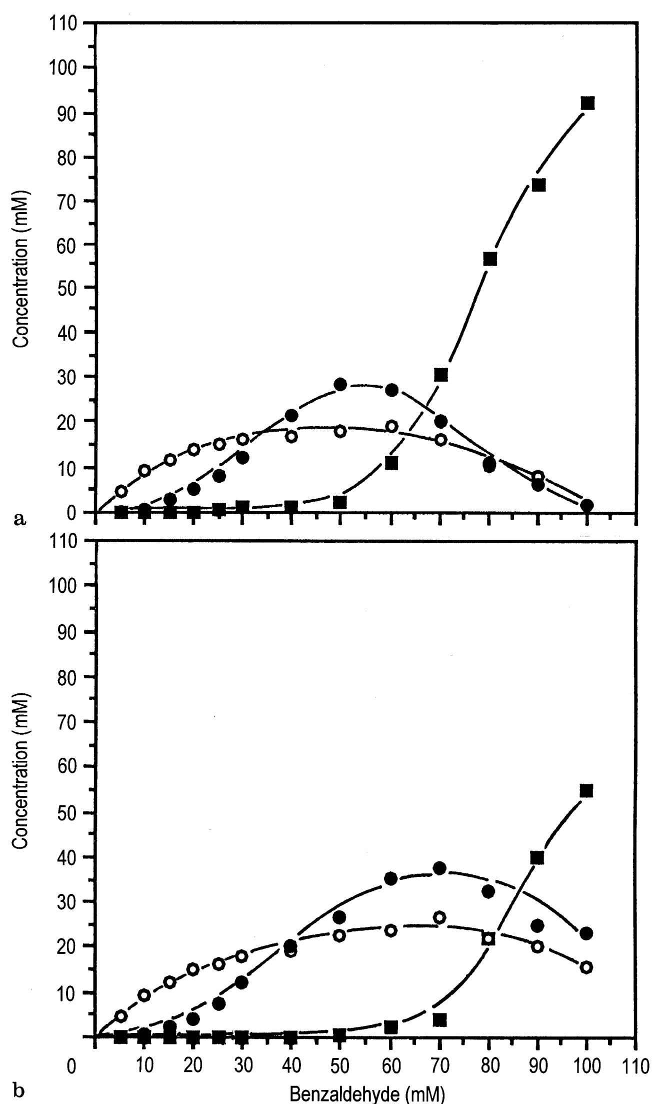


Fig. 4a,b Comparison of biotransformation products for (**a**) free cells and (**b**) immobilized cells after 16 h incubation with various initial concentrations of benzaldehyde in shake flasks at 20° C and 180 rpm: ■ benzaldehyde, ○ benzyl alcohol, ● L-phenylacetylcarbinol (*L-PAC*)

with four different benzaldehyde concentrations in a controlled fermenter following a period of adaptation for 3–4 h. A short acclimatisation phase was used with addition of 0.8 g/l/h benzaldehyde for this adaptation. During this time, it appeared that the cells adapted to the toxic substrate, which resulted in minimizing viability and/or enzyme activity loss following later extended exposure. After this acclimatisation, biotransformation kinetics were evaluated at various feeding rates of benzaldehyde until L-PAC concentrations reached their peak values.

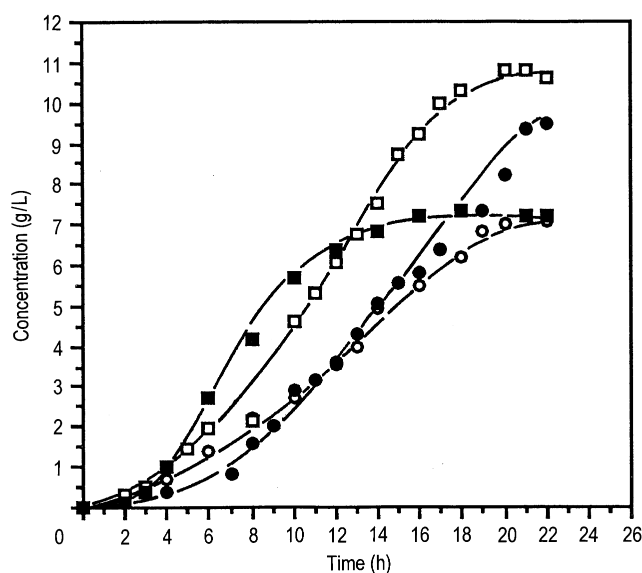


Fig. 5 Effect of various levels of benzaldehyde (○, 0.8 g/l, ● 1.5 g/l, □ 2.0 g/l, ■ 4.0 g/l) on L-PAC formation as a function of time

To maintain approximately constant benzaldehyde levels, samples were taken every hour, and benzaldehyde concentrations were measured immediately by gas chromatography. Through this analysis, levels of benzaldehyde were maintained at relatively constant values in the range of 0.8–4 g/l by controlled feeding.

As shown in Fig. 5, the highest level of L-PAC (10.8 g/l) was achieved at 2 g/l benzaldehyde. At 4 g/l benzaldehyde, 7.3 g/l L-PAC was obtained within the relatively short period of 16 h. Further evaluation of the kinetics, as summarized in Table 1, supports the conclusion that increasing the level of benzaldehyde (up to 2 g/l) resulted in higher L-PAC formation and a relative reduction in benzyl alcohol formation. Both L-PAC and benzyl alcohol production were inhibited at 4 g/l benzaldehyde.

Besides the inhibiting effect of benzaldehyde, it is possible also that benzyl alcohol accumulation could

Table 1 Summary of biotransformation products with immobilized cells with various benzaldehyde levels maintained in the fermanter

Benzaldehyde level (g/l)	L-PAC (g/l)	benzyl alcohol (g/l)	d*p*/d*t* L-PAC (g/l/h)	Reaction time (h)	Molar yield for L-PAC [a] (%)
0.8	7.0	6.4	0.35	20	44.1
1.5	9.5	6.1	0.43	22	52.8
2	10.8	6.0	0.54	20	56.4
4	7.3	4.8	0.45	16	52.3

[a] Molar conversion yield based on benzaldehyde utilized

Table 2 Effect of aeration rate and respiratory quotient on the biotransformation of benzaldehyde to L-PAC

Aeration rate (vvm)	RQ range	Maximum conc. pyruvate (g/l)	L-PAC (g/l)	Benzyl alcohol (g/l)	Molar yield for L-PAC (%)
0.3	12–20	4.2	10.7	6.0	56.2
0.6	7–12	3.8	12.4	4.9	64.6
0.75	5–7	3.5	12.5	4.7	65.7
1.0	1–4	2.2	10.1	5.2	57.1

inhibit both L-PAC and benzyl alcohol formation. When benzyl alcohol was above 6–7 g/l, there was little further production of either benzyl alcohol or L-PAC, indicating a degeneration of catalytic activity resulting from continuous contact with both toxic substrate and by-product.

Effect of respiratory quotient on L-PAC production

The metabolism of *C. utilis* is significantly affected by available oxygen, and the respiratory quotient (RQ) has been used as a good indicator of the metabolic status of the yeast. For normal respiratory growth, RQ = 1.0 is maintained with glucose as substrate while, with increasing fermentation, RQ rises. Biotransformation of benzaldehyde to L-PAC involves the production of CO_2 from decarboxylation of pyruvate to acetaldehyde and the extent of biotransformation may be indicated by the RQ value, although the effect is complex with high RQ being related also to higher levels of fermentation of glucose to ethanol.

The effect of aeration rate and RQ on L-PAC formation was evaluated with 2 g/l benzaldehyde level in order further to identify critical parameters for the biotransformation. Table 2 shows that a low aeration rate of 0.3 vvm resulted in a high RQ value. Even though this was accompanied by adequate pyruvate accumulation, a relatively high level of benzyl alcohol was formed, presumably because of higher levels of ADH (and/or other oxidoreductases). By contrast, the high aeration rate of 1.0 vvm resulted in a lower RQ and lower L-PAC production, due to decreased accumulation of pyruvate and reduced PDC activity. From Table 2 it can be concluded that conditions for which the RQ value was maintained between 5 and 7, are likely to be the most favourable to maximise L-PAC and minimize benzyl alcohol formation.

Biotransformation kinetics for L-PAC formation

A detailed biotransformation kinetic evaluation was carried out with an immobilized cell density (cell dry weight) of 15 g/l at 2 g/l benzaldehyde and 30 g/l glucose pulse feeding (Fig. 6a). Aeration was controlled to maintain the RQ value in its optimum range of 5–7, temperature was controlled at 20° C and pH at 5.0. As shown in Fig. 6b, L-PAC production occurred up to 15.2 g/l. The increased L-PAC formation compared to the previous results can be ascribed to various factors, such as programmed feeding of benzaldehyde at the optimum level (2 g/l), RQ values maintained in the range of 5–7, and pulse feeding of glucose to facilitate pyruvate production (up to 8 g/l). Profiles of enzyme activities showed that the PDC activity declined more rapidly with increasing reaction time than did ADH (Fig. 6c). The PDC activity remained at about 0.65 unit/mg protein during the early stages of the biotransformation, but declined to about 0.2 unit/mg protein by the end, indicating that final cessation of L-PAC formation resulted from depletion of pyruvate (with no further production) rather than complete loss of PDC activity. The resultant decline in benzaldehyde feeding profile is shown in Fig. 6d. At the end of biotransformation, the cells appeared to have lost metabolic activity completely (monitored by CO_2 evolution) presumably because of the increasingly inhibitory effects of benzaldehyde and biotransformation products.

Evaluation of a continuous immobilized cell process for L-PAC production

A continuous process with immobilized *C. utilis* was evaluated in a continuously stirred-tank reactor (CSTR) with low-level aeration using immobilized cells (approximate cell density = 15 g/l). Prior to benzaldehyde addition, a continuous culture was established at a dilution rate $D = 0.15\ h^{-1}$ with 60 g/l glucose-based medium, and maintained for a sufficient period to reach steady state.

As summarized in Table 3, 60 g/l of glucose was converted basically to ethanol (27.1 g/l) and pyruvate (2.1 g/l) prior to benzaldehyde addition. The enzyme activities indicated a lower PDC activity than that obtained in fed-batch culture. When benzaldehyde was added to the medium, glucose utilization decreased, as did the pyruvate and ethanol levels, and significant inhibition of ADH and PDC activities was evident. While the highest benzaldehyde feed rate of 1.5 ml/h resulted in increased L-PAC production, a "pseudo"-steady state was maintained only for 48–50 h. With 1.0 ml/h feed rate, operation stability could be

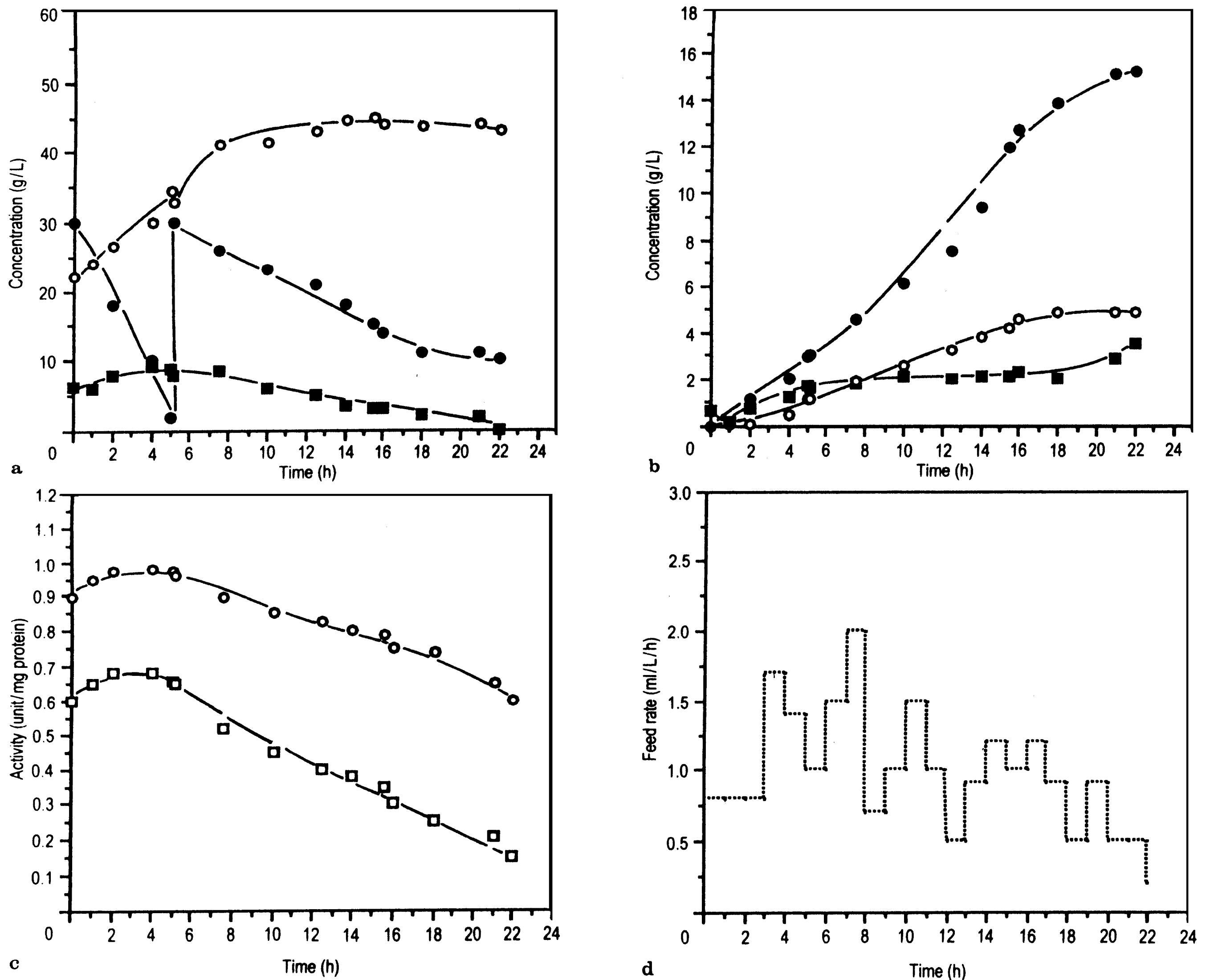


Fig. 6a–d Biotransformation time course with immobilized cells: (**a**) kinetics of (●) glucose consumption, (○) ethanol and (■) pyruvate production; (**b**) biotransformation kinetics: ■ benzaldehyde, ○ benzyl alcohol, (●) L-PAC; (**c**) enzyme profiles, ○ alcohol dehydrogenase, □ pyruvate decarboxylase; (**d**) controlled feeding rate profile for benzaldehyde

maintained for at least 110–120 h, however, the reduced benzaldehyde resulted in benzyl alcohol production exceeding L-PAC production.

From the data it is evident that a continuous L-PAC biotransformation process with immobilized cells at the higher benzaldehyde levels would have significant difficulties in long-term operation because of the steady decline in PDC activity. This is likely to result from continuous exposure to the toxic substrate and the possible inhibition effects of L-PAC and/or benzyl alcohol. The result is consistent with that of Mahmoud et al. (1990b) who reported significant inhibition effects for a semicontinuous process (operating for only a limited number of cycles) for L-PAC production using immobilized *S. cerevisiae.*

Discussion

Previous studies (Mahmoud et al. 1990a, b) have suggested that an immobilized cell process may offer significant advantages for a biotransformation involving a toxic substrate. In the present investigation of the biotransformation of benzaldehyde to L-PAC, several interesting characteristics emerged. First, in a shake-flask comparison between free and immobilized cells it was demonstrated that the immobilized cells could tolerate higher initial benzaldehyde concentrations (up to 70 mM, or 7.4 g/l) before substrate inhibition. For free cells, the level was 50 mM (or 5.3 g/l), indicating that the substrate profiles resulting from benzaldehyde diffusion into the calcium alginate beads have provided

Table 3 Kinetic parameters of continuous process with immobilized cells in 1.5 l controlled fermenter at 20° C and pH 6.0. (*BZ* benzaldehyde, *BA* benzyl alcohol, *PDC* pyruvate decarboxylase, *ADH* alcohol dehydrogenase)

BZ feed rate (ml/h)	Concentration (g/l)						
	S_{in} (glucose)	S_{out} (glucose)	P (EtOH)	P (pyruvate)	S_{out} (BZ)	P (L-PAC)	P (BA)
0	60	1.35	27.1	2.1	0	0	0
0.5	60	10.5	23.5	0.9	0.1	0.7	1.6
1.0	60	16.9	19.5	0.6	2.2	2.3	
1.5	60	35.2	10.1	0.4	0.45	4.0	3.6

Kinetic values (g/g/h)				Activities (mg protein)	
$-q_{(BZ)}$	$q_{(L\text{-}PAC)}$	$q_{(BA)}$	Productivity of L-PAC (g/l/h)	PDC	ADH
0	0	0	0	0.47	0.94
0.021	0.007	0.016	0.11	0.40	0.87
0.040	0.022	0.023	0.33	0.30	0.85
0.063	0.040	0.036	0.60	0.26	0.71

some protection against substrate inhibition. Second, it was demonstrated that the yield of L-PAC compared to the production of the major by-product, benzyl alcohol, was dependent on the benzaldehyde concentration and the available oxygen in the microenviroment (as measured by RQ). Higher benzaldehyde levels favoured L-PAC production, a result consistent with the observation by Long and Ward (1989) that the PDC of *S. cerevisiae* was more resistant to benzaldehyde than was ADH (and presumably other oxidoreductases). Highly fermentative conditions, such as high RQ, were less favourable to L-PAC production and an optimum RQ range of 5–7 (partially aerobic) was identified. The results demonstrated also that respiratory metabolism (RQ = 1–4) resulted in a marked reduction in L-PAC production presumably because of low PDC activity. Finally it was established, with optimal control of benzaldehyde and RQ levels, that an L-PAC concentration of 15.2 g/l could be achieved in 22 h in a fed-batch culture. This compares with 10 g/l L-PAC produced by immobilized *S. cerevisiae* in a semi-continuous process (Mahmoud et al. 1990b), and reflects the capacity of *C. utilis* as a suitable yeast for biotransformation of benzaldehyde, as well as the optimal conditions used. A continuous immobilized-cell process with *C. utilis* was evaluated also, and found to produce low L-PAC levels (no more than 4 g/l in sustained operation). Such a process would be unsuitable for the biotransformation of toxic substrates such as benzaldehyde.

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