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REVIEW

Production of L-Phenylacetylcarbinol by Fermentation

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In pharmaceutical industry, L-phenylacetylcarbinol (L-PAC) is used as an intermediate for the production of L-ephedrine hydrochloride—a well known bronchodilator. Certain yeast strains are known to transform benzaldehyde to produce L-PAC with the help of a specific enzyme pyruvate decarboxylase (PDC) and pyruvate. Simultaneously another by-product, benzyl alcohol is also produced by another enzyme alcohol dehydrogenase (ADH). Strains belonging to the genera *Saccharomyces* and *Candida* have been found to be more efficient L-PAC producers as comparison to other yeasts. The formation of L-PAC is determined by the growth and biotransformation conditions. In the presence of benzaldehyde, cell growth is adversely affected and L-PAC production is low. Harvested whole cells immobilized in different carriers have shown tolerance for higher benzaldehyde doses and increased L-PAC yield has been obtained with semicontinuous mode of benzaldehyde biotransformation. Strain improvement also has effectively enhanced the yield of L-PAC. Studies with isolated PDC enzyme have shown significant potential for improving the L-PAC yield.

[**Key words:** benzaldehyde, biotransformation]

L-Ephedrine hydrochloride, a traditional medicant and important pharmaceutical, is obtained from the extracts of a medicinal plant belonging to the genus *Ephedra*. L-Ephedrine hydrochloride is a unique drug in that it exhibits both α and β-adrenergic activities (1) and is a major ingredient of several pharmaceutical products used as decongestants and antiasthamatics (2). Recently, it has been reported to also control obesity (3). Natural occurence of the plant from which this drug is obtained is scarce and peculiar to certain geographical regions only. Hence, the need to develop a technology for the production of L-ephedrine hydrochloride becomes important.

Certain yeasts were found to transform, through the glycolytic pathway, aromatic aldehyde substrates to optically active acetyl aromatic carbinols and aromatic alcohols (4–7). L-Phenylacetylcarbinol (L-PAC) is produced as a result of the transformation of benzaldehyde (8). Reports and patents on the chemical synthesis of L-ephedrine from L-PAC have brought enough commercial interest in the production of L-PAC (Hilderbrandt, C & Klavehn, W., German Patent no. 548459, 1930; Hilderbrandt, C & Klavehn, W., U.S. Patent no. 1956950, 1934). Optimum conditions for the growth of yeast and for benzaldehyde biotransformation have been studied by various workers with a view to optimize the yield of L-PAC. This review attempts to present an account of the current status of L-PAC production technology.

MECHANISM OF L-PAC PRODUCTION

The cellular biochemical reactions and the enzymes involved in the metabolism of aromatic aldehydes for the production of L-PAC, and other products, have been investigated previously. Neuberg and Hirsch (8) reported that an enzyme, carboxylase, catalyses the formation of carbinols in yeast and proposed that condensation of acetaldehyde or benzaldehyde with a 'nascent' aldehyde yields the carbinol. However, the mechanism of condensation was not explained. Singer and Pensky (9) reported that purified carboxylase of wheat germ catalyses the synthesis of acetoin from acetaldehyde, pyruvate or both, in the presence of Mg^{++} and diphosphothiamine. Smith and Hendlin (10) suggested that yeast synthesizes L-PAC from pyruvic acid and benzaldehyde by the dismutation of pyruvic acid to lactic acid and an acetyl-coenzyme A complex which with benzaldehyde forms L-PAC. The reaction required carboxylase, Mg^{++}, diphosphopyridine nucleotide and coenzyme A. Other workers (11–14) have reported that the decarboxylation of pyruvic acid, a product of glycolysis, yields L-PAC and that the reaction is catalysed by the enzyme pyruvate decarboxylase (PDC). The function of PDC has also been studied in detail. It is reported to be a tetramer holoenzyme consisting of two dimeric subunits (α_2 β_2) of slightly different chain lengths and has 2–4 molecules of thiamine pyrophosphate (TPP) and Mg^{++} as obligatory co-factors (15). Catalytic function of PDC starts the nucleophilic attack of TPP on the carbonyl group of pyruvate to produce intermediates which, in the presence of benzaldehyde, form L-PAC. Detailed reaction steps have been described by Mochizuki *et al.* (16). In parallel with this reaction, benzaldehyde is also converted to benzyl alcohol by alcohol dehydrogenase (ADH) and/or other oxidoreductase(s) (5). The reaction is shown in Figs. 1 and 2.

MICROORGANISMS STUDIED

Various strains of yeasts have been investigated with reference to the production of L-PAC (Table 1). Earlier studies by Neuberg and Welde (4) and Neuberg and Libermann (5) have shown L-PAC production by brewer's yeast. Further comparative studies on the types of L-PAC producing yeasts were made by Becvarova and Hanc (17). *Hansenula anomala, Saccharomyces carlsbergensis* and *S. cerevisiae* showed higher decarboxylase

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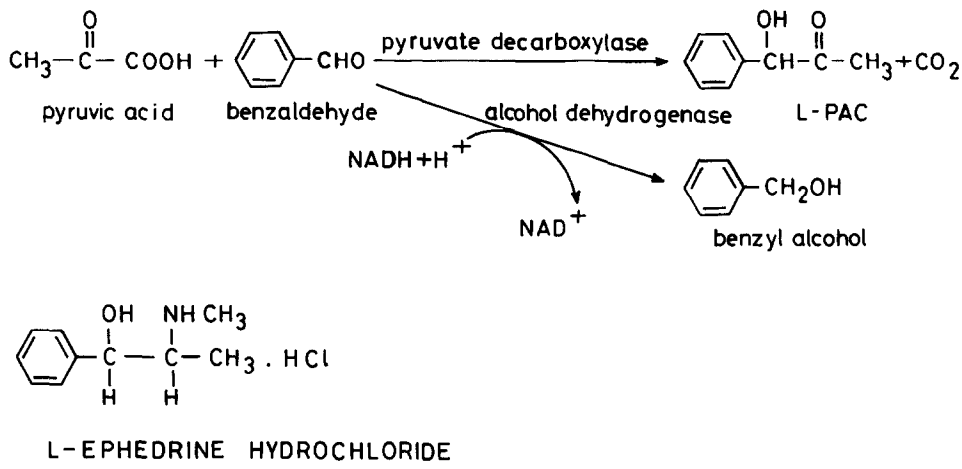

FIG. 1. Mechanism of L-PAC formation.

activity than *Torula utilis*. Nine typed yeast strains were studied by Gupta *et al.* (18). *S. cerevisiae* CBS 1171 yielded the highest amount of L-PAC. Netrval and Vojtisek (19) and Agarwal *et al.* (20) investigated several yeasts for L-PAC production and noted that *Candida* and *Saccharomyces* strains produced more L-PAC than other yeasts. The studies of Bringer-Meyer and Sahm (21) pertaining to enzyme activity and L-PAC production indicated that despite exhibiting low decarboxylase activity, *S. carlsbergensis* produced higher amounts of L-PAC than *Zymomonas mobilis* exhibiting higher decarboxylase activity. High benzaldehyde tolerance and L-PAC production by *S. cerevisiae* ATCC 834 has been reported by Mahmoud *et al.* (22–24). Long and Ward (25) and Nikolova and Ward (26, 27) studied alcohol dehydrogenase (ADH) deficient mutants of commercial baker's yeast and attempted to specify the role of ADH in relation to L-PAC production. Aldehyde resistant mutants of *Candida flaveri* and *S. cerevisiae* were found to produce substantial amounts of L-PAC (Seely, R. J. *et al.*, U.S. Patent No. 5312742, 1994). Substantially high L-PAC yield has also been obtained from *Candida utilis* (2).

COURSE OF BENZALDEHYDE BIOTRANSFORMATION AND L-PAC PRODUCTION

Growth and fermentation substrates Cane molasses, beet molasses (17), yeast autolysate, sucrose, pyruvate (28) and glucose (18), supplemented with inorganic phosphate, ammonium and magnesium salts, have been used as growth and fermentation substrates for yeast cultivation and benzaldehyde biotransformation. Glucose has been most commonly used as the carbon source for

TABLE 1. Microorganisms studied for L-PAC production

No.	Microorganism	Strain no.	Author(s) and reference no.
1.	Fresh brewer's yeast	—	Neuberg *et al.* (4, 5)
2.	*Hansenula anomala*	—	Becvarova and Hanc (17)
	Brettanomyces vini	Strain × No. 416	
	Saccharomyces carlsbergensis	—	
	S. cerevisiae	R XII	
	S. ellipsoidence	—	
	Torula utilis	—	
3.	*S. cerevisiae*	CBS 1171, NCYC 324	Gupta *et al.* (18)
	S. carlsbergensis	CBS 1485	
	S. fragilis	ATCC 12424	
	S. rouxii	CBS 732	
	S. latis	NRRL Y 1140	
	S. veronae	NCYC 412	
	S. microellipsoides	NRRL Y 1549	
	Candida sp.		Netrval and Vojtisek (19)
	Saccharomyces sp.	—	
5.	*S. cerevisiae*	—	Agarwal *et al.* (20)
6.	*S. carlsbergensis*	—	Bringer-Meyer and Sahm (21)
	Zymomonas mobilis		
7.	*S. cerevisiae*	ATCC 834	Mahmoud *et al.* (22–24)
	Zygosaccharomyces rouxii	ATCC 2615	
	Z. rouxii var. *mellis*	ATCC 10685	
8.	Commercial baker's yeast	—	Long and Ward (25) Nikolova and Ward (26, 27)
9.	*Candida flareri*	—	Seely *et al.*
	S. cerevisiae	—	US patent no. 5312742 (1994)
10.	*Candida utilis*	—	Shin and Rogers (2)

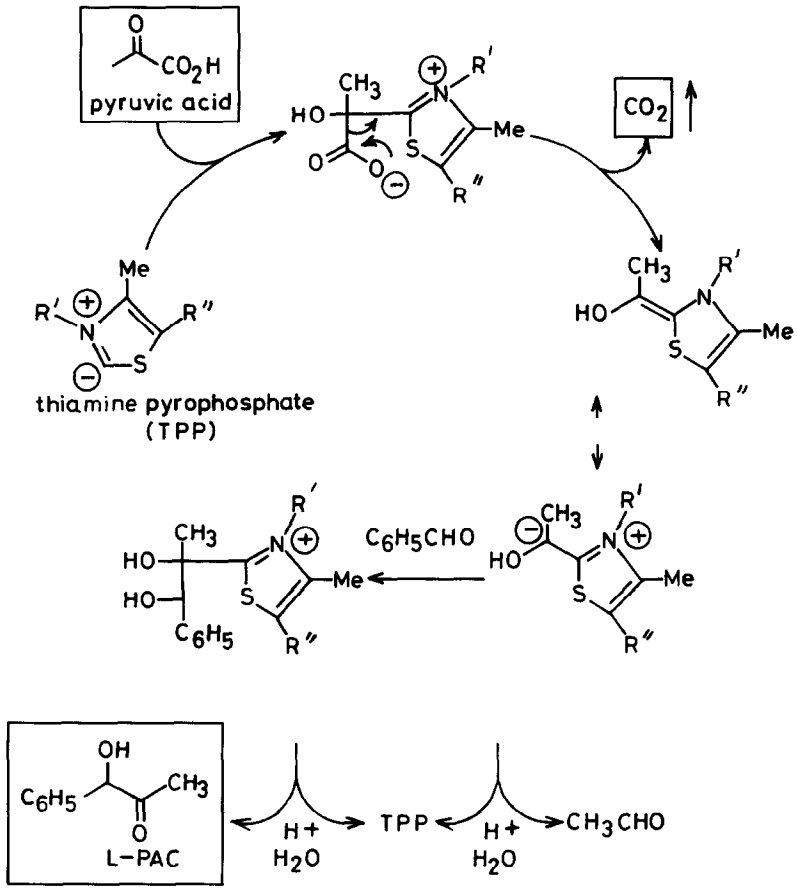

FIG. 2. Details of L-PAC formation.

growth. Agarwal *et al.* (20) devised a synthetic medium for yeast cultivation. The method of benzaldehyde addition to the culture medium significantly influenced the growth as well as the biotransformation process. Becvarova *et al.* (17, 29) found that the addition of benzaldehyde and acetaldehyde increased the yield of L-PAC because acetaldehyde completely blocked the reducing system of yeasts which otherwise caused the reduction of benzaldehyde to benzyl alcohol, at the cost of L-PAC synthesis. It was demonstrated that the dose of benzaldehyde added had a profound effect on the production of L-PAC and that the maximum production was achieved within one to four hours. Groger *et al.* (30) suggested that the amount of active acetaldehyde, formed by the decarboxylation of pyruvic acid, determined the L-PAC yield. Netrval and Vojtisek (19) also observed that the presence of sucrose and acetaldehyde increased L-PAC production.

Vojtisek and Netrval (28) critically studied the limiting factors for L-PAC production and found that, as expected, yeast PDC activity was not a limiting factor. Initial L-PAC production rate and its total yield were found to be higher in cells exhibiting lower PDC activity. An increase of approximately 30% of the total L-PAC yield was observed when 8.5% sodium pyruvate was added gradually to the fermentation medium. Significantly when the fermentation medium was supplemented with sodium pyruvate after 6 h L-PAC production restarted at the initial rate whereas in the fermentation medium with no sodium pyruvate addition there was no L-PAC production indicating that the pyruvate concentration of the medium controlled PDC activity and L-PAC production. When pyruvate concentration was depleted in the fermentation medium, the intracellular content of pyruvate determined the levels of L-PAC production. They suggested that the activity of some of the enzymes involved in metabolic pathway between sucrose and pyruvate could be considered as limiting factors for L-PAC production. Nikolova and Ward (26) also demonstrated that the level of PDC activity in the different strains was not the rate limiting factor for L-PAC production but rather that each step of the glycolytic pathway was rate limiting. Bringer-Meyer and Sahm (21) found significantly low L-PAC yield from *Zymomonas mobilis*, as compared to *S. carlsbergensis*, though the amount of PDC enzyme in the former was five times higher. They attributed this phenomenon to a lower affinity of PDC to aldehydes in *Zymomonas*. This indicated that a high PDC activity did not ensure a high L-PAC yield, it was rather quality of growth and the rate of metabolism that controlled the L-PAC yield.

Formation of the by-products Along with L-PAC, benzoic acid, benzyl alcohol (4, 5), optically active 1-phenyl-1,2-propanediol (16) and optically inactive PAC (17, 29) are also produced by the yeast during benzaldehyde biotransformation. The method of benzaldehyde biotransformation significantly influenced the formation of the product and by-products. Supplementation with acetaldehyde (17, 29) and replacement of sucrose with pyruvate (25, 26) decreased benzyl alcohol production

and increased the yield of L-PAC during benzaldehyde biotransformation. Shin and Rogers (2) noted substantial differences in L-PAC and benzyl alcohol yield from the yeast cells grown at different aeration rates. Benzyl alcohol production was favoured at 0.3 vvm aeration which generated a high respiratory quotient (10–18). High aeration of 1 vvm also reduced L-PAC production because of a low respiratory quotient (1–4). At a respiratory quotient of 5–7 and 0.75 vvm aeration L-PAC was produced in favour of benzyl alcohol.

During biotransformation, benzaldehyde concentration also determined the ratio of L-PAC to benzyl alcohol formation. At lower benzaldehyde concentrations benzyl alcohol production was higher than that of L-PAC. Formation of benzyl alcohol was higher with concentrations of benzaldehyde, below 30 mM, whereas, above 40 mM, production of L-PAC was higher (2). Mahmoud *et al.* (22) studied the production of L-PAC and benzyl alcohol in free and immobilized yeast cells. Immobilized cells produced 1.4 and 7.5 fold higher L-PAC than free cells at 2 and 6 *g/l* benzaldehyde concentrations, respectively. Benzyl alcohol production was found to be decreased substantially with increasing concentrations of benzaldehyde. It appears that the quality richness of yeast growth, concentration of benzaldehyde and mode of biotransformation, collectively control the formation of product and side product.

The role of alcohol dehydrogenases, especially in carbinol producing strains of yeasts, is of particular interest (31, 32). Nikolova and Ward (26, 27) studied *S. cerevisiae* mutants lacking some or all of the alcohol dehydrogenase isoenzymes (ADH I, II, III). Benzyl alcohol production by ADH, I, II and III deficient mutants and wild type strains was found to be identical indicating the existence of other oxidoreductases also producing benzyl alcohol during benzaldehyde biotransformation. Total alcohol dehydrogenase activity did not relate to either benzyl alcohol or ethanol production during benzaldehyde transformation. It appears that limited supply of reduced NADH is the key determinant of the whole reaction. Substantial differences were observed in recovered aromatic substrates and the reaction products as a percentage of the benzaldehyde added, indicating the production of other biotransformation side products. They also reported the presence of an isomer of PAC, 2-hydroxy-1-phenyl-1-propanone, in the reaction mixture. Moreover, the reduction of benzoin, an analogue of PAC, to hydrobenzoin was also suspected to be possible. Thus, close monitoring of the reaction products is important while trying to optimise the yield of L-PAC. An analogous extension of this study would be to use yeasts for the synthesis of modified carbinol in order to develop new ephedrine compounds as suggested by Long *et al.* (33).

Toxicity of the substrate and reaction products Gupta *et al.* (18) noted that beyond 0.15% of benzaldehyde concentration, growth and viability of the yeast cells were reduced. Long and Ward (1, 25) characterized the toxic effects of benzaldehyde and its biotransformation products. A benzaldehyde concentration of 0.5 *g/l* permitted cell growth, 1–2 *g/l* inhibited growth but the viability was maintained and 3 *g/l* reduced the cell viability. They suggested a benzaldehyde concentration of 1 *g/l* to maintain cell viability and prolong biotransformation. Detailed investigations showed that the cells grown in the presence of lower benzaldehyde concentrations exhibited low intracellular levels of both substrate and product as compared with extracellular levels of the same. However, cells grown in the presence of a higher initial benzaldehyde concentration exhibited higher intracellular benzaldehyde content than extracellular whereas biotransformation product contents were found to be the same both extracellularly and intracellularly. Studies on the stability of purified enzyme indicated that PDC was resistant to the toxicity of benzaldehyde but not to that of benzyl alcohol. L-PAC had little toxic effect on PDC. In contrast, alcohol dehydrogenase was rapidly denatured at higher benzaldehyde concentrations and also by L-PAC (1). Their study concluded that increasing the benzaldehyde concentration decreases cell viability and halts L-PAC production and decreases sucrose metabolism.

L-PAC Productivity Several attempts have been made to investigate the optimal growth and biotransformation conditions for L-PAC production by various yeast strains as indicated in the Table 2. Control of cell metabolism and method of benzaldehyde addition have been found to influence L-PAC productivity substantially. Becvarova *et al.* (17, 29), Groger *et al.* (30) and Netrval and Vojtisek (19) observed that the combination of benzaldehyde and acetaldehyde produced L-PAC up to a level of 6 *g/l*. Addition of sodium pyruvate further improved L-PAC yield to 10 *g/l* (19). Replacement of hexose by pyruvate prevents the generation of NADH by glyceraldehyde-3-phosphate dehydrogenase reaction and depresses alcohol production with an increase in L-PAC production.

Agarwal *et al.* (20) demonstrated that benzaldehyde concentration, cell age and dissolved oxygen concentration were very critical factors for determining the biotransformation of benzaldehyde to L-PAC and by-products as well. Maximum amounts of L-PAC were produced when benzaldehyde concentrations were in the range of 10 to 16 mM. When the concentration of residual benzaldehyde dropped below 4 mM, the formation of benzyl alcohol was predominant. For optimum L-PAC production, 18–24 h old cells and 75–85% dissolved oxygen saturation were recommended. Tripathi *et al.* (34) found that the whole cells, grown at higher dilution rates in glucose limited conditions, were capable of producing higher amounts of L-PAC as compared to cells grown at lower dilution rates. It was also possible to reduce formation of the by-products by raising the specific growth rate of the cells and, presumably, altering the enzymatic balance in the cells. Agarwal and Basu (35) reported a two fold increase in L-PAC production upon fed-batch cultivation of the yeast cells. These basic studies explored the possibilities of enhancing L-PAC production.

Mahmoud *et al.* (22, 23) observed that free cells could tolerate a 0.4% benzaldehyde concentration, while immobilized cells could tolerate 0.6% without inhibiting L-PAC yield in batch biotransformations. However, 0.8% and 1.0% benzaldehyde proved toxic even for the immobilized cells. They argued that the reduction of the substrate toxicity to immobilized cells could be due to the generation of a low concentration gradient surrounding the cells. In a semi-continuous process with immobilized cells of *S. cerevisiae*, they obtained a 10 *g/l* L-PAC yield. Addition of β-cyclodextrin to the fermentation medium was further helpful in mitigating substrate toxicity and the cells could tolerate a benzaldehyde concentra-

TABLE 2. L-PAC production levels obtained with different methods

No.	Method of benzaldehyde biotransformation	L-PAC yield (*g/l*)	Organism	Reference no.
1.	Batch cultivation with multiple equal simultaneous doses of benzaldehyde and acetaldehyde	4.5	*S. cerevisiae*	17
2.	Batch cultivation with benzaldehyde alone	5.2	*S. cerevisiae*	18
3.	Sucrose, benzaldehyde and acetal dehyde added to the grown cells	6.3	*S. carlsbergensis*	19
4.	Additions of sodium pyruvate, benzaldehyde and acetaldehyde	10.0	*S. carlsbergensis*	28
5.	Additions of sodium pyruvate and, subsequently only benzaldehyde doses at intervals	10.2	*S. cerevisiae*	33
6.	Semicontinuous process with immobilized cells and benzaldehyde	10.0	*S. cerevisiae*	24
7.	Fed-batch process with free cells and benzaldehyde	12.0	*S. cerevisiae*	Chech patent 222941 (1984)
8.	Biotransformation with aldehyde resistant mutant strain grown under oxygen limited or anaerobic conditions in the presence of pyruvate and benzaldehyde	12.0	*S. cerevisiae*, *Candida flareri*	US patent no. 5312742 (1994)
9.	Additions of benzaldehyde, pyruvate, TPP and Mg^{2+}	15.0	*S. cerevisiae*	16
10.	Respiratory quotent controlled (5–7) fed-batch process with benzaldehyde, glucose, and immobilized cells	15.2	*Candida utilis*	2
11.	Partially purified PDC with 2.0 molar ratio of pyruvate to benzaldehyde	28.6	*C. utilis*	15

tion of 14 *g/l* with an increased L-PAC yield (24). It was postulated that β-cyclodextrin forms a complex with benzaldehyde and releases the substrate slowly for biotransformation. Bar (36) also observed that α-cyclodextrin was helpful in protecting yeast cells from benzaldehyde toxicity, though he only investigated the production of benzyl alcohol and benzoic acid and used a lower concentration of benzaldehyde (5 *g/l*). Tripathi *et al.* (37) demonstrated higher specific L-PAC biotransformation rates in immobilized yeast cells in a continuous fluidized bed reactor with a short residence time for the substrate.

Nikolova and Ward (38) observed that selection of the cell immobilization matrix influenced the biotransformation product to by-product ratio. Cells entrapped in poly(propylene glycol) containing polymer PU-3 yielded a high quantity of L-PAC and a low quantity of benzyl alcohol.

Seely *et al.* (U.S. patent no. 5312742, 1994) obtained enhanced L-PAC production from mutant strains of *S. cerevisiae* and *Candida flareri* cultivated under oxygen-limited or anaerobic conditions. These mutants were more resistant to benzaldehyde toxicity, formed less benzylalcohol and produced at least 12 *g/l* of L-PAC. Similar L-PAC productivity was obtained by Culic *et al.* (Czech patent no. 122941, 1984). Mochizuki *et al.* (16) obtained 15 *g/l* of L-PAC by supplementing the fermentation medium with pyruvate, TPP and Mg^{++} during benzaldehyde biotransformation. Shin and Rogers (2) reported further improvement in L-PAC production (15.2 *g/l*) from immobilized *Candida utilis* by controlling benzaldehyde concentration at 2% and maintaining a respiratory quotient of 5–7, with pulse feeding of glucose in a fed-batch process. Benzyl alcohol production was approximately three times less (5 *g/l*). Investigations on L-PAC formation by partially purified PDC from *C. utilis* achieved with 7 U/ml activity and 200 mM benzaldehyde concentration with 2 molar ratio of pyruvate to benzaldehyde produced a maximum L-PAC yield of 28.6 *g/l* (190.6 mM) (15). Immobilized PDC produced 27.1 *g/l* (181 mM) of L-PAC (39). Product formation rates were substantially high in these studies and aromatic side products were not formed. The enzyme purification process, however, is liable to increase the L-PAC production cost.

Concluding remarks Species belonging to *Saccharomyces* and *Candida* were found to be the most suitable for L-PAC production. L-PAC formation efficiency was determined by the method of benzaldehyde biotransformation. Application of free and immobilized cells offered the advantage of pyruvate generation by the yeast itself. For optimal pyruvate generation, regulation of the respiratory quotient (RQ) was essential during cell growth phase. However, with this system, significant amount of benzyl alcohol was produced via the action of alcohol dehydrogenase and or other oxidoreductase(s) of the cells. L-PAC production without the formation of aromatic by-products could be very significantly enhanced with the use of purified PDC making product isolation easier. However, a higher supply of pyruvate was essential to maximize the yield of L-PAC resulting in the formation of acetoin and acetaldehyde as by-products, as only 50–60% of the possible potential theoretical conversion of pyruvate to L-PAC has been achieved. The cost of enzyme isolation, purification and pyruvate addition may render the process uneconomical. An appraisal of the commercial viability of the process is required to evolve a suitable technology for L-PAC production.

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