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Production of L-Phenylacetyl Carbinol by Whole Cells of *Hansenula polymorpha*

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Conversion of benzaldehyde to L-Phenylacetyl carbinol (L-PAC) was achieved with growing cells of *Hansenula polymorpha* in different reactors. Product formation increased (31%) with the subsequent initial reuses of the entrapped cells. Biomass production and PAC formation depleted (40 and 57%, respectively) after 4-5 continuous growth and biotransformation cycles. With the regeneration of the biocatalysts, catalytic activity of the cells was resumed. The highest yields were in a stirred tank reactor (29 g PAC) from 77 g benzaldehyde with 14 repeated uses of entrapped cells.

Key words: Benzaldehyde, biotransformation, *Hansenula polymorpha*.

L-Phenylacetyl carbinol (PAC) is an intermediate for commercial chemical synthesis of L-ephedrine, an ingredient of pharmaceutical preparations used as anti asthmatics and decongestants (Rogers *et al.* 1995). Certain microorganisms transform aromatic aldehyde substrates to produce acetyl aromatic carbinols, in presence of pyruvate generated during glycolysis (Long & Ward 1989a). This reaction is catalyzed by pyruvate decarboxylase (PDC). PAC production by growing cells of various yeast species (Netrval & Vojtisek 1982), whole cells of *Saccharomyces cerevisiae* and *Zygosaccharomyces rouxii* in growth medium (Tripathi *et al.* 1988, Mahmoud *et al.* 1990a) purified PDC from *Candida utilis* and *Zymomonas mobilis* (Shin & Rogers 1996, Goetz *et al.* 2001) and cell extracts from filamentous fungi of *Rhizopus* and *Fusarium* sp. (Rosche *et al.* 2001) have been demonstrated with various degrees of success. Commercial production of PAC should involve a fermentative growth phase to produce cell biomass with induced PDC activity followed by biotransformation phase with programmed feeding of benzaldehyde (Rogers *et al.* 1989).

Bioprocesses carried out with viable whole cells by entrapment represent an efficient system by simultaneously performing mass transfer, substrate conversion and growth (Doran & Baily 1986). Growth of whole cells, because of their altered metabolism, has been exploited for the improvement of bioprocess productivity in many instances as the Whole cells can proliferate in or on support material and can regenerate and maintain catalytic system of the cells for many repeated uses (Tanaka & Nakajima 1990). Remarkably, cells detached from their support and resuspended in medium exhibit the same altered properties as of whole cells (Doran & Baily 1986).

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Growing whole cells have been used successfully for the production of amino acids, antibiotics and biotransformation of steroids.

MATERIAL AND METHODS

Microorganism, culture condition

A strain of *Hansenula polymorpha*, isolated in our laboratory, was grown on YEMA/ agar slants. For growth and benzaldehyde biotransformation a defined medium developed by Agarwal *et al.* (1987) was used. Cells were centrifuged and washed with phosphate buffer (0.1 M, pH 6) and distilled water.

Benzaldehyde biotransformation and PAC estimation

Biotransformation studies were carried out in growth medium under aseptic conditions with different reactors as described below.

Stirred reactor

One liter, bench-top magnetic flat blade turbine type (Gallenkamp FBL-280) modular fermenter (250 ml working volume) was run at 150 rpm and 0.75 vvm aeration.

Fluidized air-bubbled cone reactor

One liter, cone reactor (250 ml working volume), bubbled with 0.75 vvm bottom aeration, was used for the study.

Fluidized air-bubbled column reactor

A30×7 cm glass column (500 ml working volume) with 0.75 vvm bottom aeration was used for the study.

Rolling shaker reactor

One liter 21 × 10 cm narrow mouth bottle (250 ml working volume) was rolled on Bellco rolling shaker at 10 rpm. No aeration was done.

Packed column reactor

A 30 × 7 cm column (one liter working volume), with medium circulation (5 ml min^{-1}) was used for the study. No aeration was done.

In all the experiments, cell beads (50 g l-1 w/v) were used for inoculating the medium and growth was continued for 20 h biomass (dry wt) was measured and biotransformation was commenced *in situ* by adding five doses of benzeldehyde, 1.1 g l-1 (10.6 mM) each, at hourly intervals. Biotransformation was continued up to 10 h (counted with the addition of first dose). Fermented broth was extracted with benzene and concentrated for isolating PAC. Quantitative estimation of PAC was done by HPLC using a PAC 18 300 Å column (3.9 × 30 cm, 15 μm spherical) and the mobile phase was acetonitrile/ water (30:70 v/v) at 1.5 ml min^{-1}. The eluate was monitored at 252 nm. The retention times of benzoic acid, benzyl alcohol, PAC and benzaldehyde were 2.3, 3.1, 3.6 and 4.9 min, respectively.

RESULTS AND DISCUSSION

Generation of cell biomass from whole cells

Multiplication of the whole cells was initiated by inoculating the reactors and biomass (dry wt) was measured after 20 h of growth. As shown in Tables 1-3, growth response of the culture differed substantially with various reactors. Cultivation with stirred and air-bubbled fluidized cone and column reactors produced higher cell biomass. Medium composition, growth condition and culture metabolism significantly influenced PAC production. Substantial pyruvate accumulation has been reported under partial fermentation condition (Shin & Rogers 1995, Tripathi *et al.* 1997a). 0.75 vvm aeration provided during growth to facilitate pyruvate accumulation and induce PDC activity for PAC production.

Growth proceeding under progressive anaerobic conditions induces PDC for pyruvate utilization to form side products, e.g. ethanol via acetaldehyde formation, and enhances alcohol dehydrogenase activity to produce more benzyl alcohol. High aeration (1 vvm) also decreases PAC production because of a low respiratory quotient (Rogers *et al.* 1998). Nikolova & Ward (1992) demonstrated that each step of glycolytic path may be rate limiting for PAC formation. Tripathi *et al.* (1988) observed that harvested free whole cells of *Hansenula polymorpha*, grown at higher specific growth rate in chemostat under glucose limited conditions, produced more PAC and less benzyl alcohol. Cell growth with the stirred and air-bubbled fluidized reactors was higher and biomass production was more (Tables 1–3). Additional aeration was not possible with roller drum and packed column reactors and biomass production with these systems were also low.

Biotransformation of benzaldehyde

High PAC formation was associated with a high level of biomass production. Benzaldehyde

Table 1. Biomass and PAC production by whole cells *Hansenula polymorpha* with aerated bioreactors

Cycles	Stirred reactor			Fluidized cone reactor		
	Dry wt (g l-1)	PAC (g l-1)	PAC (g l-1 g^{-1} Cells)	Dry wt (g l-1)	PAC (g l-1)	PAC (g l-1) g^{-1}
1	3.9	2	00.09	3.8	1.8	0.086
2	4	2.5	0.11	4	2.1	0.098
3	4.1	2.6	0.11	4.4	2.8	0.118
4	4.4	2.3	0.09	3.6	1.9	0.096
5	2.5 [a]	1.2	0.08	2.3 [a]	0.8	0.063
6	4.6	2.8	0.11	3.8	2	0.100
7	4.8	3.1	0.12	4.1	2.2	0.098
8	4.2	2.6	0.11	4.4	2.7	0.114
9	2.4 [a]	1.3	0.09	3.4	1.3	0.097
10	4.5	2.1	0.09	1.4 [a]	0.7	0.097
11	4.6	2	0.07	4	2.5	0.116
12	3.7	1.8	0.09	3.2[a]	1.2	0.069
13	3.4 [a]	0.7	0.03	3.4	1.6	0.087
14	3.8	1.7	0.08			

Table 2. Biomass and PAC production by whole cells *Hansenula polymorpha* indifferent intervals

Cycles	Fluidized column reactor			Roller drum reactor			Packed column reactor		
	Dry wt (g l-1)	PAC (g l-1)	PAC (g l-1	Dry wt (g l-1)	PAC (g l-1)	PAC (g l-1) g^{-1} Cells)	Dry wt (g l-1)	PAC (g l-1) Cells)	PAC (g l-1)
1	3.2	1.3	0.07	3.9	2.01	0.09	1.5	0.6	0.07
2	4.2	2.2	0.09	3.8	2.18	0.1	3.4	1.6	0.08
3	2.2	0.9	0.07	3.4	1.21	0.09	2.7	1.1	0.07
4	4.4	2.3	0.09	2	1.02	0.09	1.4	0.4	0.06
5	3.8	1.7	0.08	3.6	2.08	0.1	3.1	1.2	0.07
6	2.6	0.9	0.06	3.3	1.84	0.07			
7	3.4	2	0.12	2.7	1.06	0.07			
8	2.8	1.6	0.1	1.9	0.69	0.06			
9	2.2	1	0.08						

Table 3. PAC production with different bioreactors and biotransformation cycles

S. No.		Stirred Reactor	Fluidized cone Reactor	Fluidized column Reactor	Roller drum Reactor	Packed column Reactor
1	PAC (g l-1)	29.2	23.9	14.1	12	5
2	Biotransformation cycles	14	13	9	8	5
3	Biotransformation hours	140	130	90	80	50
4	PAC (g l-1 h-1*)*	0.21	0.18	0.15	0.51	0.1
5	Dry wt (g l-1)	56	41	30	25	12
6	Dry wt (cycle-1)	3.9	3.5	3.2	3	2.4
7	PAC (g-1 cells)	0.009	0.09	0.08	0.09	0.07
8	PAC (cycle-1 g l-1)	2	1.8	1.5	1.5	1

concentration and its feeding mode plays critical role in biotransformation process. Toxicity of the substrate and product/by-product; exhaustion of pyruvate; depletion of PDC activity etc. have been found to control PAC formation (Tripathi *et al.* 1997b). Agarwal *et al.* (1987) demonstrated optimum PAC production with 1.1 g benzaldehyde l-1. Rogers *et al.* (1998) observed that programmed feeding of benzaldehyde to maintain it at 1–2 g l-1 favoured higher prolonged PAC production by maintaining cell viability. Keeping in view above mentioned reports we studied PAC production with five 1.1 g l-1 hourly feeds of benzaldehyde (giving 5.5 g l-1). Rates of benzaldehyde biotransformation were rapid during first 2–3 h and continued up to 5–9 h (Mahmoud *et al.* 1990b). No increase in PAC production beyond 10 h with this concentration of benzaldehyde. Higher PAC formation was recorded with stirred reactor followed by air-bubbled fluidized cone and column reactors.

Extended exposure to benzaldehyde and/or increasing concentration of benzyl alcohol and PAC has been found to affect cell viability adversely (Roger *et al.* 1998). Loss of cell viability and biocatalytic activity of the entrapped cells have also been attributed to the deposition of inactivated cells on the bead surface (Tanaka & Nakajima 1990). Reduction in PAC production was observed with repeated biotransformation batches in all the reactors. Reactivation of the biocatalyst was necessary for its prolonged use.

Reactivation of the biocatalyst

Growth of the whole cells for one cycle, without benzaldehyde biotransformation, revived the biocatalytic activity of cells. Biocatalysts performing bioconversion in all the reactors required reactivation cycles at different intervals (Tables 1 & 2). Whole cells of *Streptomyces rimosus* are reported to produce Oxytetracycline continuously in air-bubbled reactor for 35 d with intermittent reactivation of the entrapped cells with saline or 70% (v/v) ethanol (Tanaka & Nakazima 1990). Maximum yield of PAC was obtained in stirred reactor (29 g) with 77 g of benzaldehyde where entrapped cells were used for 14 cycles.

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