INTERNATIONAL JOURNAL OF CURRENT RESEARCH IN CHEMISTRY AND PHARMACEUTICAL SCIENCES

(p-ISSN: 2348-5213: e-ISSN: 2348-5221)
www.ijcrcps.com

Research Article

OPTICALLY ACTIVE INTERMEDIATE OF PYRUVATE DECARBOXYLASE TO PHENYLACETYLCARBINOL AND ANALYSIS OF ENANTIOMERIC PURITY

SURESH K* NARAYANAN MATHIYAZHAGAN AND R.SELVAM

PG & Research Centre in Biotechnology, M.G.R. College, Hosur 635 109, Tamil Nadu, India

*Corresponding Author

Abstract

The effect of process parameters on the biotransformation of benzaldehyde to L- phenylacetylcarbinol (L-PAC).The maximum yield of L-PAC obtained was (331 mg) per 100 ml biotransformation medium (glucose 3%, peptone 0.6% and at pH 5.0) from 600 mg of benzaldehyde with 10 h of reaction at 34°C. Growing the organism in presence of 3% glucose reduced the biotransformation time to 120 min. During fermentor (3 L) studies of L-PAC production, agitation rates (250 rpm) and aeration rates (2.50 l/min) were optimised. The value of dissolved oxygen in fermented broth was observed to be almost half of the growth phase. A noticeable increase in L-PAC production was achieved when seed cultures were developed through growth recycling. Chiral HPLC analysis of purified L- PAC and PAC-diol showed 99% enantiomeric purity. The combined process is highly valued as more than 99% optically active intermediate *R*-PAC is produced during the biological step thereby eliminating the additional costs in racemic separation often encountered in chemical processes

Keywords: Phenylacetylcarbinol,HPLC, enantiomeric purity.

Introduction

Phenylacetylcarbinol (L-PAC), also known as 1-hydroxy-1-phenyl-2-propanone or Neuberg's ketol (90-63-1) or 1-hydroxy-1-phenylacetone or a-hydroxybenzylmethyl ketone. It acts as a key intermediate for the synthesis of L-ephedrine, pseudoephedrine, nor ephedrine, nor-pseudoephedrine as well as adrenaline, amphetamine, methamphetamine, phenylpropanolamine and phenyl-amine. The biotransformation of pyruvate and benzaldehyde to PAC is catalyzed by the cytosolic enzyme pyruvate decarboxylase (PDC). Pyruvate is first converted to acetaldehyde via a non-oxidative decarboxylation reaction. The resulting acetaldehyde then ligates with benzaldehyde to form PAC. Thiamine pyrophosphate (TPP) and Mg2+ are required as cofactors (**Ellaiah and Krishna, 1987**).

PAC is currently industrially produced using yeast culture fermentation by the biotransformation of benzaldehyde, giving industrial relevance to research into whole cell biotransformation for PAC production. Both active microbes as well as purified enzymes can be used as biocatalysts, with whole cell biotransformation remaining more industrially appealing. Whole microbes contain the cellular machinery and enzymes required to perform complex biotransformations. Purified enzymes may be used to catalyze a single step conversion, but as reaction complexity increases, more enzymes and cellular components must be added to the medium. Additionally, whole cells require less catalyst preparation than enzymatic processes as the required enzymes do not need to be purified. However, enzymatic processes offer the benefit of reducing by-product formation by controlling the active enzymes present in the system, whereas whole cell processes may have by-products formed in alternative metabolic pathways present in the microbe. Whole cells may also limit access of enzymes to substrate in the medium, but this may also serve to protect enzymes from inhibition.

The primary goal of the present work was to evaluate different Pyruvate Decarboxylase preparations in *Hansenula polymorpha* for enzymatic production of

Phenylacetylcarbinol. The criteria used for these evaluations are cultivation conditions for optimum production of Pyruvate dercarboxylase and study of process parameters for biotransformation with whole cell extract for production of phenylacetylcarbinol.Finally**,** the elution order and the enantiomeric excess of an enantiomeric sample of 1-hydroxy-1-phenyl-propan-2-one was determined by application of the optimized method.

Materials and Methods

Screening of yeast isolate for PAC production

The yeast strains were primarily screened for acetaldehyde tolerance after (**Agustin** and **Marcel, 2004)**.The cultures obtained after acetaldehyde-based screening was further tested by secondary screening in terms of Phenylacetylcarbinol production using submerged fermentation in 1L Erlenmeyer flask (**Shukla *et al.,* 2001**). The cells were allowed to adapt the medium for one hour in a rotary shaker incubator at 300 rpm. Five sequential doses (each 1.20 ml/l acetaldehyde + 1.20 ml/l benzaldehyde) were added after 1.0 h intervals. The process was terminated after 1.0 h of last dose and broth was used for further analysis.

Fermentation Process Conditions

Pyruvate Decarboxylase enzyme was produced via a yeast fermentation of glucose-based optimized media. Various scales and methods of fermentation were employed: (I) 0.5 L shake flask fermentations to culture of *Hansenula polymorpha*, (II) 3 L fermentation condition on fermentor. Fermentor, shake flask (Erlenmeyer), apparatus and optimized media used were sterilized by autoclaving at 121^0C for 20 mins. Media mixing and yeast (CNC-1) culturing processes were performed in a sterile environment.

Shake flask fermentation

In this fermentation 5% of yeast culture (*Hansenula polymorpha* CNC-1) was inoculated into 250 mL medium in a 500ml baffled Erlenmeyer flask. This pre-seed inoculum was grown in an orbital shaker at 30^0C and 300 rpm for 12 – 16 hours to reach an exponential phase (OD660 nm = 7−9). Processed medium in a 500 ml baffled flask and harvested and estimate.

Fermentor

Hansenula polymorpha was grown in 5L bioreactor (**Sandford, 2005**). The cultivation was started by inoculating 5% of seed culture (exponential phase) into sterile cultivation medium (addition of inoculum resulted in concentrations specified previously). The culture conditions were controlled at 34°C, pH 5.0 (using 4M H_3PO_4/KOH) and an airflow rate of 1.0 vvm. In the initial growth phase (11-13 h), the culture was maintained in fully aerobic conditions with a stirrer speed of 250 rpm. This was followed by switching to partially aerobic conditions with airflow rate and stirrer speed of 0.5 vvm and 250 rpm respectively for the next 2.5-4.0 h, when the level of DO dropped below 5% air saturation, to induce Pyruvate Decarboxylase production The harvested cells were washed twice with RO water prior to resuspension in breakage buffer to give a cell dry weight of approximately which corresponded to an optical density at 660 nm (OD660).

Whole cell Pyruvate Decarboxylase

The yeast (CNC-1) culture was harvested from both shake flask and fermentor then centrifuged at 10,000 rpm for 15 min and washed with chilled RO water for *Hansenula polymorpha*. The centrifugation and washing process were repeated three times. The washed pellet is referred to as a whole cell Pyruvate Decarboxylase used for further studies.

Recovery of Phenylacetylcarbinol

Four different solvents i.e. dichloromethane, diethyl ether, ethyl acetate and toluene were employed (sample to solvent ratio of 1:5). The sample to solvent ratio was optimized by varying the solvent volume (1-5 times of the sample). The frequency (once,twice or thrice) of the same volume of the solvent was determined by pooling up the rich toluene (toluene with extracted Phenylacetylcarbinol).

Analytical Techniques

Estimation of Phenylacetylcarbinol from the fermentation liquid, a 2 ml sample was centrifuged at 6000 *g* for **10** mm at 4°C. Supernatant was extracted four times with equal quantities of ether. Combined extracts were concentrated by evaporation at room temperature.

Colorimetric determination of L-Phenylacetyl carbinol

The colorimetric method described by **Groger and Erge,** (**1965**) was used in the determination of PAC. To the sample extract 500 µl was added 1000 µl alpha-naphthol and 1500 µl potassium hydroxide creatine solutions were pipetted out in a glass cuvette (light path length, λ = 1.0 cm) The cuvette was incubated in the spectrophotometer at 25°C for 5 min to achieve temperature equilibration and then

recorded absorbance at 580 nm (blank). Standard solution *(5* ml) in 5% ethyl alcohol (containing 30-400 ***pg*** PAC) was mixed with I ml Keratin solution (0.5%) and **1** ml a-naphthol solution (I g a-naphthol in 20 ml 2.5N NaOH).

Gas chromatography (GC)

Two microlitres of sample was injected into a gas chromatography through a 10 µl glass syringe. The gas chromatography has column and its operating conditions as follows: column material, stainless steel (2 m by 4.5 mm); packing material, 30% Silicon SE-30 on Chromosorb WHP in the mesh range of 60-80 µm; carrier gas, nitrogen (flow rate,30 cm3/min); ignition source, mixture of air and hydrogen; oven temperature, 145°C (isothermal); injector temperature, 180°C; flame ionization detector dectector temperature, 180°C. The gas chromatography was equipped with a Class-LC 10 integrator.

Residual benzaldehyde, benzyl alcohol, Phenylacetylcarbinol showed their peaks at different retention times i.e. 1.55-2.05 min, 2.58-3.03 min and 5.57-6.02 min, respectively. The peak areas were used to determine percentages of the substances by normalization method of Velankar and Heble (2003) before material balancing on the basis of benzaldehyde used. Pure form of Phenylacetylcarbinol were applied as internal standards.

***R*- and *S*- Phenylacetylcarbinol determination**

HPLC was used also to differentiate the *R*- and *S*-Phenylacetylcarbinol as well as to determine the respective concentrations. Comparison of relative peak areas of both enantiomers at 283 nm was used to calculate the enantiomeric excess (ee) of *R*-Phenylacetylcarbinol. The HPLC column Chirael OD (10 µm particle size, 250 mm length, 4.6 mm internal diameter). The mobile phase was prepared freshly based on the following composition 950 mL hexane, 50 mL isopropanol and 1 mL formic acid. The flow rate for isocratic operation was 0.8 mL/min with the run time of 30 min. The injection volume was 1 µL. The sample was prepared by vortexing 200 µL of the aqueous sample with 200 µL of ethylacetate. After separating the aqueous and organic phases by centrifugation, 150 µL of the top organic phase containing extracted Phenylacetylcarbinol was placed into a clean 1.5 mL tube and was evaporated under vaccum for 15 min at room temperature to remove ethylacetate. The sample was resuspended in 300 µL hexane, vortexed and centrifuged to remove solids. The supernatant was analysed by HPLC.

Results

Amongst the isolates sixteen was absence of grow on acetaldehyde (1.5 g/l) added YEMA plates during primary screening in Table 4.1. Out of remaining 13 isolates (acetaldehyde tolerant), 4 gave almost negligible amounts of Phenylacetylcarbinol production (≤0.6 g/l) in glucose/peptone medium using shake flasks fermentation, respectively. Four dominative isolates were producing Phenylacetylcarbinol, the product in the range of 1.42-3.12 g/l in Table 4.2. However, sugar consumption and dry biomass ranged from 1.64-2.74 and 0.24-0.82 g/l, respectively. The Isolate of CNC-1 produced higher Phenylacetylcarbinol (3.12g/l) shown as Figure 4.1 than the other isolates respectively and CNC-1 was selected for further studies.

Table 4.1. Isolation sample and primary screening of Acetaldehyde tolerant

S.No	Isolation Samples	Absence of Acetaldehyde tolerant	Presence of Acetaldehyde tolerant
		Yeast isolate - CNC	
1	**SCJ-** 1	CNC-2, CNC-4, CNC-5	CNC-1, CNC-3
2	**SCJ -**2	CNC-9, CNC-12, CNC-13, CNC-14	CNC-6,CNC-7, CNC-8, CNC-10, CNC-11
3	**SCJ -**3	CNC-17, CNC-18, CNC-20	CNC-15, CNC-16, CNC-19
4	**SCJ -**4	CNC-21, CNC-22, CNC-23	CNC-24
5	**SCJ -**5	CNC-25, CNC-26, CNC-27	CNC-28

The comparison of glucose consumption and biomass formation by the isolate CNC-1 was undertaken. The rate of sugar consumption of CNC-1 was found lower compared to other isolates. Total sugar consumption of CNC-1 1.81g/l and the rate of biomass formation of CNC-1 were found higher compared to other isolates. Total biomass of CNC-1 0.82g/l, the phenylacetylcarbinol production rates are higher than other isolate CNC1 (3.12g/l) in Figure 4.1.

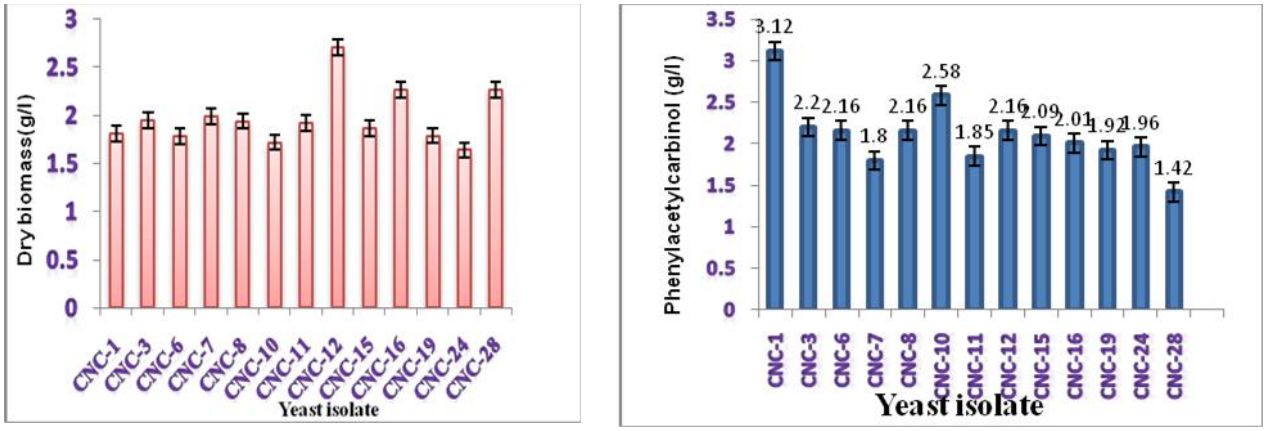

Figure 4.1 dominative Phenylacetylcarbinol producing Yeast strain in glucose medium using SSF.

Table 4.2 Optimization of fermentation conditions

Component Pyruvate decarboxylase Extract	Fermentative phase	
	Shake flask	Fermentor
	Specific enzyme activities (U/mg protein) Pyruvate decarboxylase	
Whole cell PDC	2.36±0.16	2.94±0.11

Comparatively higher values of Pyruvate decarboxylase were found in whole cell PDC for (Shake flask 2.36±0.16 U/mg protein), (Fermentor 2.94±0.11 U/mg protein).Table 4.2. Comparison in phenylacetylcarbinol extraction was investigated using different solvents. The results are given in Table 4.26a &(Figure 4.3 a, b, and c. Comparatively higher value of distribution coefficient (41.53) and percent extraction (97.64) was observed when toluene was employed. The data shown in Table 4.3 b highlight the effect of volume ratio (fermented broth to toluene) on phenylacetylcarbinol extraction. The higher value of distribution coefficient (48.12) and % extraction (98.0) was found after the use of 1:2 ratios. The data presented in Table 4.26cshow the efficiencies of different frequencies of toluene usage. Comparatively higher value of distribution coefficient (48.48) and % extraction (98.11) was obtained after the use of single volume of toluene.

Table 4.3.a Comparative efficiencies of different phenylacetylcarbinol extracting solvents

Solvent	Distribution coefficient (k)	% Extraction
Dichloromethane(control)	40.16	97.57
Diethyl ether	28.0	96.55
Ethyl acetate	22.18	95.61

Table 4.3.b Effect of fermented broth to toluene ratio on phenylacetylcarbinol extraction

Fermented broth/ toluene	Distribution coefficient (k)	% Extraction
1:5 (control)	41.56	97.70
1:4	44.51	97.80
1:3	46.20	97.91
1:2	48.10	98.01
1:1	15.75	94.1

Table 4.3.c Effect of usage frequency of toluene on phenylacetylcarbinol extraction

Frequency (Times)	Distribution coefficient (k)	% Extraction
1	41.57	97.72
2	44.61	97.88
3	46.23	97.92
4	46.21	97.89

After protein removal Phenylacetylcarbinol was extracted into chloroform and was analysed with the chiral column for estimation of the enantiomeric excess .the retention time for the R and S enantimers under these GC conditions is 9.90 and 12.47 min respectively. It conformed that the peaks to emerge corresponds to the S- enantiomer and the second one to the R- enantiomer. A typical reaction mixture obtained from the reaction in Ethyl acetate was extracted using Ethyl acetate at a pressure of 2009 psi and a temperature of 40.degree. C. for a duration of 10 minutes. Subsequent gas chromatography analysis indicated that the phenylacetylcarbinol had been successfully isolated from the original reaction mixture. The vessel was then stirred at room temperature for 24 h. After 24 h, the reaction vessel was slowly de-gassed. The vessel contents and residue was washed three times with diethyl ether and filtered. Gas chromatography analysis revealed 12% conversion to phenylacetylcarbinol. Finally This vessel was pressurised to 1500 psi by pumping dried liquid carbon dioxide into the vessel. The vessel was then stirred at room temperature for 3 h. After 3 h, the reaction vessel was slowly de-gassed. The vessel contents and residue was washed three times with dichloromethane and filtered. Gas chromatography analysis revealed 51% conversion to phenylacetylcarbinol. Chiral GC showed a ratio of 97.57, 96.55% ee. Figure 4.2

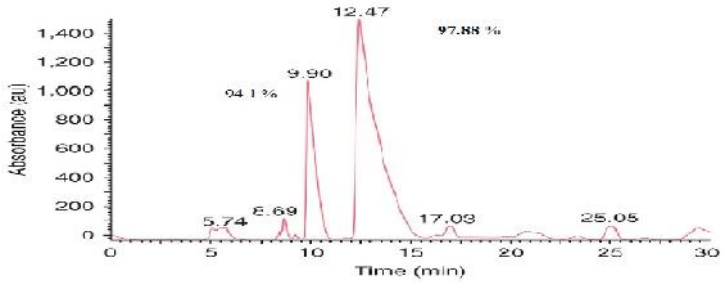

Figure 4.2 Chiral analysis of Phenylacetylcarbinol by GC

To confirm the optical purity of the compounds, they were subjected to chiral HPLC analysis. The chromatograms after chiral separation showed that both phenylacetylcarbinol produced and purified had more than 99% enantiomeric excess giving a single peak with same optical rotation as analysed by chiralyzer diode array detector Figure 4.3.

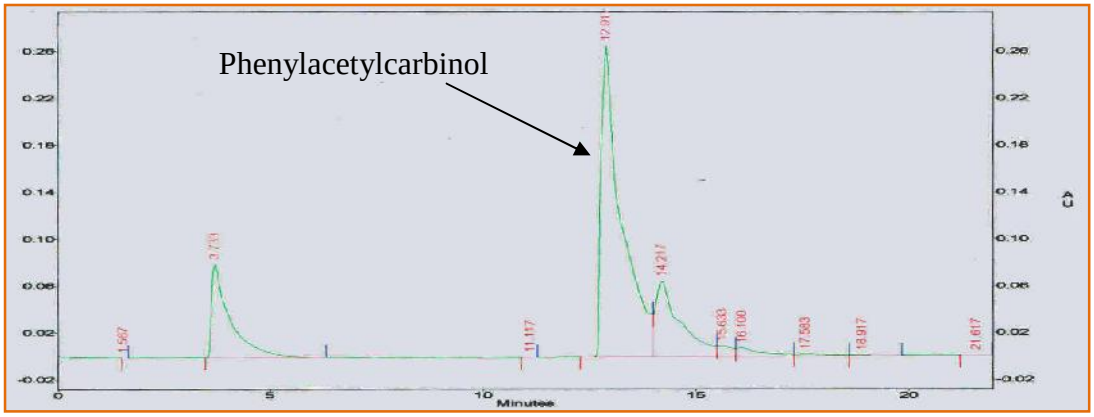

Figure 4.3. HPLC chromatogram showing the reaction for Phenylacetylcarbinol. Revealed a reaction had occurred, leading to a small conversion of Phenylacetylcarbinol to a product eluted at 13 mins. Enantiomeric purity determination of 99% (R) enantiomer.

After carrying out the screening for acylation biotransformation of Phenylacetylcarbinol, it was decided to scale up these reactions in order to isolate the products identified by HPLC analysis. The enantiomeric excess for the cyanohydrins as determined by chiral HPLC was compared with that obtained by mixing the optically pure (*S*)-phenylacetylcarbinol (e.e..99%) with the (*R*)-phenylacetylcarbinol (e.e.73.1%) in various proportions (from 1: 1 to 1:5). The enantiomeric excess values expected on the basis of optical rotations were reasonably close but it should be pointed out that purification by column chromatography was a long and tedious process, and although the presence of phenylacetylcarbinol.

Discussion

In the previous study, the rates of sugar uptake, biomass formation and L-PAC production decreased while benzyl alcohol increased gradually using GCU-36. **Robert *et al.*,** (**1990**) pointed out a gradual decrease in biomass formation due to benzaldehyde toxicity. The toxicity, beside many other factors also seems to be the major cause of decrease in sugar uptake. The decreasing rate pattern of L-PAC during present study is substantiated with (**Long** and **Ward, 1989**). The decrease in the rate of L-PAC production was likely to be due to deficiency of pyruvates, PDC inactivation or both. Increase in benzyl alcohol with decrease in L-PAC production marks the production of the former product at the expense of the later, suggesting induction of ADH enzyme at PDC inactivation. A similar kind of finding of rate of benzyl alcohol production has already been reported by **Shin** and **Rogers,** (**1995**). In present study the isolate CNC-1, identified as *Hansenula polymorpha* was considered as the best strain on account of quantitative assay and time course study.

In previous study higher value of L-PAC (8.33 g/l) was found at pH 5.0. The concentrations decreased gradually at lower or higher pH values. Benzyl alcohol production increased with increase in pH, becoming maximal (2.17 g/l) at pH 6.0. The minimal concentration of residual benzaldehyde (0.14 g/l) was observed at pH 5.0.The better L-PAC productivity signifies the optimal time intervals towards the minimal production of side-products e.g. benzyl alcohol. A higher L-PAC production (8.33 g/l) was noticed at optimal initial pH 5.0, suggesting positive changes in ionization of amino acids at the active site of PDC. Similarly, the lowest PAC value at pH 6.0 is likely to be due to the breakage of bonds in the PDC and subsequent denaturization. Benzyl alcohol production decreased gradually at pH (6.0-4.0), indicating the gradual inactivation of ADH enzyme at the pH values. However, the present work is not in good agreement to **Hauer *et al.,*** (**2006**).A slight rise in pH was found in in the fermentation process during present study. It may be due to uptake of protons by pyruvate to make L-PAC and acetaldehyde to make acetoin (**Rosche *et al*., 2005**).

The methods reported for estimation of L-PAC and its byproducts include spectrophotometric (**Gupta *et al.,* 1979**), polarographic and titrimetric methods (**Smith** and **Hendlin, 1953**), which suffer from poor sensitivity due to possible interferences, especially while analysing a complex system like biotransformation broth. In older methods U.V. absorbance as well as the iodoform method have been used for estimation of benzaldehyde and benzyl alcohol (**Smith** and **Hendlin, 1954**). Application of the Voges Prauskaur reaction has been tried for estimation of L-PAC (**Groeger** and **Erge, 1965**). According to **Nikolova** and **Ward,** (**1991**) a GC method with a DB-1- 15XW column having 1:5 lm 100% methylpolysiloxane film could give retention times for benzaldehyde, benzyl alcohol and L-PAC of 1.32, 2.19 and 5.45 min respectively. Separation of L-PAC has also been reported with a column of silica megabore coated with 1 lm thickness of 25% cynopropyl, 25% phenyl, 50% methyl polysiloxane and helium as a carrier gas **Nikolova** and **Ward,** (**1992**). Estimation of benzaldehyde and benzyl alcohol on a GC using helium as a carrier gas and colorimetric estimation of L Pac also have been reported **Long** and **Ward, (1989).**

References

Agustin,A. and Marcel,O. 2004. Exposure of Saccharomyces cerevisiae to acetaldehyde induces sulfur amino acid metabolism and polyamine transfer genes, which depend on Met4p and Haa1p transcription factors, respectively. App. Environ.Microbiol **70**(4): 1913-1922

Ellaiah,P. and Krishna,K.T. 1987. Studies on the production of phenylacetylcarbinol from benzaldehyde by Saccharomyces cerevisiae. Indian Drugs **24**: 192-195.

Groeger,D., Schmauder,H.P. and Mothes, K. 1966 Isolation of L-phenyl acetyl carbinol Zeitschrift fuer Allgemeine Mikrobiologie **6**: 275-287 (C.A.-67: 81081k).

Groger , D. and Erge, D. 1965. Analysis for PAC. *Die Pharrnazie.* **20;** 92

Gupta,J.K., Helding, L.G. and Jorgensen,O.B. 1976. Effect of sugars, hydrogen ions concentration and ammonium nitrate on the formation of citric acid by Aspergillus niger. Acta. Microbiol. Acad. Sci. Hung **23**(1): 63-67.

Hauer,B., Breuer,M., Rogers,P., Sandford,V. and Rosche, B. 2006. Process for production of R-

phenylacetylcarbinol by an enzymatic process in two phase system. US Patent No. 7,074,966

Long,A. and Ward,O.P. 1989. Biotransformation of aromatic aldehydes by Saccharomyces cerevisiae: Investigations on reaction rate. J. Indust. Microbiol **4**: 49-53.

Nikolova,P. and Ward,O.P. 1991. Production of L-phenylacetylcarbinol by biotransformation: product and byproduct formation and activities of keto enzymes in wild type and ADH-isoenzyme mutants of Saccharomyces cerevisiae. Biotechnol Bioengin **20**: 493-498.

Robert,W.C., Mahmoud,W.M. and Abdul-Halim,M.M.E. 1990.Production of L-phenylacetylcarbinol by immobilized yeast cells. II. Semicontinuous fermentation. Biotechnol. Bioengin **36**: 55-63.

Rosche, B., Sandford, V. and Breuer, M. 2002. Enhanced production of *R*-phenylacetylcarbinol (*R*-PAC) through enzymatic biotransformation Journal of Molecular Catalysis B: Enzymatic. (20) 2; 109–115

Sandford,V., Breuer,M., Hauer,B., Rogers,P.L. and Rosche,B. 2005. (R)-phenylacetyl carbinol production in aqueous/organic two-phase system using partially purified pyruvate decarboxylase from Candida utilis. Biotechnol. Bioengin **91**(2): 190-198.

Shin, H. S. and Rogers, P. L. 1995. Biotransformation of benzaldehyde to phenylacetylcarbinol, an intermediate in L-ephedrine production, by immobilized Candida utilis. Applied Microbiology and Biotechnology **44** : 7-14.

Shukla, V. B. and Kulkarni, P. R. 2000. L-Phenylacetylcarbinol (L-PAC): biosynthesis and industrial applications. World Journal of Microbiology and Biotechnology **16**:499-506.

Smith, P. F. and Hendlin, D. 1953. Mechanism of phenylacetylcarbinol synthesis by yeast. J. Bacteriol **65**: 440-445.

