Pak. J. Bot., 44: 361-364, Special Issue March 2012.

STUDIES ON THE PRODUCTION OF *L*-PHENYLACETYLCARBINOL BY *CANDIDA UTILIS* IN SHAKE FLASK

MARIYAM ABID KHAN, IKRAM-UL-HAQ, MUHAMMAD MOHSIN JAVED*, M.A. QADEER, NAEEM AKHTAR AND SYED ALI IMRAN BOKHARI

Institute of Industrial Biotechnology, GC University, Lahore, Pakistan
**Corresponding author E-mail: mmj_bot@yahoo.com; Ph No. 042-9911634*

Abstract

The present study reveals the production of *L*-phenylacetylcarbinol (*L*-PAC) by *Candida utilis* in 250ml shake flask. Different fermentation media supplemented with various carbon sources were optimized along with different incubation temperatures and inocula to achieve the optimum level of biomass. M1 medium containing (g/L) clarified molasses (30.0 Brix) 250.0, urea 10.0 and $MgSO_4$ 20.0 (pH 6.0) gave the optimum biomass level ($240x10^6$ cells/ml) after the inoculation of 14 hours old vegetative inoculums (15% v/v containing $240x10^6$ cells/ml) after 6.0 hours at 30^0C. Biotransformation of benzaldehyde into *L*-PAC was then carried out with different dose patterns along with supplementation of acetaldehyde. Maximum *L*-PAC production (4.15 g/L) was observed when 316µl of benzaldehyde was fed in six instalments such as 68, 62, 56, 50, 43, 37 µl in equal interval of 60 minutes at 30^0C with orbital shaking of 150rpm.

Introduction

L-PAC acts as a chiral precursor of *L*-ephedrine, pseudoephedrine and norephedrine. *L*-ephedrine is a major component of many medicines used as decongestants and antiasthamatics (Shin & Rogers, 1995). *L*- Ephedrine can be prepared by three methods: conventional extraction from species of Ephedra plant, a synthetic chemical method which includes resolution of the racemic mixture, and a process which involves the biotransformation of benzaldehyde to *L*-PAC by various species of yeasts, later being the most ideal method from industrial point of view. Extraction, purification and isolation of these pharmaceutical compounds from *Ephedra* plants is tedious, lengthy, difficult, expensive and undesired products may also produce. Biotransformation process is more convenient, easy to handle, cheap and simple. This process can be easily carried out under relatively mild conditions of temperature and pH (Schmid *et al*., 2002; Cherry & Fidantsef, 2003).

The biosynthesis of *L*-PAC takes place by the condensation of an 'active acetaldehyde' (from pyruvic acid) and benzaldehyde, catalyzed by the cytosolic enzyme pyruvate decarboxylase (PDC EC 4.1.1.1) in the presence of two cofactors Thiamine pyrophosphate (TPP) and Mg^{2+}(Oliver *et al*., 1999). PDC of *C. utilis* is highly stable against benzaldehyde and acetaldehyde as compared to the other yeast PDCs resulting in relatively fast and highest production of *L*-PAC with minimum formation of by-products especially acetoin (Rosche *et al*., 2003b; Satianegara *et al*., 2006; Gunawan *et al*., 2007). Production of *L*-PAC utilizing whole cell biotransformation is achieved from of benzaldehyde and molasses at industrial level most commonly. To date, production of *L*-PAC reported is by stirred fermentation. Glucose, sucrose and molasses, as carbon source, have been used successfully for the production of *L*-PAC. Similarly corn steep liquor, yeast hydrolysate, yeast extract, peptone and urea have been reported as nitrogen sources for the production of *L*-PAC (Oliver *et al*., 1997). Biotransformation pH ranges from 4-6 in different cases (Smith & Hendlin, 1953; Voets *et al*., 1973; Long & Ward, 1989a, b) and temperature from 25 to 30°C depending on different organisms (Smith and Hendlin, 1953; Voet *et al*., 1973; Netrval & Vojtisek, 1982; Nikolova & Ward, 1991).

Methods

Pure culture of *Candida utilis* NRRL Y-900 was obtained from culture bank of Institute of Industrial Biotechnology, GC University, Lahore. The culture was maintained on YEPD agar slants containing (g/L) glucose 20.0, peptone 20.0, yeast extract 10.0 and agar 20.0 (pH 5.0). Fifty milliliters of fermentation medium (Table 1) was transferred to 250ml Erlenmeyer flask and was inoculated with 15.0% (v/v) inoculum ($240x10^6$ cells/ml or OD_{595}=0.4) of age 14 h. The flask was incubated at 50-240 rpm at 25-37°C to achieve a cell density of $120x10^6$-$480x10^6$ cells/ml (OD_{595}=0.1-0.6). At this stage, dosing of benzaldehyde was started. Total number of doses ranged from 5-8 and the interval between them ranged from 45-60min. All the experiments were run parallel in triplicates. After that the fermented broth was used for *L*-PAC estimation. *L*-PAC formed was extracted from the fermentation broth using toluene (sample to volume ratio of 1:2) in a separating funnel. The sample was then used for the estimation of *L*-PAC. *L*-PAC was estimated by polarimetry method (Netraval & Vojtisek, 1982; Becvarova *et al*., 1963). Optical rotation was recorded and *L*-PAC (g/L) was calculated as follows:

g/L= 0R x 2 (dilution factor) x 1.11 (density of L-PAC)

Table 1. Composition of fermentation media.

Sr. #	Medium	Composition (g/L)	Reference
1.	M1	Urea 10.0, $MgSO_4$ 10.0 and clarified molasses (30.0 Brix) 200.0 (pH 6.0)	modified Ellaiah & Krishna (1988)
2.	M2	Yeast extract 10.0, peptone 20.0 and glucose 10.0 (pH 6.0)	modified Shukla *et al*., (2001)
3.	M3	Glucose 40.0, yeast extract 10.0, $(NH_4)_2$ SO_4 10.0, KH_2PO_4 3.0, $Na_2HPO_4.12H_2O$ 2.0, $MgSO_4.7H_2O$ 1.0, $CaCl_2$ 0.5 and $FeSO_4$ 0.05 (pH 6.0)	modified Shin & Rogers (1995)

Results and Discussion

In this study three different media M1, M2 and M3 were analyzed for the production of *L*-PAC (Table 2). Use of M1 medium resulted in slowest rate of growth {7.5 hours needed to achieve $120x10^6$ cells/ml (OD_{595}= 0.2)} but the production of *L*-PAC was 2.18 g/L while the other two media M2 and M3 were found to enhance the growth rate of yeast {4.5 and 5.5 hours, respectively, to attain $120x10^6$ cells/ml (OD_{595}= 0.2)} but resulted in no production of *L*-PAC (Table 2). This may be due to that complex media like molasses contain a large amount of macro and micronutrients, vitamins, ionic nutrients and growth factors which specifically stimulate the production of *L*-PAC.

Best production of *L*-PAC (2.27g/L) was found at 30°C and employing the temperatures of 25, 34 and 37°C gave 1.03, 1.92 and 1.60 g/L of *L*-PAC (Table 3). This goes with the findings of Rogers (1990) that the rate of production of *L*-PAC is 1.5 times more at 30°C than at 20°C and at this temperature the enzymatic activity and metabolic functions are at maximum.

The maximum yield of *L*-PAC (3.11g/L) was achieved at pH of 6.0 (Table 4) as the same was also reported by Leksawasdi *et al*., (2003) and Chen *et al*., (2005). So it was concluded that any decrease or increase of pH form 6.0 may cause inactivation of the PDC, inhibition of metabolic functions and decrease the solubility of the substrates in the medium resulting in lower production of *L*-PAC.

Biotransformation of benzaldehyde into *L*-PAC at different cell densities was investigated for the production of *L*-PAC. Highest level of *L*-PAC (3.37 g/L) was obtained when dosing of benzaldehyde was started at $240x10^6$ cells/ml (OD_{595}=0.4) (Table 5). The results were found to be in concordance to with Long and Ward (1989b) and Oliver *et al*. (1999) that dosing at lower cell densities (lower than 240x106 cells/ml) results in lower production of *L*-PAC because the biomass could be in adaptation to benzaldehyde and unable to tolerate the toxic effects of benzaldehyde. While dosing at higher cell densities (more than $240x10^6$ cells/ml) also results in lower production of *L*-PAC due to the toxic effects of accumulated by-products and other metabolites on the cells and depletion of sugar leading to less sugar available for the production of pyruvate to be converted in to *L*-PAC.

Different agitation rates were investigated for the production of *L*-PAC. By employing an agitation rate of 150 rpm *L*-PAC production of 3.38g/L was achieved (Table 6). As agitation is directly related to aeration and PDC becomes activated in partially aerobic conditions so the application of these agitation rates enhanced biomass production but PDC had not been induced properly so the production of *L*-PAC decreased. Production of *L*-PAC was investigated by varying the pattern of benzaldehyde dosing. Highest production of *L*-PAC (3.52 g/L) was achieved by adding benzaldehyde in the form of 8 doses of unequal volumes in descending order with interval of 45 minutes between the doses (Table 7).

Table 2. Effect of fermentation medium on the production of *L*-PAC by *Candida utilis* NRRL Y-900A in 250 ml shake flask.

Medium	Time to achieve 120 x 10^6 cells/ml (OD_{595} = 0.2) (h)	*L*-PAC (g/L)
M1	7.5	2.18 ± 0.069^a
M2	4.5	0 ± 0^b
M3	5.5	0 ± 0^b
LSD ($p \leq 0.05$)		0.079

Table 3. Effect of temperature on the production of *L*-PAC by *C. utilis* NRRL Y-900A in 250ml shake flask.

Temperature (°C)	Time to achieve 120 x 10^6 cells/ml (OD_{595} = 0.2) (h)	*L*-PAC (g/L)
25	9.75	1.03 ± 0.069^d
30(Control)	8.25	2.27 ± 0.068^a
34	7.0	1.92 ± 0.058^b
37	7.5	1.60 ± 0.55^c
LSD ($p \leq 0.05$)		0.118

Table 4. Effect of initial pH on the production of *L*-PAC by *C. utilis* NRRL Y-900A in 250 ml shake flask

pH	Time to achieve 120 x 10^6 cells/ml (OD_{595} = 0.2) (h)	*L*-PAC (g/L)
4.0	7.5	0 ± 0^i
4.2	7.5	0.77 ± 0.025^h
4.5	7.33	1.17 ± 0.059^g
4.8	7.5	1.78 ± 0.90^f
5.0(control)	8.25	2.25 ± 0.063^{cd}
5.2	8.33	2.14 ± 0.069^{de}
5.5	8.5	2.33 ± 0.11^c
5.8	9.25	2.49 ± 0.055^b
6.0	9.5	3.11 ± 0.086^a
6.2	9.5	2.06 ± 0.132^e
LSD ($p \leq 0.05$)		0.133

Table 5. Effect of benzaldahyde dosing at different cell densities on the production of *L*-PAC by *C. utilis* NRRL Y-900A in 250 ml shake flask.

Cell density (cell/ml)/ OD_{595}	Time to achieve cell density or OD (h)	*L*-PAC (g/L)
$60x10^6$ (OD_{595}=0.1)	2.75	2.17 ± 0.064^d
$120x10^6$ (OD_{595}=0.2)	4.5	3.26 ± 0.061^a
$180x10^6$ (OD_{595}=0.3)	5.25	3.28 ± 0.057^a
$240x10^6$ (OD_{595}=0.4)	6.0	3.36 ± 0.063^a
$360x10^6$ (OD_{595}=0.5)	6.25	2.91 ± 0.063^b
$480x10^6$ (OD_{595}=0.6)	7.0	2.71 ± 0.053^c
LSD ($p \leq 0.05$)		0.107

Table 6. Effect of agitation rate on the production of *L*-PAC by *Candida utilis* NRRL Y-900A in 250 ml shake flask.

Agitation rate (rpm)	Time to achieve 240 x 10^6 cells/ml (OD_{595} = 0.4) (h)	*L*-PAC (g/L)
50	8.25	0.78± 0.017[d]
100	7.5	0.89± 0.011[d]
150(control)	6.0	3.38± 0.055[a]
180	5.0	3.03± 0.060[a]
200	3.5	2.25± 0.063[b]
240	3.0	1.62± 0.063[c]
LSD ($p \leq 0.05$)		0.089

Table 7. Effect of pattern of benzaldehyde dosing on the production of *L*-PAC by *C. utilis* NRRL Y-900A in 250ml shake flask.

Pattern of benzaldehyde dosing	Times to achieve 240x10^6 cells/ml (OD_{595} = 0.4) (h)	*L*-PAC (g/L)
1x372	6.0	0±0[h]
2x186	6.0	0.28±0.05[g]
4x93	6.0	0.65±0.05[f]
6x62	6.0	1.15±0.05[e]
8x46.5	6.0	1.71±0.059[d]
10x37.2	6.0	2.29±0.062[b]
*68, 62, 56….(control)	6.0	3.52±0.027[a]
**25, 31, 37…..	6.0	2.06±0.063[c]
LSD ($p \leq 0.05$)		0.087

*68, 62, 56, 50, 43, 37, 31, 25(µl) descending order
** 25, 31, 37, 43, 50, 56, 62, 68(µl) ascending order
[No. of doses x vol. of doses(µl)]

Table 8. Effect of number of benzaldehyde doses on the production of *L*-PAC by *C.utilis* NRRL Y-900A in 250ml shake flask.

Number of doses	Times to achieve 240x10^6 cells/ml (OD_{595} = 0.4) (h)	*L*-PAC (g/L)
5*	6.0	3.22±0.020[c]
6**	6.0	3.62±0.055[a]
7***	6.0	3.54±0.040[b]
8**** (control)	6.0	3.55±0.03[b]
LSD ($p \leq 0.05$)		0.072

*68, 62, 56, 50, 43 (µl)
**68, 62, 56, 50, 43, 31(µl)
***68, 62, 56, 50, 43, 37, 31(µl)
****68, 62, 56, 50, 43, 37, 31, 25(µl)

Table 9. Effect of time interval between benzaldehyde doses on the production of *L*-PAC by *C.utilis* NRRL Y-900A in 250ml shake flask.

Interval (min)	Times to achieve 240x10^6 cells/ml (OD_{595} = 0.4) (h)	*L*-PAC (g/L)
15	6.0	1.67±0.019[d]
30	6.0	3.06±0.063[c]
45 (control)	6.0	3.59± 0.080[b]
60	6.0	3.81±0.065[a]
75	6.0	3.53±0.041[b]
LSD ($p \leq 0.05$)		0.106

Number of benzaldehyde doses was optimized for increased production of *L*-PAC. Maximum amount of *L*-PAC (3.62 g/L) was achieved on the addition of 6 doses (316 µl or 0.63% or 6.3 g/L benzaldehyde) of unequal volume in descending order in the medium (Table 8). The results obtained were found to be in concordance with that of Long and Ward (1989a), Shukla *et al*., (2001) and Kumar *et al*. (2006) who also reported the use of 0.6% benzaldehyde for the production of *L*-PAC using different *Candida* species, *S. cereviciae* and *S. cereviciae*, respectively. But the results were not in concordance with that of Mahmoud *et al*., (1990a,b) who observed that free cells could tolerate a 0.4% benzaldehyde concentration. It can be concluded from the above results that strain of *C. utilis* can withstand benzaldehyde levels up to 0.63%. Increase or decrease from this value gives lower production of *L*-PAC due to by-product formation and toxicity.

Time interval between the doses was investigated for maximum production of *L*-PAC and an interval of 60 minutes was found to be appropriate for the highest production (3.81 g/L) of *L*-PAC (Table 9). It can be inferred from the obtained results that *C. utilis* has the ability to consume benzaldehyde properly when an interval of 60 minutes is kept between the doses. Interval of less than 60 minutes (15, 30, 45 minutes) could lead to the toxic effect of benzaldehyde on cells while interval of more than 60 minutes (75 minutes) could result in the production of *L*-PAC derivatives like PAC-diol due to the activity of some alcohol dehydrogenases, thus minimizing *L*-PAC production.

Production of *L*-PAC was investigated by supplementation of benzaldehyde doses with acetaldehyde. Improved production of *L*-PAC (4.15 g/L) was obtained when acetaldehyde was added along with benzaldehyde doses (Table 10). This goes with the findings of Becvarova *et al*., (1963), Groeger *et al*., (1966), Netrval & Vojtisek (1982) and Oliver *et al*., (1997) that addition of benzaldehyde along with acetaldehyde in the medium increased the production of *L*-PAC by reducing the production of benzyl alcohol, acting as hydrogen acceptor and inhibiting hydrogenation of benzaldehyde to benzyl alcohol and diverting more benzaldehyde towards *L*-PAC formation. As acetaldehyde acts as a competitor of benzaldehyde for active sites of ADH so it is more readily available to ADH than benzaldehyde.

Table 10. Effect of supplementation of benzaldehyde doses with acetaldehyde on the production of *L*-PAC by *C.utilis* NRRL Y-900A in 250ml shake flask.

Acetaldehyde supplemented (µl)	Times to achieve 240x10^6 cells/ml (OD_{595} = 0.4) (h)	*L*-PAC (g/L)
No acetaldehyde (control)	6.0	3.82±0.05[a]
*Acetaldehyde added	6.0	4.15±0.05[b]
LSD ($p \leq 0.05$)		0.116

*Acetaldehyde added as benzaldehyde:acetaldehyde (50%) with ratio 1:1.5 (Groeger *et al*., 1966).

Conclusion

It is concluded from the study that the present wild culture of *Candida utilis* NRRL Y-900 has the potential of producing 4.15g/L of *L*-PAC from 6.3g/L of benzaldehyde in 250ml shake flask which shows 65 % of biotransformation capacity. This production can be enhanced in a bioreactor along with random and site directed mutagenesis of *C. utilis*.

Acknowledgements

I thank Dr. Ikram-ul-Haq for his comments, Dr. Qadeer, Dr. Mohsin and Dr. Syed Ali Imran Bokhari for their support and help. This work was supported by Alpha chemicals, Lahore.

References

Becvarova, H., O. Hanc and K. Macek. 1963. Course of transformation of benzaldehyde by *Saccharomyces cerevisiae. Folia Microbiol*., 8: 165-169.

Chen, A.K., M. Breuer, B. Hauer, P.L. Rogers and B. Rosche. 2005. pH shift enhancement of *Candida utilis* pyruvate decarboxylase production. *Biotechnol. Bioeng*., 92(2): 183-188.

Cherry, J.R. and A.L. Fidantsef. 2003. Directed evolution of industrial enzymes: an update. *Curr. Opin. biotechnol*., 14: 438-443.

Ellaiah, P. and K.T. Krishna. 1988. Effect of aeration and alternating current on the production of phenylacetylcarbinol by *Saccharomyces cerevisiae. Indian J. Technol*., 26: 509-510.

Groeger, D., H.P. Schmauder and K. Mothes. 1966. Isolation of *L*-phenylacetylcarbinol. *Z. Allg. Mikrobiol*., 6: 275-287.

Gunawan, C., G. Satianegara, A.K. Chen, M. Breuer, B. Hauer, P.L. Rogers and B. Rosche. 2007. Yeast pyruvate decarboxylases: variation in biocatalytic characteristics for *R*-phenylacetylcarbinol production. *FEMS Yeast Res*., 7: 33-39.

Kumar, M.R., M.A. Chari and M.L. Narasu. 2006. Production of *L*-phenylacetylcarbinol (*L*-PAC) by different novel strains of yeasts in molasses and sugar cane juice as production medium. *Res. J. Microbiol*., 1(5): 433-437.

Leksawasdi, N., M. Breuer, B. Hauer, B. Rosche and P.L. Rogers. 2003. Kinetics of pyruvate decarboxylase deactivation by benzaldehyde. *Biocatal. Biotrans*., 21: 315-320.

Long, A. and O.P. Ward. 1989a. Biotransformation of aromatic aldehydes by *Saccharomyces cerevisiae* investigation of reaction rates. *J. Ind. Microbiol*., 4: 49-53.

Long, A. and O.P. Ward. 1989b. Biotransformation of benzaldehyde by *Saccharomyces cerevisiae:* characterization of the fermentation and toxicity effects of substrates and products. *Biotechnol. Bioeng*., 34(7): 933-941.

Mahmoud, W.M., A.M.M. El-Sayed and R.W. Coughlin. 1990a. Effect of β- cyclodextrin on production of *L*-phenylacetylcarbinol by immobilized cell of *Saccharomyces cerevisiae*. *Biotechnol. Bioeng*., 36: 256-262.

Mahmoud, W.M., A.M.M. El-Sayed and R.W. Coughlin. 1990b. Production of *L*-phenylacetylcarbinol by immobilized yeast cell: I. Batch fermentation. *Biotechnol. Bioeng*., 36: 47-54.

Netrval, J. and V. Vojtisek. 1982. Production of phenylacetylcarbinol in various yeast species. *Eur. J. Appl. Microbiol. Biotechnol.,* 16: 35-38.

Nikolova, P. and O.P. Ward. 1991. Production of *L*-phenylacetylcarbinol by biotransformation: product and by-product formation and activation of the key enzymes in wild-type and ADH isoenzyme mutants of *Saccharomyces cerevisiae*. *Biotechnol. Bioeng*., 38: 493-498.

Oliver, A.L., B.N. Anderson and F.A. Roddick. 1999. Factors affecting the production of *L*-phenylacetylcarbinol by yeast: A case study. *Adv. Microb. Physiol*., 41: 1-45.

Oliver, A.L., F.A. Roddick and B.N. Anderson. 1997. Cleaner production of phenylacetylcarbinol by yeast through productivity improvements and waste minimization. *Pure Appl. Chem*., 69(11): 2371-2385.

Rogers, P.L. 1990. ICI Internal Report. Orica Limited, Ascot Vale, Victoria, Australia.

Rosche, B., M. Breuer, B. Hauer and P.L. Rogers. 2003b. Screening of yeasts for cell free production of *R*-phenylacetylcarbinol. *Biotechnol. Lett*., 25: 841-845.

Satianegara, G., M. Breuer, B. Hauer, P.L. Rogers and B. Rosche. 2006. Enzymatic *R*-phenylacetylcarbinol production in a benzaldehyde emulsion system with *Candida utilis* cells. *Appl. Microbiol. Biotechnol.*, 70: 170-175.

Schmid, A., F. Hollmann, J.B. Park and B. Buhler. 2002. The use of enzymes in the chemical industry in Europe. *Curr. Opin. biotechnol*., 13: 359-366.

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

Shukla, V.B., U.P. Veera, P.R. Kulkarni and A.B. Pandit. 2001. Scale-up of biotransformation process in stirred tank reactor using dual impeller bioreactor. *Biochem. Eng. J*., 8: 19-29.

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

Voets, J.P., E.J. Vandamme and C. Vlerick. 1973. Some aspects of the phenylacetylcarbinol biosynthesis by *Saccharomyces cerevisiae. Z. Allg. Mikrobiol*., 13: 355-366.

(Received for publication 12 February 2011)