CHEMICAL ENGINEERING TRANSACTIONS
VOL. 27, 2012
A publication of AIDIC
The Italian Association of Chemical Engineering
Online at: www.aidic.it/cet
Guest Editors: Enrico Bardone, Alberto Brucato, Tajalli Keshavarz

ISBN 978-88-95608-18-1; **ISSN** 1974-9791
DOI: 10.3303/CET1227028

Selection of Yeasts for the Production of L-phenyl-acetil-carbinol Bybiotransformation in Shake Flasks

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L-phenylacetylcarbinol (L-PAC) is an intermediate in the production of L-ephedrine and pseudoephedrine, which are pharmaceutical compounds used as decongestants and anti-asthmatics. L-PAC can be produced by chemical synthesis from cyanohydrins but the biotransformation route for its production from benzaldehyde is preferred industrially. Several microorganisms in nature are capable of producing L-PAC, from bacteria to fungi. There are a large number of yeasts that carry out this biotransformation route, which is why they are the most studied organisms in the production of L-PAC. This work aims to select the best yeast producer among 22 potential strains belonging to the genera *Saccharomyces*, *Kluyveromyces*, *Pichia* and *Candida* and to increase L-PAC production by improving process conditions in shake flasks. The production medium contained initially (in grams per liter): glucose 25, peptone 20, yeast extract 10; $MgSO_4.7H_2O$ 1; $CaCl_2.2H_2O$ 0.05, Na_2HPO_4, citric acid 10.7 and benzaldehyde 1. L-PAC production was performed at 200 rpm, at 30°C for 1 day. The substrate, the product and by-products were analysed in HPLC column (Hipersil ODS C18). Among all the species/strains studied five were selected as good producers, one specie of *Kluyveromyces* and four species of *Saccharomyces*. The results indicated that the best time to add benzaldehyde in the production medium was at 3 h of cultivation, which is during the exponential cell growth phase. Beside, yeasts were inhibited by high concentrations of benzaldehyde. The maximum concentration of L-PAC obtained was 0.6 g / L at 4.5 h cultivation.

1. Introduction

L-Phenylacetylcarbinol (L-PAC) is an intermediate in the production of L-ephedrine and pseudoephedrine, which are pharmaceutical compounds used as decongestants and anti-asthmatics. Some reports also indicate its potential use in obesity control (Astrup et al., 1992). It is currently produced via microbial transformation process using different yeast species with benzaldehyde as the main substrate, involving the condensation of an “active acetaldehyde” (from pyruvic acid produced by the yeast) and benzaldehyde. The production of the L-PAC is catalyzed by the enzyme pyruvate decarboxylase (PDC) and is associated with by-product formation, viz. benzyl alcohol, due to the activity of an alcohol dehydrogenase (ADH) and/or oxidoreductases (Figure 1). Some traces of benzoic acid as a by-product have also been reported (Khan and Daugulis, 2011). L-PAC can be produced by chemical synthesis from cyanohydrins (Brusse et al., 1988; Jackson et al., 1990) but the biotransformation route from benzaldehyde is preferred industrially.

Please cite this article as: Miguez M., Nunes P., Azeredo N., Pedraza S., Vasconcelos M., Viana O., Coelho M.A.Z. and Amaral P., 2012, Selection of yeasts for the production of l-phenyl-acetil-carbinol bybiotransformation in shake flasks., Chemical Engineering Transactions, 27, 163-168 DOI: 10.3303/CET1227028

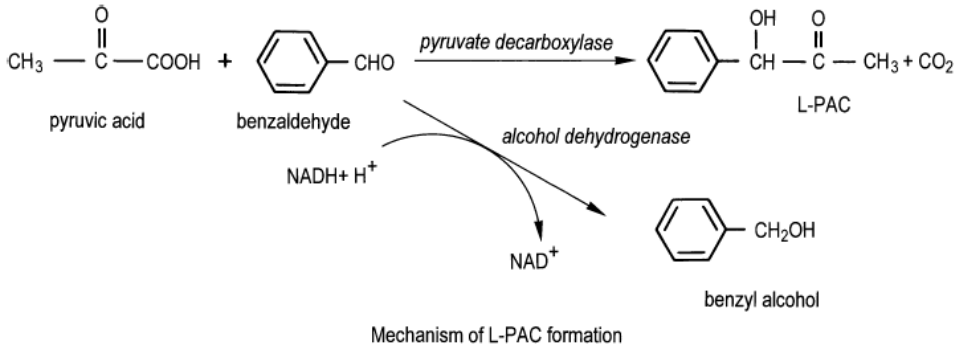

Figure 1: Mechanism of L-PAC formation. (Shin and Roger,1995)

Saccharomyces cerevisiae manifests the capacity to catalyse the conversion of a range of substituted benzaldehydes to corresponding L-acetyl aromatic carbinols and substituted aromatic alcohols are also formed. The role of purified yeast alcohol dehydrogenase in converting benzaldehyde and substituted benzaldehydes to the corresponding alcohols has been conclusively established (Long and Ward, 1989).

According to Shin and Rogers (1995), three principal factors leading to the cessation of PAC production in a fermentation process include: (i) depletion of pyruvate at the end of the biotransformation phase, (ii) deactivation of PDC caused by prolonged exposure to benzaldehyde and/or endproduct inhibition and (iii) progressive reduction in cell viability due to benzaldehyde as well as accumulated concentrations of benzyl alcohol and PAC.

Therefore, this work aims to select a good L-PAC yeast producer and to increase its production by evaluating the non-toxic concentration of benzaldehyde and the best time to add it to the culture.

2. Materials and methods

2.1 Microorganisms producing L-PAC

Saccharomyces pastorianus 40090 was obtained Fiocruz (INCQS, RJ, Brazil). *Saccharomyces cerevisiae* (baker's yeast) was obtained in a local market (Fleischamann Industry). *Saccharomyces cerevisiae* ATCC 32167 and *Saccharomyces cerevisiae* S228c ATCC 26108. All other strains (designated IMUFRJ) were obtained from Institute of Microbiology at the Federal University of Rio de Janeiro, Brazil. The strains were maintained on a solid medium containing 2 % glucose, 0.5 % yeast extract, 0.3 % malt extract, 0.5 % sodium phosphate monobasic, 2 % agar, cultivated for 48 h at 28 °C, stored at 4 °C.

2.2 Media and Culture Conditions

Pre-inoculum was prepared in 500 mL Erlenmeyer flasks containing 100 mL of YPD medium (20 g glucose, 20 g peptone, and 10 g yeast extract per liter of distillate water) and a seed culture from the solid medium. After 24 h of growth in a shaker at 200 rpm and 30 °C the culture broth was centrifuged at 1600 x *g* for 10 min and the cells were used to inoculate the production medium.

The L-PAC production medium consisted of: 20 g peptone, 25 g glucose, 10 g yeast extract, 1 g $MgSO_4 \cdot 7H_2O$, 0.05 g $CaCl_2 \cdot 2H_2O$, 35 g $Na_2HPO_4 \cdot 12H_2O$, and 10.7 g citric acid per liter of distillate water (Zhang et al., 2008). Yeast cell and benzaldehyde were added to this medium for microbial transformation at 200 rpm and 30 °C. Samples were taken every hour, where a 2-mL aliquot was subjected to removal of biomass by centrifugation at 1600 x *g* for 7 min. The clear supernatant was subjected to substrate/ products and glucose analysis.

2.3 Analytical methods

HPLC analysis was preformed on Hypersil C_{18} column (5 μm, 250×4.6 mm) with acetonitrile/water (30:70) as the mobile phase (1.0 mL/min). The product phenylacetylcarbinol and substrate benzaldehyde were detected with UV-detection at 283 nm (Rosche et al., 2001) with a retention time of 7 and 11 min, respectively. The byproduct benzylalcohol was detected at 254 nm with a retention time 6 min. Then, the substrate concentration and the byproduct concentration were determined by comparison with a standard sample. Glucose concentration in the sample of a mixture supernatant was determined by glucose oxidase method (Raabo and Terkildsen, 1960).

2.4 Biomass determination

Culture samples were collected for analysis of cell concentration at a spectrophotometer (optical density at 570 nm, OD_{570}) and the OD_{570} was converted to g cell dry per liter by a predetermined factor (Oliveira et al., 2010).

3. Results and discussion

Twenty two yeast strains were chosen based on yeast metabolism. Ethanol producing yeast were preferred since active pyruvate decarboxylase is necessary. As Table 1 shows, all yeasts were able to grow in the presence of benzaldehyde but only five of them were good L-PAC producers.

Table 1: L-PAC production by yeast strains

CRN[a]	Yeast species	μ[b] (h^{-1})	ΔX[c] (g/L)	L-PAC (g/L)
	Saccharomyces cerevisiae (Fleischamann Ind.)	0.345	3.89	0.27
	Saccharomyces cerevisiae (ATCC 32167)	0.427	2.06	0.06
S228c	*Saccharomyces cerevisiae* (ATCC 26108)	0.283	2.42	0.07
INCQS 40090	*Saccharomyces pastorianus* (ATCC 2366)	0.338	3.30	0.39
IM-UFRJ 50916	*Candida colliculosa* (DR888)	0.283	5.21	0.01
IM-UFRJ 51503	*Candida guilliermondii (*ATCC 6260)	0.167	3.99	0.01
IM-UFRJ 51680	*Debaryomyces yamadae* (ATCC 56471)	0.181	3.30	0.01
IM-UFRJ 51710	*Kluyveromyces aestuarii* (S 12-1)	0.311	1.04	0.07
IM-UFRJ 50815	*Kluyveromyces marxianus* (DR159V)	0.137	3.83	0.74
IM-UFRJ 50893	*Kluyveromyces marxianus* (DR721)	0.141	2.09	0.03
IM-UFRJ 50800	*Kluyveromyces thermotolerans (*DR113A)	0.085	5.87	0.19
IM-UFRJ 50407	*Pichia anomala*	0.251	3.17	0.12
IM-UFRJ 51654	*Pichia anomala*	0.123	4.20	0.01
IM-UFRJ 51534	*Pichia ohmeri -like*	0.330	0.91	0.17
IM-UFRJ 50816	*Saccharomyces cariocanus* (DR159Br)	0.141	3.59	1.75
IM-UFRJ 51599	*Saccharomyces cerevisiae* (DBVPG 6039 CBS 1395)	0.159	2.16	0.03
IM-UFRJ 51600	*Saccharomyces cerevisiae* (DBVPG 6175 CBS 1782)	0.256	2.22	0.79
IM-UFRJ 51601	*Saccharomyces cerevisiae* (DBVPG 6250 CBS 4734)	0.245	1.54	0.97
IM-UFRJ 51603	*Saccharomyces cerevisiae* (DBVPG 6302 CBS)	0.171	3.52	0.19
IM-UFRJ 51605	*Saccharomyces cerevisiae* (DBVPG 6251CBS 1250)	0.076	5.21	0.14
IM-UFRJ 51607	*Saccharomyces dairensis* (DBVPG 6747 CBS 7127)	0.207	3.99	0.36
IM-UFRJ 51609	*Saccharomyces spencerorum* (DBVPG 6746 CBS 3019)	0.139	3.89	0.06

[a]CRN: Collection reference number ……..

[b]μ: specific growth rate at exponential phase of growth

[c]ΔX: maximum biomass minus initial biomass concentration

The strains *Kluyveromyces marxianus* IMUFRJ 50815, *Saccharomyces. cariocanus* IMUFRJ 50816, *Saccharomyces cerevisiae* IMUFRJ 51600, *Saccharomyces cerevisiae* IMUFRJ 51601 and *Saccharomyces pastorianus* INCQS-40090 produced respectively 0.74 g/L, 1.75 g/L, 0.79 g/L, 0.97g/L and 0.39 g/L of PAC and were chosen for further experiments.

Several authors have already shown that benzaldehyde is highly toxic to cells (Rogers et al., 1997; Zhang et al., 2008) and suggest that pulse feeding increases product formation. Therefore, in the present study, the time of benzaldehyde addition was evaluated in order to let the cells adapt to the production medium before benzaldehyde addition. The addition of 1 g of benzaldehyde per L was carried out 0, 1.5, 3.0 and 4.5 h after the inoculation of the microorganisms in the production medium (without benzaldehyde). In this study, four experiments were performed for the five strains where benzaldehyde was added once during the fermentation, at the times mentioned. Table 2 shows the results for the five strains.

Table 2: L-PAC production by the best strains in experiments where benzaldehyde was added.

Strain/ Addition time	Time of PAC_{max}[a] (h)	PAC_{max}[a] (g/L)	Benzaldehyde (g/L)[b]	Glucose (g/L)[b]	Benzoic acid (g/L)[b]	Benzyl alcohol (gL)[b]	Biomass formed (g/L)[b]
Kluyveromyces marxianus IMUFRJ 50815							
0 h	4.5	0.35	0.02	5.9	0.15	0.54	4.21
1.5 h	4.5	0.52	0.00	7.4	0.11	0.53	4.60
3.0 h	4.5	0.55	0.14	5.5	0.06	0.49	5.29
4.5 h	24.0	0.11	0.00	3.8	0.19	0.40	6.94
Saccharomyces. cariocanus IMUFRJ 50816							
0 h	7.0	0.47	0.01	10.6	0.13	0.75	3.08
1.5 h	7.0	0.36	0.11	17.7	0.26	0.58	3.51
3.0 h	7.0	0.28	0.03	9.3	0.19	0.71	4.53
4.5 h	24.0	0.02	0.00	0.0	0.14	0.61	4.60
Saccharomyces cerevisiae IMUFRJ 51600							
0 h	4.0	0.37	0.00	8.2	0.27	0.61	3.57
1.5 h	4.0	0.59	0.00	0.2	0.16	0.41	4.75
3.0 h	5.0	0.61	0.11	0.0	0.13	0.37	7.08
4.5 h	ND[c]	ND[c]	0.00[d]	0.0[d]	0.22[d]	0.92[d]	9.06[d]
Saccharomyces cerevisiae IMUFRJ 51601							
0 h	6.0	0.26	0.00	0.1	0.20	0.63	6.24
1.5 h	4.5	0.52	0.00	0.1	0.11	0.60	5.57
3.0 h	4.5	0.20	0.28	0.2	0.10	0.45	7.13
4.5 h	5.0	0.06	0.45	0.1	0.06	0.30	7.45
Saccharomyces pastorianus INCQS 40090							
0 h	6.0	0.24	0.00	0.2	0.31	0.63	3.80
1.5 h	6.0	0.51	0.00	0.0	0.16	0.55	5.43
3.0 h	4.5	0.67	0.00	0.1	0.12	0.38	6.25
4.5 h	6.0	0.09	0.61	0.0	0.13	0.41	7.65

[a] Time of PAC_{max} : time of maximum PAC production
[b] Concentration at the time of PAC_{max}
[c] ND: not detected by the method.
[d] Concentrations at 24 h of process.

For the best producers (*Kluyveromyces marxianus* IMUFRJ 50815, *Saccharomyces cerevisiae* IMUFRJ 51600 and *Saccharomyces pastorianus* INCQS-40090) the best time for benzaldehyde addition was 3 h, being 1.5 h also a good result. For this experiment (benzaldehyde addition at 3 h) some benzaldehyde still remains at the medium and almost all glucose was consumed. Therefore, the best time for benzaldehyde addition was found to be 3 h, which corresponds to the exponential cell growth phase, as Figure 2 depicts. At this point the cells are already adapted to the medium and more biocatalyst is available. In comparison to the experiments where benzaldehyde was added at 1.5 h, the experiments performed with benzaldehyde addition at 3 h had less by-products at the time of PAC_{max} (benzyl alcohol and benzoic acid) (Table 2).

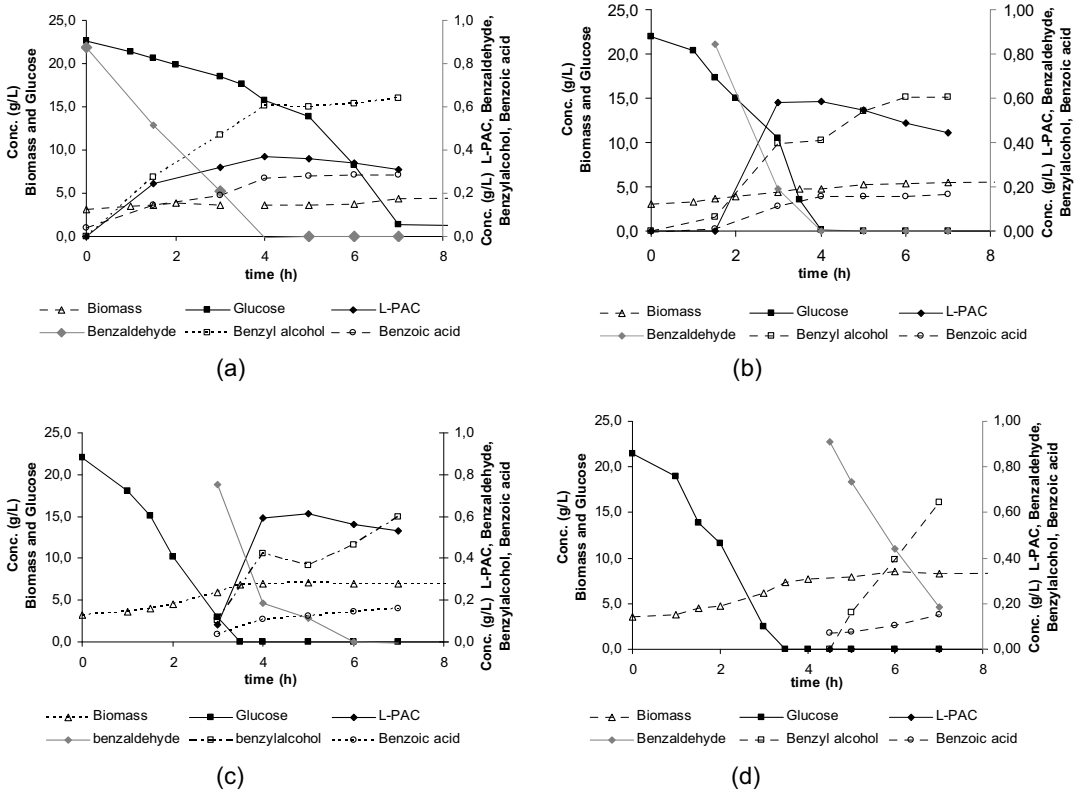

Figure 2: Kinetics of the experiments performed with S. cerevisiae IMUFRJ 51600 with different times of benzaldehyde addition: (a) 0 h; (b) 1.5 h; (c) 3.0 h; (d) 4.5.

The time of addition proved to be an essential parameter in the process. By comparing the experiments of 1.5 h and 3 h, with 0 h benzaldehyde inhibited cell growth, as can be seen in Figure 2, which shows the kinetics of the experiments with *S. cerevisiae* IMUFRJ 51600. The later benzaldehyde addition (4.5 h) showed the worst conversion because at this stage the cells are already in stationary phase of growth, most of the glucose is already consumed for cell growth and none is left for the bioconversion. Long and Ward (1989) also following an equilibration period of 1 h, the fermentation was initiated by addition of aromatic aldehyde. The shake flask reaction was carried out for 1 h at 30 and the concentration of L-PAC formed with 1 g/L the benzaldehyde was 0.1 g/L.

4. Conclusion

In this study three yeast strains were selected as best producers for L-PAC production: *Kluyveromyces marxianus* IMUFRJ 50815*, Saccharomyces cerevisiae* IMUFRJ 51600 and *Saccharomyces pastorianus* INCQS-40090. Benzaldehyde showed to be toxic to the cells, reducing growth when added at the beginning of the process. The addition of benzaldehyde in 3 h of cultivation was the best result obtained. It was possible to obtain 0.67 g/L of L-PAC in 4.5 h.

Acknowledgments

This project was financially supported by the Nortec Química of Rio de Janeiro.

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