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Application of Plackett–Burman Design for Medium Constituents Optimization for the Production of L-phenylacetylcarbinol (L-PAC) by *Saccharomyces Cerevisiae.*

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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. This work aims to select the best culture medium and process conditions to produce L-PAC in shake flasks and 1 L bioreactor. Statistical experimental designs were applied for the optimization of medium constituents for L-PAC production by *Saccharomyces cerevisiae.* The production medium (PM) contained initially (grams per liter) glucose 25, peptone 20, yeast extract 10; $MgSO_4.7H_2O$ 1; $CaCl_2.2H_2O$ 0.05, $Na_2HPO_4.12H_2O$ 35, citric acid 10.7 and Benzaldehyde 1. Using Plackett–Burman design (PB-12), peptone, citric acid and $Na_2HPO_4.12H_2O$ were identified as significant variables which highly influenced L-PAC production. The optimum medium (OM) was constituted by glucose 40, peptone 5, yeast extract 10; $MgSO_4.7H_2O$ 5; $CaCl_2.2H_2O$ 0.01, $Na_2HPO_4.12H_2O$ 35, citric acid 2 and Benzaldehyde 4. In this optimum condition L-PAC production was 4.92 g/L. Using this medium constitution, in bioreactor it was possible to obtain 7.5 g/L of L-PAC. This result was 44% superior than with the medium without optimization.

1. Introduction

In bacterial and yeast systems, pyruvate is readily decarboxylated and the resulting hydroxyethyl- thiamin diphosphate carbanion couples to benzaldehyde to generate (L)-phenylacetylcarbinol (L-PAC), which is the commercial precursor to ephedrine (Meyer et al., 2011).The L-PAC production is catalyzed by 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.

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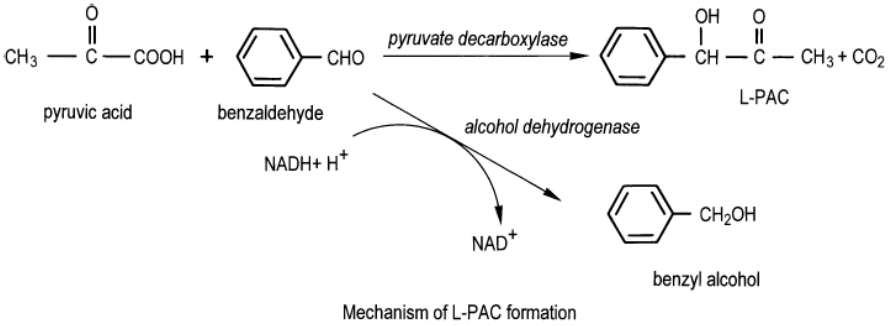


Figure 1 Mechanism of L-PAC formation. (Shin and Roger,1995)

Therefore, this work aims to select the best culture medium and process conditions to produce L-PAC in shake flasks and in 1 L bioreactor.

2. Materials and methods

2.1 Microorganism

Saccharomyces cerevisiae (DBVPG 6175 CBS 1782) was obtained from Institute of Microbiology at the Federal University of Rio de Janeiro, Brazil. The strain was maintained on a solid medium containing 2% glucose, 0.5% yeast extract, 0.3% malt extract, 0.5% monobasic sodium phosphate , 2% agar, after being cultivated for 48 h at 28°C and was stored at 4°C.

2.2 Media and Culture Conditions

The inoculum was prepared in 500 mL Erlenmeyer flasks containing 200 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 of peptone, 25 g of glucose, 10 g of yeast extract, 1 g of $MgSO_4.7H_2O$, 0.05 g of $CaCl_2.2H_2O$, 35 g of $Na_2HPO_4.12H_2O$, and 10.7 g of citric acid per liter of distillate water (Zhang et al., 2008). Yeast cells were added to this medium for a 7 hour microbial transformation in 500 mL flasks with 3 g / L of cell and 100 mL of medium shaken at 200 rpm or in a 1 L bioreactor with 30-50 g / L of cell 750 mL of medium agitated at 350 rpm. In both systems temperature was maintained at 30°C and benzaldehyde was added after 3 h of cultivation in flasks and 1,5 h of cultivation in bioreactor. A Tecnal bioreactor (TEC-BIO-C) equipped with 2 Rushton turbines and air submerged sparger system was used. Initially aeration was maintained at 2 vvm and after benzaldehyde addition it was ceased to prevent volatilization (Miguez et al 2012) 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 performed on Hypersil C18 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).

2.5 Experimental design and optimization

A total of six medium components were screened in fifteen experimental runs and the corresponding Plackett–Burman (Plackett and Burman, 1946) experimental design matrix for screening of important variables for L-PAC production is shown in Table 1. A 2^3 full factorial design was carried out to verify the

effects and interactions of glucose, cell and benzaldehyde initial concentrations. In this design, a set of 11 experiments, including three replicates at the central point, was performed. The range and the levels of the variables herein investigated are given in Table 2. "STATISTICA" (version 7.0) software was used for regression and graphical analyses of the data obtained.

3. Results and Discussion

Identification of important medium constituents was performed using Plackett–Burman design (PB-12). This statistical tool was selected for the optimization of culture medium to increase the production of L-PAC and reduce producing costs by minimizing the salts added to the medium in shake flasks. Table 1 shows the results for the experimental design. A p-value inferior to 0.05 for the three variables viz Peptone (X_1), $Na_2HPO_4.12H_2O$ (X_5) and citric acid (X_6) indicates that these are significant variables for L-PAC production, which can be visualised in the Pareto chart (Figure 2a). The results indicate that it is possible to minimize the concentrations of peptone from 20 to 5 g/L, $CaCl_2.2H_2O$ from 0.05 to 0.01 g L and Citric acid from 10.7 to 2 g/L. The Pareto chart for L-PAC production (Figure 2a) shows that the concentration of $MgSO_4.7H_2O$ is not statistically significant. However, when 1 g / L of $MgSO_4.7H_2O$ is used L- PAC production is rather low (1.02 g/L) compared with production of L -PAC (2.07 g/L) with 5 g/L. Although the statistical analysis shows that $MgSO_4.7H_2O$ do not influence the production of L-PAC, the ion Mg^{+2} is very important to favour the metabolic pathway to L-PAC production since it is required for the enzymatic transformation of benzaldehyde and pyruvate in L- PAC, avoiding the formation of benzyl alcohol.
The optimal salt composition determined by PB- 12 was $MgSO_4.7H_2O$ 5 g/L, $CaCl_2.2H_2O$ 0.01 g/L, Na_2HPO_4 .12 H_2O 35 g/L, citric acid 2 g/L and benzaldehyde 4 g/L.

Table 1 Plackett–Burman experimental design matrix for screening of important variables for L-PAC, Benzyl alcohol and Benzoic acid production

	X_1[a,b]	X_2[a,b]	X_3[a,b]	X_4[a,b]	X_5[a,b]	X_6[a,b]	L-PAC[a]	Benzyl alcohol[a]	Benzoic acid[a]
1	+1 (20)	-1 (2)	+1 (5)	-1 (0.01)	-1 (5)	-1 (2)	0.37	0.00	0.47
2	+1 (20)	+1 (10)	-1 (1)	+1 (0.1)	-1 (5)	-1 (2)	0.65	0.13	0.34
3	-1 (5)	+1 (10)	+1 (5)	-1 (0.01)	+1 (35)	-1 (2)	2.02	0.59	0.07
4	+1 (20)	-1 (2)	+1 (5)	+1 (0.1)	-1 (5)	+ 1 (10)	0.11	0.00	0.42
5	+1 (20)	+1 (10)	-1 (1)	+1 (0.1)	+1 (35)	-1 (2)	2.17	0.47	0.10
6	+1 (20)	+1 (10)	+1 (5)	-1 (0.01)	+1 (35)	+ 1 (10)	0.18	0.00	0.42
7	-1 (5)	+1 (10)	+1 (5)	+1 (0.1)	-1 (5)	+ 1 (10)	0.09	0.00	0.40
8	-1 (5)	-1 (2)	+1 (5)	+1 (0.1)	+1 (35)	-1 (2)	1.92	0.54	0.08
9	-1 (5)	-1 (2)	-1 (1)	+1 (0.1)	+1 (35)	+ 1 (10)	0.33	0.00	0.02
10	+1 (20)	-1 (2)	-1 (1)	-1 (0.01)	+1 (35)	+ 1 (10)	0.37	0.00	0.01
11	-1 (5)	+1 (10)	-1 (1)	-1 (0.01)	-1 (5)	+ 1 (10)	0.42	0.00	0.03
12	-1 (5)	-1 (2)	-1 (1)	-1 (0.01)	-1 (5)	-1 (2)	1.02	0.26	0.04
13	12.5	0 (6)	0 (3)	0 (0.055)	0 (20)	0 (6)	0.38	0.00	0.37
14	12.5	0 (6)	0 (3)	0 (0.055)	0 (20)	0 (6)	0.36	0.00	0.39
15	12.5	0 (6)	0 (3)	0 (0.055)	0 (20)	0 (6)	0.58	0.00	0.03

[a]unit: g/L,
[b]X_1- Peptona, X_2 - Yeast Extract, X_3 $MgSO_4.7H_2O$, X_4 $CaCl_2.2H_2O$, X_5 $Na_2HPO_4.12H_2O$, Citric Acid.

In order to verify the influence of the salts in the formation of byproducts benzyl alcohol (Figure 2b) and benzoic acid (Figure 2c) were also considered as dependent variables. The variables that influenced the production of benzyl alcohol were also the citric acid concentration with a negative effect and sodium phosphate dodecahydrate concentration with a positive effect. For benzoic acid as the response variable the most influent variables were peptone and magnesium sulphate heptahydrate. Therefore, as citric acid and sodium phosphate exerted the same influence on product and byproducts formation, the product formation should be stimulated and the formation of benzyl alcohol inhibited by using another strategy. In the case of benzoic acid it is possible to reduce the concentration of peptone in order to inhibit its undesirable production.

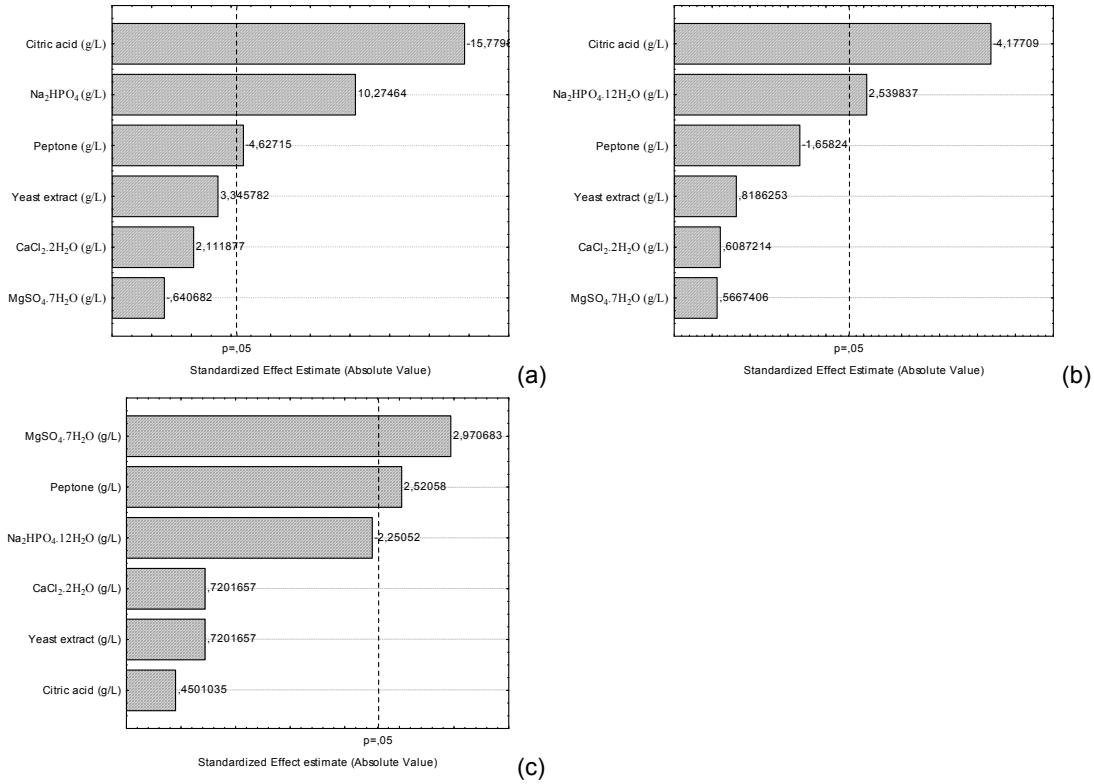


Figure 2 Pareto chart for the effect of various variables on (a) L-PAC, (b) Benzyl alcohol and (c) Benzoic acid production based on Plackett Burman design

Using the salt composition optimized by PB-12, with the addition of benzaldehyde after 1.5 h of fermentation, an experimental design with 2 levels and 3 dependent variables (2^3) was conducted in order to find the best condition for initial cell concentration, benzaldehyde and glucose concentrations for the L-PAC production shake flasks. To ensure that the microorganism is still in exponential phase of growth, producing enough pyruvate, the addition of benzaldehyde was reduced to 1.5 h since more cells were present (Miguez et al. 2012). Table 2 shows the results indicating that high concentrations of L-PAC are obtained when high initial glucose and benzaldehyde concentrations are used. On the oder hand, the inferior cell concentration used (30 g/L of biocatalyst) was more adequate for the production. This is probably due to increased demand for glucose when higher cell concentration is present.

Mujahid et al. (2012) obtained a rather similar result for *Saccharomyces cerevisiae* GCU 36, with a maximum concentration of 2.58 g / L of L-PAC and an inoculum of 30 g / L of cells, 50 g / L glucose and 5 additions 1.2 mL / L benzaldehyde and 1.2 mL / L of acetaldehyde with intervals of 1 hour.

The Pareto chart (Figure 3) shows that benzaldehyde and glucose concentrations have a significant effect on the response variable (L- PAC) with a positive effect, meaning that increasing the concentration of these components an increase in the production of L–PAC is possible. Therefore, it is important to study higher concentrations of benzaldehyde and glucose. In the case of cell concentration, it was observed that this variable exerted a negative effect on the production of L-PAC.

Table 2 Experimental design and results of the 2^3 full factorial design for L-PAC production evaluation.

	Glucose[a]	*[ ]i* Cel.[a]	Benzaldehyde[a]	$PAC_{máx}$[a]
1	-1 (40)	-1 (30)	-1(4)	0,4
2	-1 (40)	-1 (30)	1(6)	1,64
3	-1 (40)	1 (50)	-1 (4)	0,05
4	-1 (40)	1 (50)	1 (6)	1,14
5	1 (80)	-1 (30)	-1 (4)	0,72
6	1 (80)	-1 (30)	1 (6)	4,92
7	1 (80)	1(50)	-1 (4)	0,41
8	1 (80)	1(50)	1 (6)	2,86
9	0 (60)	0(40)	0 (5)	0,23
10	0 (60)	0(40)	0 (5)	0,53
11	0 (60)	0(40)	0 (5)	0,69

[a]unit: g/L

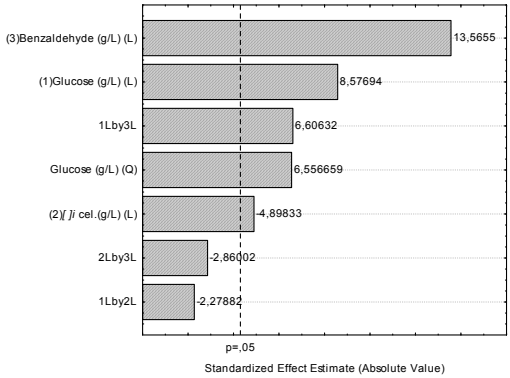
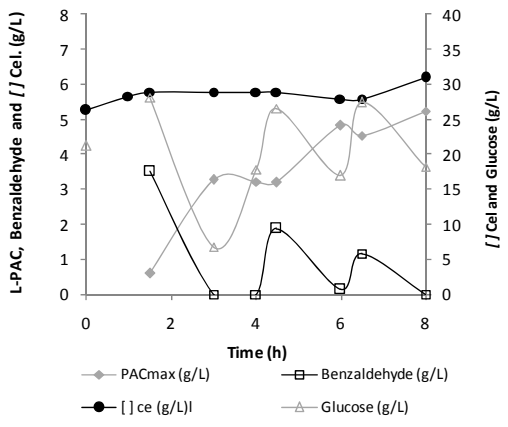


Figure 3 Pareto chart showing the effect of dependent variables on L-PAC production.

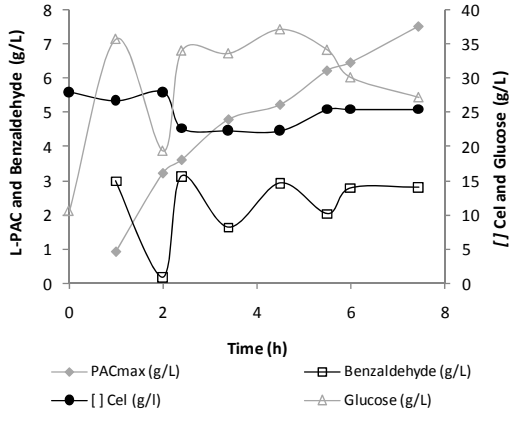
By the results obtained in shake flasks, L-PAC production was evaluated in a 1 L bioreactor in a feed batch process with the initial production medium (PM) and the optimized one (OM). Benzaldehyde and glucose were fed intermittently after 1.5, 4.5 , and 6.5 h of process in PM and 1, 2.5, 4.5 and 6 h in OM and these substrates were monitored by quantitative analysis in order to maintain benzaldehyde concentration inferior to 6.0 g / L and glucose superior to 40 g/L. Khan et al. (2012) using *Candida utilis* determined that the best interval between additions of benzaldehyde would be 1 h. Shorter intervals would have a toxic effect and would favour the production of higher alcohol dehydrogenase, which would reduce L- PAC production.

Figure 4 shows that different batches performed has enabled an increase in L-PAC production with an increase of benzaldehyde concentration.

The best result (7.5 g/L) was obtained with the medium composition determined by the PB-12 and with four feeds of benzaldehyde and three glucose feeds (Figure 4b).



(a)



(b)

Figura 4 Kinetics of the experiments performed in bioreactor with glucose and benzaldehyde feeding with different medium: (a) PM; (b) OM;

The best experimental conditions presented an yield ($Y_{P/S}$) of 0.91 g of L-PAC / g of benzaldehyde with an efficiency of η =74% (efficiency based in the theoretical $Y_{P/S}$, would be $Y_{P/S}$ =1,23.

4. Conclusions

By using the experimental design PB12 it was possible to reduce peptone, citric acid and sodium chloride concentrations in the medium. However, the magnesium sulphate concentration was increased. In shake flaks, the factorial design 2^3 showed that 30 g of biocatalyst per litter was the best initial cell concentration,

which resulted in a L-PAC production 2.4 times higher than with 3 g of cells/L (4.92 g of L-PAC/L). The feed batch operation in the bioreactor using this best initial cell concentration and the optimized medium, 7.5 g of L-PAC per litter was achieved, which represents a 44% increase when comparing to the non-optimized medium.

Acknowledgments

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