Potential application of raw glycerol from biodiesel in citric acid production by *Yarrowia lipolytica*

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A possible application of glycerol from biodiesel is the production of high-value compounds through microbial fermentation. In this work was optimized the culture medium by experimental design to produce citric acid by strain *Y. lipolytica* (IMUFRJ 50,682), using glycerol as carbon source and varying the carbon/nitrogen ratio. The parameters used were: glycerol, ammonium sulfate, yeast extract and stir. Was found a production of 0.55 g /L citric acid for the test that used 200 g/L glycerol, 0.5 g/L yeast extract, 0 g/L ammonium sulfate, the agitation of 250 rpm, in deionized water and non-buffered medium. For the second experimental design the factors used were glycerol and yeast extract. Was found a production of 2.51 g/L citric acid for the test that used 150 g/L glycerol and 0.1 g/L yeast extract, using agitation of 250 rpm, in deionized water and non-buffered medium. Repeating this test in the mineral buffer was obtained a production of 10.64 g/L citric acid. Using these same concentrations of glycerol and yeast extract were performed 2 experiments using the co-product of biodiesel production. The first study in non-buffered mineral medium reached a production of 3.65 g/L citric acid and the second obtained 4.18 g/L citric acid using a buffer medium.

1. Introduction

Glycerol has been produced from the production of biodiesel, which is a promising renewable energy source designed to replace oil (Silva *et al*. 2009; Rymowicz *et al*. 2006; Papanikolaou *et al*. 2002). The substance 1,2,3-propanetriol, function belongs chemical alcohol, is present in industrial applications. One of the promises for the implementation of glycerol from the biodiesel production is the production of high-value compounds by microbial fermentation (Silva *et al*. 2009; Papanikolaou *et al*. 2008). Several microbial metabolites can be produced from glycerol, including citric acid (Papanikolaou *et al*. 2008; Rymowicz *et al*. 2008; Rymowicz *et al*. 2006; Papanikolaou *et al*., 2002).

Citric acid is a weak organic acid, which can be produced on an industrial scale by filamentous fungus Aspergillus niger by submerged fermentation process with sucrose as carbon source (Papanikolaou *et al*. 2002; Rymowicz *et al*. 2006, Levinson *et al*. 2007; Rymowicz *et al*. 2008, Silva *et al*. 2009).
The yeast *Yarrowia lipolytica* is unique in its ability to produce and excrete into the culture medium a wide variety of organic acids, including intermediate tricarboxylic acid cycle citric acid, isocítrico, α-ketoglutaric and pyruvic (Barth and Gaillardin, 1997; Fickers *et al*., 2005). Studies indicate a high production of citric acid by *Y. lipolytica* from the glycerol biodiesel' production. Levinson *et al*. (2007) show that the composition of the medium and the carbon/nitrogen ratio are important in the production of citric acid and isocitric.
The purpose of this study was to optimize the culture medium to produce citric acid by *Yarrowia lipolytica* IMUFRJ 50,682, using PA glycerol and glycerol from the biodiesel production as carbon sources and varying the carbon / nitrogen.

2. Materials and Methods

2.1. Microorganism

The microorganism used was a wild strain of *Yarrowia lipolytica (*IMUFRJ 50682) selected from an estuary in Rio de Janeiro, Brazil (Haegler & Mendonça, Haegler, 1981) that kept in YPD medium with 2% glucose, 2% peptone, 1% yeast extract and 2% Agar-Agar at a temperature of 4 º C

2.2. Medium composition

A factorial design 2^{k-1}, with k = 4 and three central points, was performed by varying the concentration of glycerol, yeast extract, ammonium sulfate and agitation. The table 1 presents the experimental design adopted. The culture medium were prepared with the components of non-buffered mineral medium in g/L: KH_2PO_4,12; $Na_2HPO_4.7H_2O$,12; $MgSO_4.7H_2O$, 1,5; $CaCl_2.2H_2O$, 0,15; $FeCl_3.6H_2O$, 0,15; $ZnSO_4.7H_2O$, 0,02; $MnSO_4.H_2O$, 0,06.

Table 1 - Code and real values of the variables of the experimental design 2^{k-1}, and k = 4.

Test	Glycerol		Yeast extract		Ammonium sulfate		Agitation	
	Code	Value (g/L)	Code	Value (g/L)	Code	Value (g/L)	Code	Value (rpm)
1	-1	40	-1	0,5	-1	0	-1	160
2	+1	200	-1	0,5	-1	0	+1	250
3	-1	40	+1	10	-1	0	+1	250
4	+1	200	+1	10	-1	0	-1	160
5	-1	40	-1	0,5	+1	10	+1	250
6	+1	200	-1	0,5	+1	10	-1	160
7	-1	40	+1	10	+1	10	-1	160
8	+1	200	+1	10	+1	10	+1	250
9	0	120	0	5,25	0	5	0	205
10	0	120	0	5,25	0	5	0	205
11	0	120	0	5,25	0	5	0	205

2.3. Conditions of cultivation

The experiments were conducted in flasks of 1 liter containing 400 ml of culture medium and inoculated with YPD medium described above at a concentration of 1g / L and incubated at 28 ° C in a rotary shaker (Tecnal Digimec BTC9090) for 5 days.

2.4. Determination of organic acids

Samples of 5 mL were collected from the culture medium and centrifuged in a Sigma 2K centrifuge 15 for further analysis of organic acids. The concentration of citric acid was determined by enzymatic kit (Biopharm).

3. Results and Discussion

Table 2 presents the results of citric acid production by enzymatic method for the experiments of the first experimental design. It is apparent that there was significant production only in tests 1 and 2, which contains the largest C / N ratios, ie, when the source of nitrogen is more limiting. The kinetics of production is shown in Figure 1.

Table 2 - Maximum production of citric acid to each test with an enzymatic method

Test	1	2	3	4	5	6	7	8	9	10	11
Citric Acid (g/L)	0,59	0,55	0,013	0,015	0,007	0,003	0,024	0,02	0,013	0,017	0,012

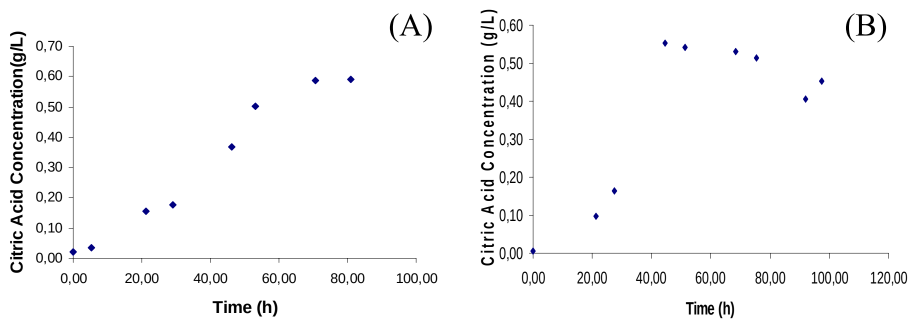

Figure 1: Production of Citric Acid (A) Test 1 and (B) test 2

The second experimental design was drawn from the test 2, because in terms of productivity test 2 was better. A factorial design 2^k, with k = 2, was performed by varying only the concentration of glycerol and yeast extract. Table 3 shows the experimental design adopted a total of 6 tests. Tests 5 and 6 are the central points. Initially, the culture media were prepared in non-buffered mineral medium, described above in materials and methods section.

Table 3 - Code and real values of the variables of the experimental design 2^k, and k = 2

Tests	Glycerol		Yeast extract		Agitation
	code	value (g/L)	code	value (g/L)	value (rpm)
1	-1	150	-1	0,1	250
2	1	250	-1	0,1	250
3	-1	150	1	0,9	250
4	1	250	1	0,9	250
5	0	200	0	0,5	250
6	0	200	0	0,5	250

Table 4 presents the results of maximum concentration of citric acid obtained in each test analyzed by enzymatic method. The highest concentration of citric acid was obtained in test 1, which was 2.51 g / L in the process time of 75.83 h. Test 1 was formulated with the least amount of substrate glycerol and yeast extract, benefiting from the cost-benefit.

Table 4 - Maximum production of citric acid to each test of the 2 experimental design with an enzymatic kit

Test	1	2	3	4	5	6
Citric Acid (g/L)	2,51	1,62	0,90	0,74	1,37	1,13
Time Process (h)	75,83	45	75,83	75,83	75,83	75,83

From the results of the second experimental design and noting that the test 1 provided a higher acid concentration an experiment was made by replacing the glycerol PA by glycerol from the biodiesel under the same conditions of test 1 of the second experimental design, ie, glycerol concentration of 150 g / L, yeast extract equal to 0.1 g / L, using 250 rpm agitation and non-buffered mineral medium. For this test there was a production of 3.65 g / L of citric acid as shown in figure 2.

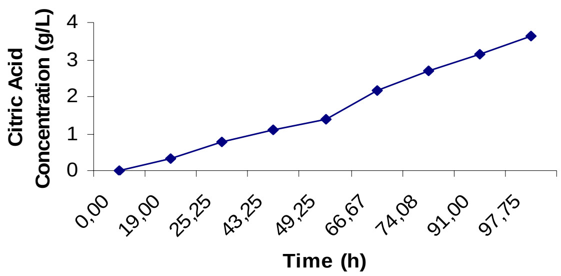

Figure 2. Production of citric acid using the same values of the factors of the test 1 of the second experimental design using glycerol from the biodiesel production in non-buffered mineral medium.

New tests were performed using the same values of the test 1of the second experimental design to evaluate the influence of mineral medium buffered in the production of citric acid. The composition of the new buffered mineral medium consisting of KH_2PO_4: 12 g/L; Na_2HPO_4. 7 H_2O: 22.66 g/L; $MgSO_4$. 7 H_2O: 1.5 g/L; $CaCl_2$. 2 H_2O: 0.2 g/L; $FeCl_3$. 6 H_2O: 0.15 g/L; $ZnSO_4$. 7 H_2O: 0.02 g/L; $MnSO_4$. H_2O: 0.06 g/L. In this batch of experiments, the glycerol production of biodiesel was also tested. Using the glycerol PA in buffered mineral medium was produced 10.65 g /L of citric acid as shown in figure 3.

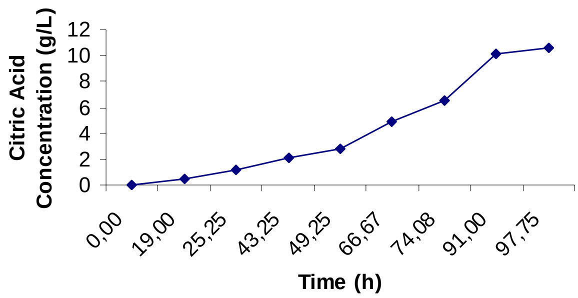

Figure 3. Production of citric acid using the same values of the test 1 of the second experimental design using glycerol PA in mineral medium buffered.

There was a production of 4.18 g / L of citric acid to the experiment that used the co-product generated in the production of biodiesel in mineral medium buffered, as shown in Figure 4.

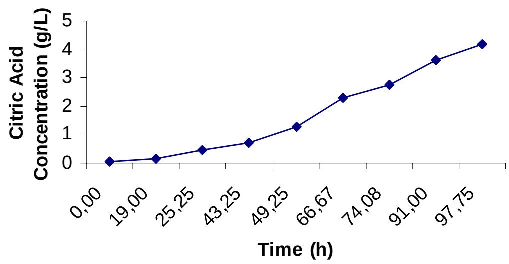

Figure 4. Production of citric acid using the same values of the factors of the test 1 using glycerol biodiesel in mineral medium buffered

4. Conclusion

The strain *Yarrowia lipolytica* (IMUFRJ 50682) showed to be a significant producer of citric acid from glycerol PA and glycerol from the biodiesel production. Through this study, was observed that the carbon / nitrogen ratio was an important parameter in the production of citric acid and to improve citric acid production medium must be capable of limiting nitrogen. The concentration of salts that make up the mineral medium also influenced the final production of citric acid. It was observed that in both buffered mineral medium using glycerol PA and glycerol from biodiesel the concentration of citric acid was higher. For the test 1of the second experimental design using glycerol PA among non-buffered mineral the production was 2.51 g / L. However, for this same test, but applying mineral medium buffered the maximum concentration of citric acid was 10.65 g / L. The same behavior was observed when was used the glycerol from the production of biodiesel. In non-buffered mineral medium the production was of 3.65 g/L and buffered mineral medium was produced 4.15 g / L.

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