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Production of Citric Acid by *Yarrowia lipolytica* in Different Crude Glycerol Concentrations and in Different Nitrogen Sources

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Increased demand for biodiesel leads to an abundant amount of glycerin in the market. The value of the glycerin has much to offer in reducing the cost of biodiesel production and minimize environmental problems caused by the producers of this biofuel. Strains of the yeast *Yarrowia lipolytica* has the ability to grow in culture media containing glycerin stemming from the biodiesel industry and produce citric acid, which has a large industrial application. The aim of the present work was to study citric acid production by *Y. lipolytica* IMUFRJ 50682 using crude glycerol stemming from biodiesel industries as carbon source. It was tested different initial concentrations of glycerol and ammonium sulfate added to the culture medium. When the tests were performed to verify the influence of the concentration of glycerol used in nitrogen deficiency in the production of citric and isocitric acids, it was observed that the production of citric and isocitric acids was equal to 12.94 g/L and 6.66 g/L, respectively, in 160 h of fermentation for the test that contained 45 g/L of glycerol, showing that the strain Y. *lipolytica IMUFRJ 50682* requires less carbon source to maximize the production of citric acid, generating a higher yield in terms of citric acid. In tests with the addition of ammonium sulfate to determine the influence of nitrogen source in the production of the interest, the results show that the addition of ammonium sulfate to the culture medium directs the metabolic pathway for the production of isocitric acid because it was producted only 1.46 g/L citric acid and 16.79 g/L isocitric acid in 93 hours fermentation.

1. Introduction

Biofuels are alternative fuels to fossil fuels, which in addition to being renewable and biodegradable, they also have the advantage of reducing oil imports and reducing the emission of carbon monoxide, hydrocarbons, soot and particulate. An example of biofuel that is being widely produced is biodiesel and during its obtained process, other compounds are also generated as a by-product. The amount of these additional products generated is growing due to increased production and glycerol is the main byproduct of this production process, accounting for 10 % of biodiesel produced (DasariI et al., 2005).
The large amount of glycerol obtained during the production of biofuels has led to search for alternatives to the use of this byproduct. In order to avoid environmental problems with accumulation of glycerol and reduce costs of biodiesel production, the use of glycerol as a carbon source for the production of compounds of commercial interest has been very attractive. Through microbial process, organic acids can be produced with glycerol as the carbon source, including citric acid (Da Silva et al., 2009; Papanikolaou et al., 2008).

Citric acid, the official name of 2-hydroxy-1 ,2,3-propanetricarboxylic, is an intermediate of the Krebs Cycle and is produced industrially by the filamentous fungi *Aspergillus niger* by submerged fermentation with sucrose as a carbon source (Da Silva et al., 2009; Levinson et al., 2007; Rymowicz et al., 2006; Rymowicz et al., 2008). In 2007, the world production of citric acid increased to 1.7 Mt (BCC Research, 2011) and the consumption of this acid increases around 5 % per year, mainly due to its use in food products (70 %), pharmaceutical (12 %), cosmetics and in other industrial products (18 %) as acidifier, antioxidant, flavoring, preservative and plasticizer (Silva et al., 2010).

Due to increased demand for citric acid, alternative processes involving cultivation of yeast species are being used for their production. Specifically *Yarrowia lipolytica*, *Candida quillermondii* and *Candida oleophila* have been used for the production of citric acid (Rymowicz et al., 2006). It becomes necessary to use a cheap raw material and available in industrial production because citric acid is a commodity. For this reason, the yeast *Yarrowia lipolytica* has been extensively used for production of organic acids through various renewable sources or waste materials as substrates, for example, glycerol from biodiesel production.

The cultivation condition for the production of citric acid and its excretion by *Y. lipolytica* is the limitation of cell growth by the excess of carbon and nitrogen deficiency in the culture medium (Fickers et al., 2005). After nitrogen deficiency in the stationary phase of growth, the yeast metabolic activity is kept and it still assimilates carbon and producing citric acid (Morgunov et al., 2004).

The use of *Candida* or *Yarrowia* has some advantages over the filamentous fungi: higher conversion rate, increased productivity, improved process control due to the unicellular nature of yeasts (Fickers et al., 2005). A disadvantage of this process is the significant amount isocítric acid produced during fermentation, which can reach levels above 50 % of the total acid produced (Anastassiadis et al., 2002). Isocitric acid has buffering and chelating capacity lower that citric acid. Additionally, the crystallization of citric acid during the purification process is hampered by contamination with isocitric acid in concentrations higher than 5 %. Therefore, the challenge in this process is to minimize the simultaneous production of isocitric acid.

Therefore, the aim of the present work was to study citric acid production by *Y. lipolytica* IMUFRJ 50682 using crude glycerol stemming from biodiesel industries, by using this renewable substrate as carbon source. Different initial concentrations of glycerol and ammonium sulfate in the culture medium were tested in order to increase citric acid production and decrease isocitric acid secretion.

2. Materials and Methods

2.1 Materials

The components of culture media used were peptone and yeast extract (Oxoid - Hampshire, UK), Glucose (Vetec - RJ, Brazil), agar-agar (Vetec - RJ, Brazil). The crude glycerin used in this study was kindly supplied by Mr. Charles Khalil (M.Sc.), Senior Consultant CENPES Petrobras, Brazil.

2.2 Microorganism

The microorganism used in this study was the wild-type strain *Yarrowia lipolytica* IMUFRJ 50682 selected an estuary in Rio de Janeiro, Brazil (Haegler & Mendonca-Haegler, 1981).

2.3 Crude glycerol treatment

Crude glycerol was derived from the transesterification reaction of soybean oil and ethanol catalyzed by NaOH.

Before being added to the citric acid production medium, the pH of the glycerin phase was corrected with H_2SO_4 (1 N) to 7 to eliminate the free alkalinity. It was then subjected to heating (up to 120 °C for approximately 1 h) under agitation to eliminate the ethanol. The sulfate resulting from the neutralization was separated by decantation in a separatory funnel during 24 h.

2.4 Inocullum and Culture conditions

The inoculum was prepared with YPD medium % ((w/v) glucose, 2; peptone 2, yeast extract, 1), inoculated and incubated in a rotary shaker at 28 °C, 160 rpm agitation for 72 h. After this period biomass from this inoculum was used to inoculate the citric acid production medium in sufficient amount for an initial cell concentration of 2 g dry weight of cells per litre.

The media composition for citric acid biosynthesis is described in Table 1. These media also contained minerals, such as KH_2PO_4 (12 g/L), $Na_2HPO_4.7H_2O$ (22.66 g/L), $MgSO_4.7H_2O$ (1.5 g/L), $CaCl_2.2H_2O$ (0.2 g/L), $FeCl_3.6H_2O$ (0.15 g/L), $ZnSO_4.7H_2O$ (0.02 g/L), $MnSO_4.H_2O$ (0.06 g/L) (Papanikolau *et al.*, 2002).

The production of citric acid was carried out in 1 L flasks containing 0.4 L of medium in a rotary shaker at 28 °C and 250 rpm with daily sampling for determination of biomass concentration, pH and concentration of citric and isocítric acids.

2.5 Analytical methods

2.5.1 Quantification of cell growth

Cell concentration was determined in a Shimadzu spectrophotometer by optical density measurements at 570 nm and these values were converted to mg dry weigh/ml using a conversion factor previously determined.

2.5.2 Determination of organic acids

The quantification of citric and isocitric acids was performed by high performance liquid chromatography (Waters 1525), ultraviolet detector and a zinc lamp at 214 nm. The analytical column used was octadecylsilane (YMC-Pack ODSAQ, S-5 mm, 12nm). The mobile phase consisted of 10% methanol solution (70%) and 90% KH_2PO_4 buffer solution (pH 2.45).

2.5.3 Quantification of glycerol

The determination of glycerol was carried out using high performance liquid chromatography (HPLC), equipped with an Aminex HPX-87H column (Bio-Rad) and refractive index detector, with the mobile phase, 0.005 M sulfuric acid (0.80 mL/ min) at a temperature of 60 ° C.

2.5.4 pH

The pH of the culture medium free of cells was determined using a pHmeter Digimed brand, model DM-22

Table 1: Composition of citric acid production media

Test	Glycerol (g/L)	Yeast Extract (g/L)	Ammonium Sulphate (g/L)
1	45	0.1	0
2	160	0.1	0
3	45	0.1	0.7

2.6 Calculation of fermentation parameters

For comparison of all trials, production parameters, such as citric acid yield from the consumption of glycerol ($Y_{P/S}$) conversion factor of glycerol in cells ($Y_{X/S}$), citric acid yield relative to biomass formed ($Y_{P/X}$), volumetric productivity of citric acid (Q_{CA}) and ratio of citric and isocitric acids production ($R_{CA}/_{ICA}$) were determined. The equations used to calculate these parameters are described below.

$$Y_{P/S} = -\frac{P_{CA}}{S_f - S_o}\ , (g/g) \quad (1)$$

$$Y_{X/S} = -\frac{X_f - X_o}{S_f - S_o}\ , (g/g) \quad (2)$$

$$Y_{P/X} = \frac{P_{CA}}{X_f - X_o}\ , (g/g) \quad (3)$$

$$Q_{AC} = \frac{P_{CA}}{V * t}\ , (g/L.h) \quad (4)$$

$$R_{CA/ICA} = \frac{P_{CA}}{P_{ICA}} \quad (5)$$

where P_{CA} is the maximum concentration of citric acid produced; P_{ICA} is the citric acid concentration at time t of maximum production of citric acid; S_f is the glycerol concentration at time t of maximum production of citric acid; S_0 is the initial concentration of glycerol; X_f is cell concentration at time t of maximum production of citric acid, X_0 is the initial cell concentration, V the initial volume of liquid culture and t is the fermentation time for maximum citric acid production.

3. Results and Discussion

Data in literature indicate that the composition of the culture medium and the carbon to nitrogen ratio are important parameters in the production of citric and isocitric acids, being necessary to establish optimal values for these parameters in order to improve the production of citric acid (Levinson et al., 2007). Therefore, the effect of citric acid production medium composition was studied.

This study was conducted to evaluate the influence of the initial crude glycerol concentration in the citric acid production. Figure 1 shows cell growth and pH profiles as well as the production of citric and isocítric acids, of the experiments performed with initial crude glycerol concentrations of 45 g/L (Test 1) and 160 g/L (Test 2). It is possible to observe that the cell concentration is higher when less crude glycerol is added. There was a reduction in cell concentration in 87 h of process in test 1, restarting later. In test 2, it was observed that up to 87 h, cell concentration was reduced and oscillated between 0.67 g/L and 4.72 g/L. This oscillation can be attributed to the presence of intense foaming. This is due to the biosurfactant production since this strain produces emulsifier activity in similar conditions (Amaral et al., 2006).

The initial and final pH values were similar for both trials. The production of citric acid gradually increased in the stationary phase of growth and maximum production was obtained in the end of the process (160 h) for Tests 1 and 2. The citric acid production was limited in the first 90 h when higher crude glycerol was used. After that, when no foaming was apparent, citric acid started to be produced (Figure 1b).

The isocitric acid production was favored by major initial glycerol concentration and, as can be seen from Figure 1, in this experiment isocitric acid concentration remained almost constant between 70 h and 95 h of fermentation.

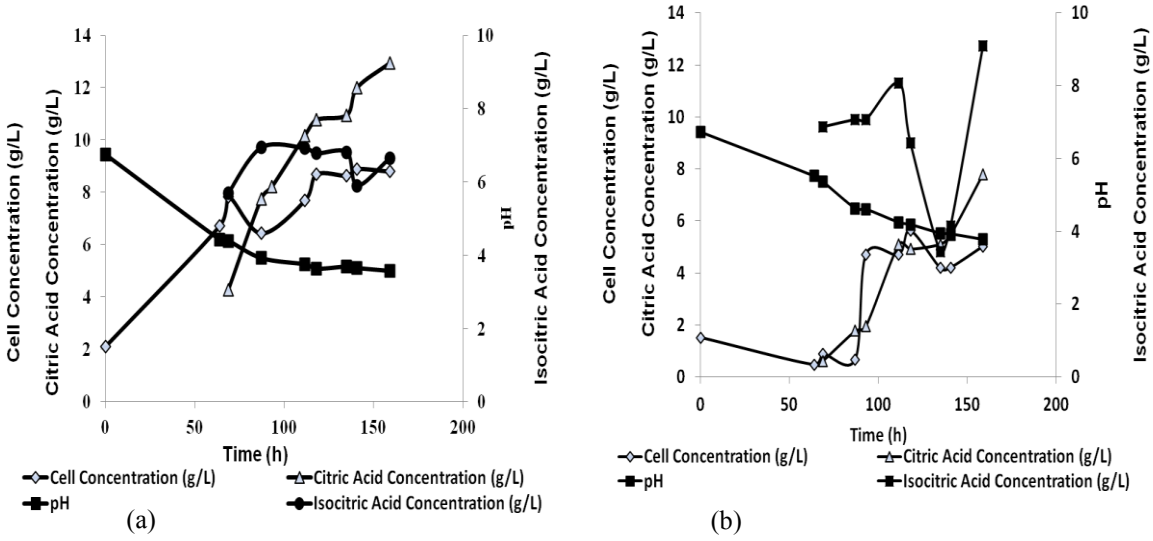

Figure 1: Kinetics of cell concentration, citric and isocitric acids concentrations and pH with 45 (a) and 160 (b) g/L (crude glycerol per liter of citric acid) production medium

Table 2 presents the results obtained after 160 h of citric acid production process, when maximum citric acid production was reached. The concentration of citric acid as well as the Q_{CA} and $Y_{P/S}$ were higher for the test conducted at lower concentrations of glycerol. The consumption of glycerol detected was 30.72 g/L to test 1. In the test 2, the presence of around 90 g/L of residual glycerol was detected at the end of the process, indicating that the *Y. lipolytica* IMUFRJ 50682 was not able to consume the all this carbon source, probably due to cell inhibition by this high glycerol concentration.

Table 2: Results of tests using different initial concentrations of glycerol without ammonium sulphate

Initial Glycerol Concentration (g/L)	P_{CA} (g/L)	Q_{CA} (g/L.h)	$Y_{P/S}$	$Y_{X/S}$	$Y_{P/X}$	P_{ICA} (g/L)	$R_{CA/ICA}$
45	12.94	0.08	0.42	0.22	1.94	6.66	1.94
160	7.8	0.05	0.18	0.08	2.22	9.09	0.86

According to Fickers et al. 2005, the cultivation condition for the production of citric acid and its excretion by *Y. lipolytica* is the limitation of cell growth by the excess of carbon and nitrogen deficiency in the culture medium, but the results of this study contradict the information obtained in the literature, since the maximum citric acid production was favored at lower concentrations of glycerol tested.
In order to check the influence of the nitrogen source in citric and isocitric acids production, 0.7 g/L of ammonium sulfate was added (Test 3, Table 1). Crude Glycerol concentration used in this test was 45 g/L since this of the best condition for citric acid production. When 0.7 g/L of ammonium sulfate was added to the culture medium, the change in pH was lower than the first two tests. The cell concentration ranged from 2.01 g/L to 9.68 g/L, similar to that observed without the addition of ammonium sulfate. However, citric acid production was reduced (Table 3). The accumulation of isocitric acid was favored with the addition of ammonium sulphate, representing a production 2.4 times higher than in tests 1 and 2. Comparing the $R_{CA/ICA}$ of the three tests, it is observed that test 1 had higher ratio. The low production of citric acid and glycerol consumption of 26.85 g/L for the third test justify the low value of $Y_{P/S}$.

Table 3: Experimental results of tests using glycerol concentration 45 g/L and 160 g/L without the addition of ammonium sulfate and 45 g/L with the addition of ammonium sulfate in 93 h of production

Initial glycerol Concentration (g/L)	P_{CA} (g/L)	Q_{AC} (g/L.h)	$Y_{P/S}$	$Y_{X/S}$	$Y_{P/X}$	P_{AIC} (g/L)	$R_{AC/ISC}$	Process Time (h)
45 g/L com 0.7 g/L de sulfato de amônio	1.46	0.02	0.05	0.29	0.19	16.79	0.09	93
45	8.21	0.09	-	-	-	6.92	1.19	93
160	1.95	0.02	-	-	-	7.07	0.28	93

4. Conclusion

The composition of the culture medium influences the production of citric and isocitric acids by *Yarrowia lipolytica* IMUFRJ 50682. The concentration of citric acid was favored by the lower initial concentration of glycerol tested in this study and in the absence of ammonium sulfate. When this nitrogen source was added to the fermentation broth, there was a reduction in the production of citric acid and increased isocitric acid production.

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