EVALUATION AND OPTIMIZATION OF GROWTH AND CITRIC ACID PRODUCTION BY *Yarrowia lipolytica* NRRL Y-1095 USING GLYCEROL AS CARBON SOURCE AS AN ALTERNATIVE TO USE BIODIESEL BYPRODUCT

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ABSTRACT

The aim of the present study was to optimize growth and citric acid production by *Yarrowia lipolytica* NRRL Y-1095 using glycerol as the sole carbon source, like an alternative to use biodiesel glycerol, a promising and cheap carbon source. Fermentations were performed in Erlenmeyer flasks to optimize growth and citrate production from glycerol. The fermented broth was analyzed by HPLC equipped with a UV and RI detector to evaluate isocitrate, citrate and glycerol consumption. The growth medium was optimized in flasks and in batch fermentation. The present study have optimized media conditions for the growth phase of *Yarrowia lipolityca* NRRL Y-1095 using experimental design and surface response methodology, obtaining 6.18 g.l^{-1} of dry cell weight (DCW) and up to 22 g.l^{-1} DCW in bioreactor after 96 h. Six fermentations were performed in a feed batch reactor with varying aeration and agitation. Dissolved oxygen was an important factor and a 0.5 yield of citric acid was obtained from feed batch fermentation, where up to 59 g.l^{-1} of citric acid was obtained. Glycerol is a cheap alternative to citric acid production since biodiesel glycerol production is growing rapidly and becoming an environmental problem.

1 Introduction

Citric acid is widely used in the food, chemical and pharmaceutical industries due to its properties as an acidifier, preservative, pH adjuster and antioxidant (Antonucci et al., 2001; Venter et al., 2004; Anastassiadis et al., 2005; Jianlong et al., 2007). It represents more than 70% (w/w) of all food acidulates and one of the most widespread metabolites produced through biotechnological methods on an industrial scale, with an annual demand of more than 1.2 million tons (Papanikolaou & Aggelis, 2009). The current price of citric acid is about \$1.3 per kg (Rymowicz et al., 2008) and its production reached over 1.7 million tons in 2011 worldwide (Rywińska et al., 2011), and represents over a 2 billion dollars industry (Soccol et al., 2006; Sauer et al., 2008). Also, there is an estimated 3.5–4.0% annual growth in demand of citric acid (Vandenberghe et al., 2004). Because of the large and ever-increasing demand for citric acid, alternative approaches of its production are required. Among various approaches, the use of yeast strains for production of citric acid is the most studied one (Rymowicz et al., 2008).

One commonly reported disadvantage associated with citric acid production by yeast is the significant amount of isocitric acid produced by yeasts during fermentation, which can reach as much as 50% of the total acid production, negatively affecting the citric acid yield (Antonucci et al., 2001; Rymowicz et al., 2008) and also interferes with citric acid crystallization when contamination is over 5%. Furthermore, citric acid production is preferably carried out by two stage fermentation, growth phase or trophophase, and citric acid production or idiophase (Moresi, 1994; Anastassiadis et al., 2002; Antonucci et al., 2001; Anastassiadis & Rehm, 2006), in which nitrogen and phosphorous deficient medium stimulates citrate accumulation (Rane & Sims, 1996, Rywińska et al., 2006).

In recent years, there has been considerable interest in finding less expensive carbon sources for citric acid production (Anastassiadis & Rehm, 2006; Moeller et al., 2007). Many substrates have been assessed for the production of citric acid, such as sugarcane molasses, beet molasses, glucose and sucrose, but fermentations using *Aspergillus niger* are generally carried out using refined sugar, which is expensive (Jianlong et al., 2007). However, *Yarrowia lipolytica* has the capacity to ferment various carbon sources to citric acid, such as n-alkanes, fatty acids, glucose, ethanol and glycerol (Moresi, 1994; Antonucci et al., 2001; Kamzolova et al., 2003; Crolla & Kennedy, 2004; Anastassiadis & Rehm 2006; Papanikolaou et al., 2006; Sauer et al., 2008).

Biodiesel production is on the rise throughout the world as a consequence of the prediction of fossil fuels extinguishing in the future (Fischer & Schrattenholzer, 2001; Silva et al., 2009). As from 10 kg of biodiesel produced, one kg of glycerol is left as the byproduct, a world market of 900 million tons of crude glycerol is expected from biofuel production alone in 2010 (Crolla & Kennedy, 2004; Vollenweider & Lacroix, 2004; Rymowicz et al., 2008). Alternatives for the use of this byproduct as substrate have been studied, since the costs of cleaning or disposal as a residue are included to the biodiesel price. So its conversion into other molecules may reduce the production costs of biodiesel (Papanikolaou et al., 2002; Yazdani & Gonzalez 2007; Silva et al., 2009). The very low cost (or even none) of crude glycerol makes it very attractive as carbon source, and its use minimizes environmental problems (Rywińska et al., 2009). Crude glycerol has also been used for the biosynthesis of citric acid by *Y. lipolytica* (Papanikolaou et al., 2002; Levinson et al., 2007; Rymowicz et al., 2008; Papanikolaou & Aggelis 2009). Nevertheless, despite the increasing supply of crude glycerol, only a few reports have been published thus far on the production of added-value microbial metabolites from this substrate (Rymowicz et al., 2008; Rywińska et al., 2009; Papanikolaou & Aggelis, 2009; Makri et al., 2010). The aim of the present study was to optimize growth and citric acid production by *Y. lipolytica* NRRL Y-1095 using glycerol as the sole carbon source.

2 Material and Methods

2.1 *Yarrowia lipolytica* Strain and its culturing

Yarrowia lipolytica NRRL Y-1095 used in this study was obtained from the Universidade Estadual de *Campinas*, Brazil. The culture was maintained at 8ºC in YM agar slants, with periodic transference to new media, or in long-term maintenance at -86ºC, using GYMP. The yeast strain was reactivated in 300 ml conical flasks with 50 ml of sterile YM broth (pH 6.5), at 28ºC and shaken at 200 rpm.

2.2 Growth Optimization

Growth optimization was performed using experimental design methods and surface response methodology using same conditions as item 2.1. Independent variables glycerol and yeast extract (YE) were tested, as glycerol was the major carbon and energy source. Experimental range and levels of the independent variables used are displayed in Table 1. The mineral medium for the fermentations consisted of the following (g L^{-1}): KH$_2$PO$_4$ (7.0), Na$_2$HPO$_4$ (2.5), CaCl$_2\cdot$ 2H$_2$O (0.05), FeCl3 (0.65), ZnSO4 (1.2), CuSO4.5H2O (0.31) and MnSO4 (0.27).

2.3 Citrate Production

To determine the influence of aeration and agitation over citric acid production, six fermentations were carried out using an Infors fermentor with a 13 l capacity reactor (with 4 l of working volume), with Rushton type turbines with 6 blades, 3 baffles, at 28ºC, pH controlled at 5.5 using NaOH 10 mol l^{-1} and foam controlled using FG-1520 silicon oil-based antifoam, automatically activated whenever foam was formed and 0.5 ml.h^{-1} limitation. A pO$_2$ probe was used to perform the oxygen readings. Air injected was filtered using a 0.22 μm PTFE filter.

Table 1 Growth – Range and values used in full factorial composite design.

Independent Variables		Range and Levels		
		-1	0	1
Glycerol (g L^{-1})	X_1	25	50	75
Yeast Extract (g L^{-1})	X_2	3	5	7

Table 2 Coded and observed values from growth experimental design.

Flask	X_1	X_2	Glycerol, g L^{-1}	Yeast Extract, g L^{-1}	Biomass, g L^{-1}	Residual YE, g L^{-1}	Residual Glycerol, g L^{-1}
1	-1	-1	25	3	4.37	0.21	9.92
2	-1	0	25	5	5.63	0.3	3.11
3	-1	1	25	7	5.87	0.49	4.51
4	0	-1	50	3	4.96	0.17	34.77
5	0	0	50	5	6.19	0.28	30.87
6	0	1	50	7	6.32	0.44	40.32
7	1	-1	75	3	4.07	0.17	52.88
8	1	0	75	5	5.39	0.24	53.62
9	1	1	75	7	4.66	0.44	51.84
10	0	0	50	5	6.19	0.24	30.87
11	0	0	50	5	6.20	0.26	30.84

X_1 Glycerol and X_2 Yeast Extract

Table 3 Dissolved oxygen in culture medium in six fermentations.

	0 h	72 h	96 h	144 h
1 – 1.0 vvm x 500 rpm	100%	45%	0%	0%
2 – 1.5 vvm x 500 rpm	98.1%	44%	0%	0%
3 – 2.0 vvm x 500 rpm	99.4%	52%	0%	0%
4 – 1.0 vvm x 800 rpm	100%	92%	79.4%	88.3%
5 – 1.5 vvm x 800 rpm	99.6%	96.6%	83.6%	82.4%
6 – 2.0 vvm x 800 rpm	97.2%	96.6%	83.2%	81.4%

The reactor filters and hoses were sterilized in an autoclave for 30 min at 121 ºC. Growth medium was formulated based on growth optimization, and contained 50.0 of glycerol, 5.0 g.l^{-1} of YE, 7.0 g.l^{-1} of KH$_2$PO$_4$, 2.5 g.l^{-1} of Na$_2$HPO$_4$, 0.05 g.l^{-1} of CaCl$_2$· 2H$_2$O, 0.65 g.l^{-1} of FeCl$_3$, 1.2 g.l^{-1} of ZnSO$_4$, 0.31 g.l^{-1} of CuSO$_4$.5H$_2$O and 0.27 g.l^{-1} of MnSO$_4$. The seed culture (same as growth medium, 10% v/v) was cultivated 24 h and inoculated in the reactor. After 72 h of growth, 320 ml of the medium was aseptically withdrawn from the vase and a sterile concentrated production medium made up of 400 g of glycerol (320 ml), 1.6 g of MgSO$_4$·7H$_2$O, 0.6 g of CaCl$_2$· 2H$_2$O, 0.4 g of KH$_2$PO$_4$ was then added. Thiamine was sterilized by filtration (0.22 µm PTFE filter) directly to the reactor to a final concentration of 5.0 mg.l^{-1}. Samples were taken aseptically every 24 h for a total of 168h.

2.4 Analytical Methods

The cells were harvested by centrifugation at 10,000 g for 10 min, and supernatant were kept frozen in anticipation of HPLC analysis. Cell concentrations were measured using a spectrophotometer at 610 nm and calculated by linear regression to dry biomass. Citric acid, isocitric acid and glycerol analysis were performed using high performance liquid chromatography (Dionex Ultimate 3000) with a 20 µL loop injection volume, equipped with an in-line vacuum degasser, Ultimate 3000 Autosampler, UV/Vis detector at 210 nm (UV Ultimate 3000 and RS Variable Wavelength Detector) and a Shodex Refractive Index detector. A Dionex Acclaim OA (150 mm x 4 mm) column was used to determine citric and isocitric acid, with a 1.0 ml.min^{-1} mobile phase of 100 mM of KH$_2$PO$_4$ acidified with methanesulphonic acid to 2.5 pH. Glycerol was determined by a Dionex AS-1 column (250 mm x 4.6 mm), with H$_2$SO$_4$ 0.005 M as the mobile phase at 0.6 ml min^{-1}. Nitrogen (YE) present at the end of the fermentation was

analyzed using the micro-Kjeldahl method (Lowenheim, 1972).

2.5 Experimental design and statistical analysis

A full factorial design was performed to test the effects on dry cell weight (DCW). All experiments were performed at least in triplicate. The Statistica 7.0 software package (StatSoft, USA) was used for the experimental design and regression analysis of the data. Response surface and contour plots were generated to understand the interaction of the different variables. ANOVA and regression analysis were performed, with polynomial regression only carried out with significant factors (p-value < 0.05). The polynomial model equation was tested by the coefficient of determination (R²) and the statistical significance was determined by an F-test. The significance of the regression coefficients was tested by the t-test.

3 Results and Discussion

3.1 Growth optimization

The growth experiments in flasks represented by a surface response graph (Figure1), which revealed the optimal concentrations of the main substrates (glycerol and yeast extract). There was significant interaction between these two variables ($p > 0.05$). ANOVA revealed $R^2 = 0.99$ for DCW.

Even after 72 h of cultivation, considerable amounts of nutrients remained. Residual glycerol and YE were quantified and plotted on surface response graphs (Figure 2). ANOVA revealed $R^2 = 0.98$ for residual yeast extract and $R^2 = 0.98$ for residual glycerol. The treatments with coded and experimental values and respective observed values are displayed in Table 2.
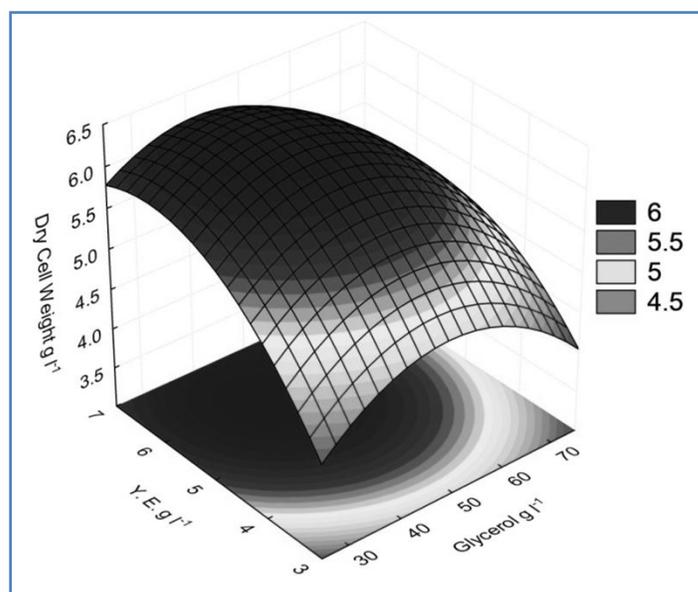

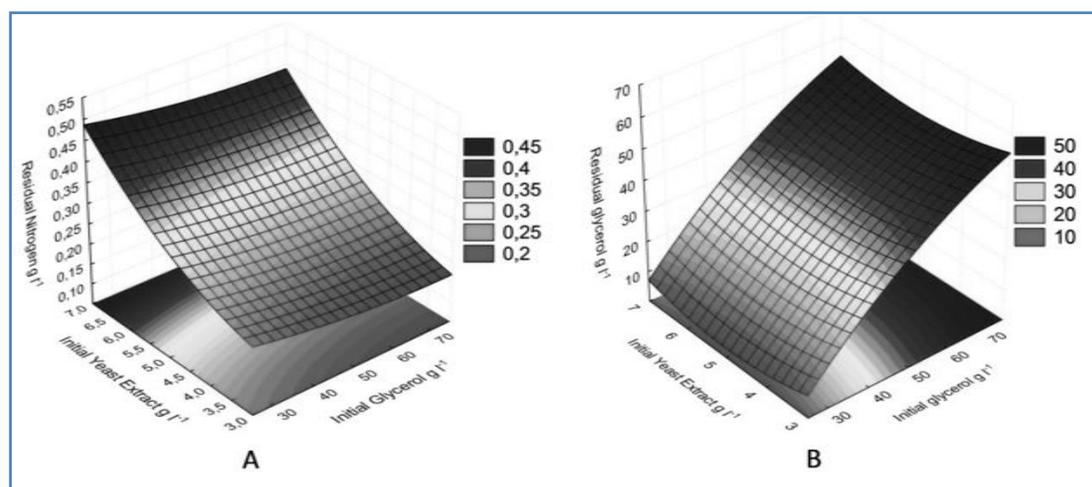Figure 1 Adjusted surface response from *Y. lipolytica* NRRL Y-1095 growth using glycerol and YE.

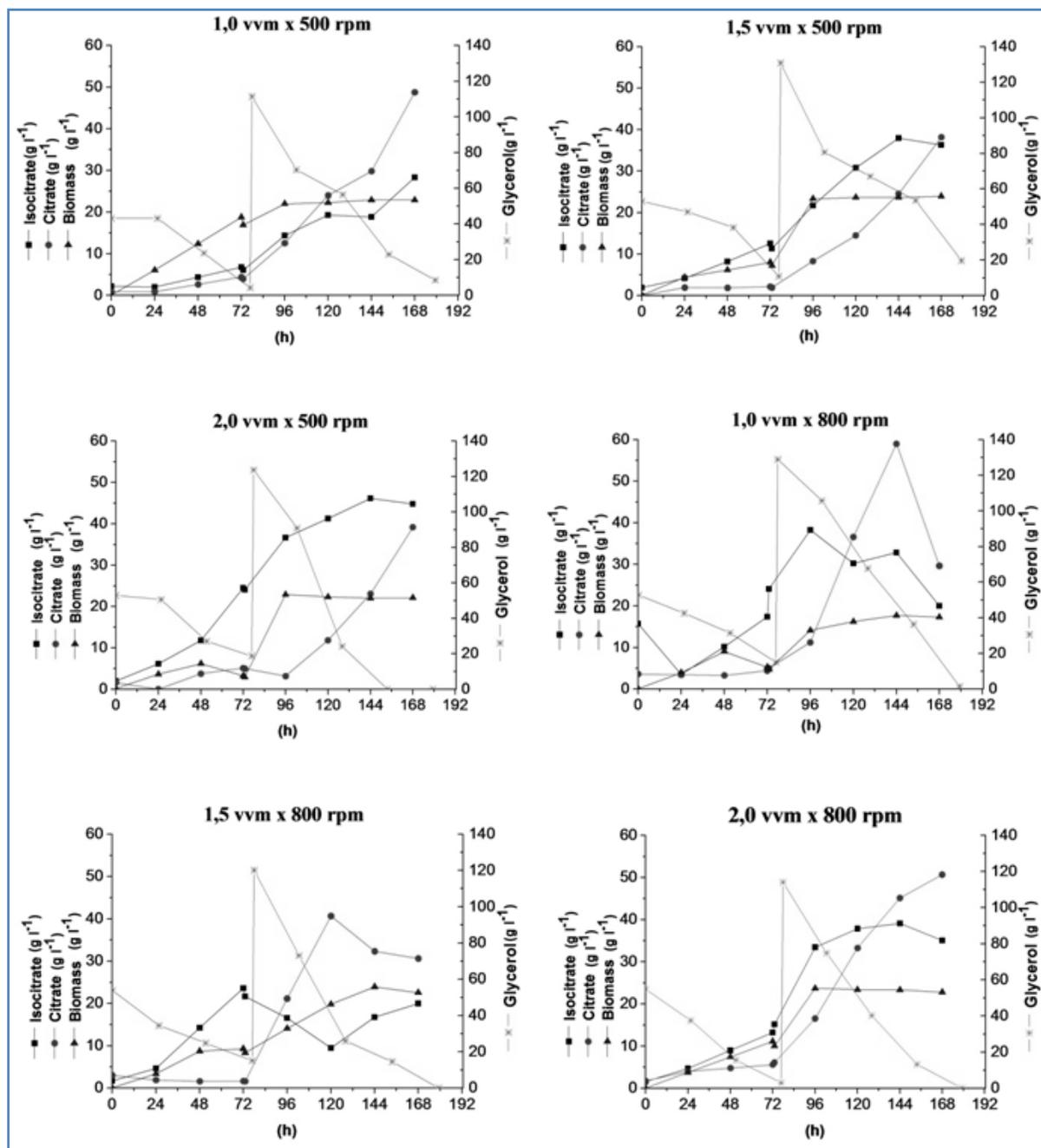Figure 2 Residual nitrogen (A) and glycerol (B) g L^{-1} after 72 h Fermentation.

Figure 3 Citric and isocitric acid in six different aeration and agitation conditions.

The decision was made to use 5.0 g.l^{-1} of YE and 50 g.l^{-1} of glycerol, which corresponded to the optimal area in the surface response and resulted in 6.18 g.l^{-1} of biomass in the flasks. Papanikolaou et al. (2002) using raw glycerol as carbon source and a different strain obtained 6.5 g.l^{-1} of biomass after 88 h using unbuffered medium, and 7.8 g.l^{-1} after 118h using buffered media. This amount of time might not be interesting to a biotechnological process. Although the present study opted

to use more glycerol and yeast extract, adjusts may be done to use less substrate and still have a high yield, since there was residual substrate left. Also, ammonium sulfate was not considered in the optimization. Regarding biomass growth in the fermentor, there was stability between 48 h and 72 h of cultivation in most experiments, but after adding the production medium, the yeast continued to grow and stabilized at close to 22 g.l^{-1} dry biomass. Even if controlled, the foam in

the early fermentation process leads to the accumulation of biomass on the vessel wall, which affects both growth and the readings of biomass in the culture medium, explaining differences among aerations used. Crolla & Kennedy (2004) also noticed that even after the end of the exponential growth phase, biomass continued to increase until the end of fermentation. Moreover, there was little difference among the four agitation speeds used in relation to total biomass. Kamzolova et al. (2003) reported increase of biomass at increasing air saturation from 5% to 60% at low iron concentration.

3.2 Citric acid production

The results of six fermentations with varying aeration (vvm) and agitation (rpm) are displayed in Figure 3. The best result for citrate production was obtained on the fourth fermentation at 144 h (800 rpm; 1.0 vvm), which reached 59 g.l^{-1} of citric acid produced. The second best result was obtained on the sixth fermentation (2 vvm; 800 rpm), with 45.13 g.l^{-1} of citric acid produced. The use of large volumes of aeration may have disturbed production due to increased foam in the reactor, requiring larger amounts of defoam. However, a better ratio of citrate to isocitrate (2:1) was obtained in the fermentation with less aeration (1.0 vvm; 800 rpm) within a shorter time (120 h). In other fermentations, lower agitation led to less citrate production. The concentrations of dissolved oxygen in the six fermentations are displayed in Table 3.

Citric acid production is stimulated by an increase in aeration in submerged fermentation. A lack of oxygen in the culture medium can lead to high proportions of isocitrate production, whereas no effect in citrate production was found at higher iron concentration using 20 to 60% air saturation. Raising air saturation from 5% to 60%, using low iron concentration, led to enhanced citrate biosynthesis (Kamzolova et al., 2003).

Using 500 rpm, the yeast generally consumed all the oxygen and, due to the large amount of biomass, not enough oxygen was available in the culture medium. This may explain the larger amount of isocitric acid in relation to citric acid in fermentations using this agitation. However, the use of 800 rpm resulted in a greater amount of available dissolved oxygen, which explains the greater amount of citric acid and higher ratio of citrate to isocitrate. Increasing the dissolved oxygen concentration from 20% to 80% saturation in the production of citric acid by *Yarrowia lipolytica* NRRL Y-1095 using a cell-recycle reactor leads to an increase in volumetric productivity as well as an increase in the ratio of citrate to isocitrate (Rane & Sims, 1996). Increasing aeration intensity and aeration rate has been reported to influence positively citric acid production in various yeast strains (Anastassiadis & Rehm, 2006). Crolla & Kennedy (2004) used four different agitation speeds in the synthesis of citric acid from n-paraffin using *Y. lipolytica* NRRL Y-1095. With fixed aeration (1.0 vvm), the authors also determined that the agitation influences the formation of biomass and citric acid synthesis. Best results occurred when using 800 rpm to 1000 rpm, with the best

productivity achieved using 800 rpm. Rywińska et al. (2010) reported a maximum citric acid concentrations (157.5 and 155.2 g.l^{-1}), obtained from 300 g.l^{-1} of glycerol in fedbatch cultures production. However, periodic feeding with 200 g.l^{-1} of the substrate was more efficient in yield.

Conclusions

Glycerol was found to be the most important factor in flask fermentation, both in the production of citric acid and isocitric acid. The use of regular flasks did not favor the accumulation of citrate due to restrictions to the transfer of oxygen to the culture medium. Fermentation in reactor produced greater amounts of citrate. Using 1.0 vvm aeration and 800 rpm, 59 g.l^{-1} of citric acid and 29 g.l^{-1} of isocitric acid (2:1 ratio) were produced. The use of glycerol for the production of citric acid by *Yarrowia lipolytica* NRRL Y-1095 may be a profitable alternative.

Acknowledgments

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