

## **A Cost Effective Fermentative Production of Glutathione by *Saccharomyces cerevisiae* with Cane Molasses and Glycerol**

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### **ABSTRACT**

*This work aimed to evaluate the effect of sugar cane molasses and glycerol on glutathione (GSH) fermentation by Saccharomyces cerevisiae ATCC 7754 in flask culture using response surface methodology. Under optimized conditions (80 g/L of molasses and 60 g/L of glycerol), the highest GSH and biomass concentration achieved were 119.6 mg/L and 25.3 g/L, respectively. Further studies done in 5 L bioreactor resulted 241.3 mg/L GSH after 96 h in batch fermentation without amino acids addition and the concentration of biomass was 12.1 g/L. In batch fermentation with the addition of the three amino acids (4 mM cysteine, glycine and glutamic acid at 32 h), biomass reached to 25 g/L and GSH, 236.1 mg/L at 96 h of fermentation. The strategy of precursor amino acids addition is a key aspect in increasing the synthesis of GSH.*

**Key words:** Glutathione, Fermentation, Fed Batch Culture, Amino Acids, Response Surface Methodology

### **INTRODUCTION**

Glutathione (GSH) is a biologically active tripeptide consisting of L-glutamate, L-cysteine and glycine (L- $\gamma$ -glutamyl-L-cysteinylglycine) (Zhang et al. 2007). It is the mainly abundant non-protein thiol compound present in living organisms, from prokaryotes to eukaryotes (Rollini and Manzoni 2006). GSH is considered to be one of the most important self-generated defense molecules (Maris et al. 2000). In humans, GSH deficiency can be associated with many diseases, such as liver cirrhosis, pulmonary diseases, gastrointestinal and pancreatic inflammations, diabetes, neurodegenerative diseases, and aging (Navarro et al. 1999). GSH is widely used as a drug and has great potential to be used in food additives and in the cosmetic preparations, if the price can be decreased further

(Li et al. 2004). GSH can be produced by enzymatic methods and also by fermentation. In the latter method, the yeasts *Saccharomyces cerevisiae* and *Candida utilis* are used to produce GSH at industrial scale (Navarro et al. 1999). *S. cerevisiae* is a versatile and simple eukaryotic model organism. This facultative anaerobe is able to live on various carbon sources, including fermentable and non-fermentable substances (Magherini et al. 2009).

To produce GSH cost-effectively on an industrial scale by yeast fermentation, more economical carbon and nitrogen sources should be investigated. Molasses, the by-product of sugar manufacturing, is cheap and abundant material. The carbohydrates (glucose, sucrose, and fructose) present in molasses suggest that it could be an excellent carbon source. On the other hand, the additional nutrients, such as vitamins and

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minerals, in molasses endow it with superior performance (Beshay and Moreira 2005). The use of renewable waste substrates is an environmentally-friendly choice that also contributes to the reduction of waste treatment costs and increases the economic value of by-products. Glycerol is a versatile carbon and energy source with many possible applications in industrial fermentation (Silva et al. 2009). Glycerol is not only cheap and abundant, but its higher degree of reduction than sugars offers the opportunity to obtain reduced chemicals, such as succinate, ethanol, xylitol, propionate, hydrogen, etc. at higher yields than those obtained from the sugars (Dharmadi et al. 2006). In yeast fermentations due to the Crabtree effect, respiratory enzymes are inhibited and ethanol is formed when the substrate concentration is above a critical value, which results in a decrease in biomass productivity (De Deken 1966). This problem can be overcome by fed-batch fermentation in which essential nutrients can be fed incrementally to the bioreactor during the cultivation. In order to optimize the fermentation process to achieve a maximum biomass yield and a high biomass concentration, a suitable control strategy has to be developed (Wen et al. 2006). GSH biosynthesis has close relationship with three constituent amino acids. Cysteine addition accompanied by glycine and glutamic acid can improve GSH production (Wen et al. 2005). However, cysteine addition would cause growth inhibition, while the introduction of glutamic acid and glycine can weaken the inhibition effect (Wang et al. 2007). The objective of this study was to develop an economic method for the GSH production using *S. cerevisiae* ATCC 7754. The initial approach was to use molasses and glycerol as inexpensive carbon sources. A feedback control strategy and the effect of precursor amino acids addition were investigated.

## MATERIAL AND METHODS

### Microorganism and Culture Media

*Saccharomyces cerevisiae* ATCC 7754 was used throughout this study. Stock cultures were maintained at 4°C on Yeast Malt (YM) agar. The inoculum was carried out on rotary shaker, using Erlenmeyer flasks (250 mL) containing 50 mL of medium. The culture conditions to obtain the

inoculum were 30°C, 150 rpm, 24 h, and YM medium.

### Batch Cultivation in Flask Culture

Sugar cane molasses was purchased from Usina Éster (Brazil) containing 46% of total reducing sugars (TRS) with 42.8% of sucrose and 3.2% of reducing sugars. Molasses and glycerol were sterilized separately to avoid medium darkness due to sugars reactions (caramelization and Maillard). To study different molasses and glycerol concentrations on GSH production, a central composite rotatable design (CCRD) 2<sup>2</sup> was used with  $\alpha$  equal to 1.41, resulting in 11 trials (four axial points and three central points). Table 1 shows the coded and real levels of the variables studied with the results of biomass and GSH concentrations. The other compounds of the medium were (g/L): corn steep liquor 50, whey protein 50, yeast extract 10, magnesium sulphate 10 and lecithin 5 mM. All the chemicals and reagents used were of analytical grade. The inoculum concentration was fixed at 5% (v/v). The fermentation process was developed in Erlenmeyer flasks (250 mL) containing 50 mL of medium at 20°C, pH 5.0 and 300 rpm (Santos et al. 2007) for 96 h with samples taken at 0, 24, 48, 72 and 96 h of incubation.

### Batch and Fed-Batch Cultivation in Bioreactor

Fermentations were performed in New Brunswick Bioflo III (5 L) bioreactor. It was sterilized with the initial culture medium, except for the carbon sources, which were sterilized separately. The medium optimized in flask culture by CCRD was used in bioreactor for all the experiments. In the batch experiments, 3.5 L of medium was used. In fed-batch experiments, the flow rate was determined according to the working volume of the bioreactor. The initial working volume was 1.5 L and carbon sources were fed at constant flow (30.6 mL/h) up to 3.5 L at the end of the cultivation (96 h). The feeding was started after sugar depletion according to literature (Li et al. 1998; Liang et al. 2008a). Carbon sources and feeding solutions were fed into bioreactor using peristaltic pump. The initial pH was fixed at 5.0 for all the experiments. Antifoam agent (Dow Corning FG-10) was automatically added to the vessel to prevent foam formation. Firstly, a batch fermentation (assay 1) using the conditions optimized by CCRD in flask cultures

was done with 500 rpm and 1.1 vvm, process conditions previously optimized by Santos (2008). In assay 2, the feeding was done with molasses (40 g/L) and glycerol (30 g/L), while the assay 3 was done with double of these concentrations. Finally, batch experiments evaluated the influence of amino acids addition in different concentrations and cultivation times. For assay 4, 4 mM of cysteine, glycine and glutamic acid were added at 32 h (Wang et al. 2007) and for assay 5, 2 mM of cysteine at 6 h and 3.35 mM of cysteine, 18 mM glycine and 10 mM glutamic acid were added at 36 h (Wen et al. 2006). The amino acids solutions were sterilized through Millipore membranes (0.2 µm).

### Analytical Methods

The pH of the cultures was measured potentiometrically by a pH meter. The optical density of the sample (properly diluted) was measured using a spectrophotometer (at 600 nm) to determine the cell concentration. The supernatants after centrifuging were used as blank to eliminate the interference caused by media colors. The optical density was positively related to the cell dry mass with an experimentally determined calibration curve.

Sucrose present in the molasses was converted into an equal mixture of glucose and fructose by acid hydrolysis. The total reducing sugars (TRS) consumption along the fermentations was measured in terms of glucose concentration by a colorimetric glucose oxidase peroxidase method. The intracellular GSH was extracted from the cells by 40% ethanol for 2 h at 30°C and the concentration was determined according to the method described by Owens and Belcher (1965) and Santos et al. (2007). The absorbance of the

sample was measured at 412 nm, and the concentration of GSH was obtained from a standard curve using commercial L-glutathione reduced (Fluka, Japan). The GSH yield (mg/L.h) was obtained from the GSH production per hour and percent of GSH (%) related the GSH mass (g) in relation to cell (g) for a specific fermentation. The data obtained are means of triplicate samples.

### Experimental Design

A central composite rotatable design (CCRD) was used in flask culture assays to obtain the optimum levels of the carbon source conditions. The two factors investigated were molasses and glycerol concentrations (independent variable). Three replications of the center point and four axial points were performed to obtain a second-order model for prediction of GSH concentration (dependent variable). The design matrix, the variables, and their levels in coded and natural units are shown in Table 1. The following second-order polynomial model was predicted by a multiple regression procedure. Equation 1 represents this empirical model related to the response.

$$Y = a_0 + \sum_{i=1}^n a_i x_i + \sum_{i=1}^{n-1} \sum_{j=i+1}^n a_{ij} x_i x_j \quad (1)$$

where  $Y$  is the predicted response in GSH concentration,  $i$  and  $j$  take value from 1 to the number of variables ( $n$ ),  $a_0$  is the intercept term,  $a_i$  values are the linear coefficient,  $a_{ij}$  values are the quadratic coefficient, and  $x_i$  and  $x_j$  are the level of the independent variables. For the analysis of the data at 72 h of incubation, the software Statistica 7.0 (Statsoft 2325, Tulsa, OK) was used, and the adequacy of the model was evaluated through analysis of variance (ANOVA).

**Table 1** - Matrix of the assays of the CCRD  $2^2$  and results of biomass concentration (g/L) and GSH production (mg/L) for 72 h of incubation.

| Assay | Molasses (g/L)* | Glycerol (g/L)* | Biomass (g/L) | GSH (mg/L) |
|-------|-----------------|-----------------|---------------|------------|
| 1     | -1 (13.1)       | -1 (13.1)       | 14.9          | 66.1       |
| 2     | +1 (76.9)       | -1 (13.1)       | 19.5          | 97.9       |
| 3     | -1 (13.1)       | +1 (76.9)       | 25.3          | 78.6       |
| 4     | +1 (76.9)       | +1 (76.9)       | 22.3          | 119.6      |
| 5     | -1.41 (0)       | 0 (45)          | 13.7          | 51.0       |
| 6     | +1.41 (90)      | 0 (45)          | 22.7          | 117.8      |
| 7     | 0 (45)          | -1.41 (0)       | 15.4          | 63.9       |
| 8     | 0 (45)          | +1.41 (90)      | 16.9          | 76.0       |
| 9     | 0 (45)          | 0 (45)          | 20.0          | 106.1      |
| 10    | 0 (45)          | 0 (45)          | 20.2          | 109.3      |
| 11    | 0 (45)          | 0 (45)          | 21.1          | 108.7      |

\* real values in parenthesis

## RESULTS AND DISCUSSION

### Batch Cultivation in Flask Culture Using CCRD 2<sup>2</sup>

An increase of the final pH was observed using agro-industrial substrates (molasses, corn steep liquor and cheese whey protein) for GSH production. This was probably related to proteolysis of the microorganism, a natural phenomenon that occurred after complete exhaustion of the substrate, especially when yeast did not utilize a different carbon source. As a consequence of the amino acids degradation, leading to the ammonia formation, this could be responsible for the pH increase. The final pH was higher than the initial pH in all the assays, reaching until 8.06 at the end of the cultivation. Increases in pH were also found in other studies using agro-industrial substrates such as molasses, corn steep liquor, yeast hydrolyzed (Valduga 2005) and hydrolyzed casein (Mantzouridou et al. 2002). The TRS were totally consumed within 24 h of incubation and the consumption of carbon present in the molasses was expressed as a function of the glucose concentration after hydrolysis. It was not possible to quantify glycerol consumption during the cultivation. The CCRD matrix and results of biomass concentration (g/L) and GSH production (mg/L) obtained at 72 h of incubation are shown in Table 1. The highest biomass concentration (25.3 g/L) was obtained in assay 3 and in assays with lower carbon sources concentrations (1, 5 and 7) resulted lowest biomass concentration. Among the 11 assays, GSH concentration ranged between 51.1 and 119.6 mg/L, showing the importance of carbon source suitable for GSH production. It was also found that at the same molasses concentration and with the variation of glycerol concentrations (assay 7 and 8), the GSH concentration showed a small variation (increased 15.9%). In the assays with the same glycerol concentration and with variation of the molasses concentrations (assays 5 and 6), the

GSH concentration changed considerably (increasing 56.7%), showing that GSH production was strongly influenced when molasses was used as the carbon source. In most assays, the GSH concentration decreased after 72 h of incubation. Since the goal of this experimental design was to obtain a model to describe the behavior of GSH concentration as a function of the parameters studied for the higher GSH production, the statistical analysis was performed at 72 h of cultivation.

The results for GSH concentration (Table 1) were analyzed by regression and variance (ANOVA) analysis. A second order model was fitted to evaluate the main effects of the two factors. The parameters were considered significant when the p-values less than 10% ( $p < 0.1$ ). The ANOVA (Table 2) indicated that the model was significant and adequate to represent the actual relationship between the response and the significant variables with very small p-value (0.0021). The coefficient of determination ( $R^2$ ) was 0.9192, which indicated that only 8.08% of the overall variation was not explained by the model. The result of  $F_{calculated}$  was 8.1 times higher than  $F_{tabulated}$ , indicating that the model (coded equation) was representative of the actual relationship among the selected reaction parameters (Eq. 2). The independent variable  $x_2$  (glycerol concentration) in the investigated range, did not have significant effect on the response and was eliminated from the model and incorporated to the residual as well as the interaction between  $x_1$  and  $x_2$ . Clearly, a higher molasses concentration led to higher GSH concentration. The model with the coded variables representing the statistically significant GSH concentration (mg/L) in terms of molasses and glycerol concentrations in the range studied is shown in Equation 1, where  $x_1$  is the cane molasses concentration and  $x_2$  the glycerol concentration.

$$\text{GSH (mg/L)} = 108.03 + 20.90 \cdot x_1 - 8.47 \cdot x_1^2 - 15.70 \cdot x_2^2 \quad (2)$$

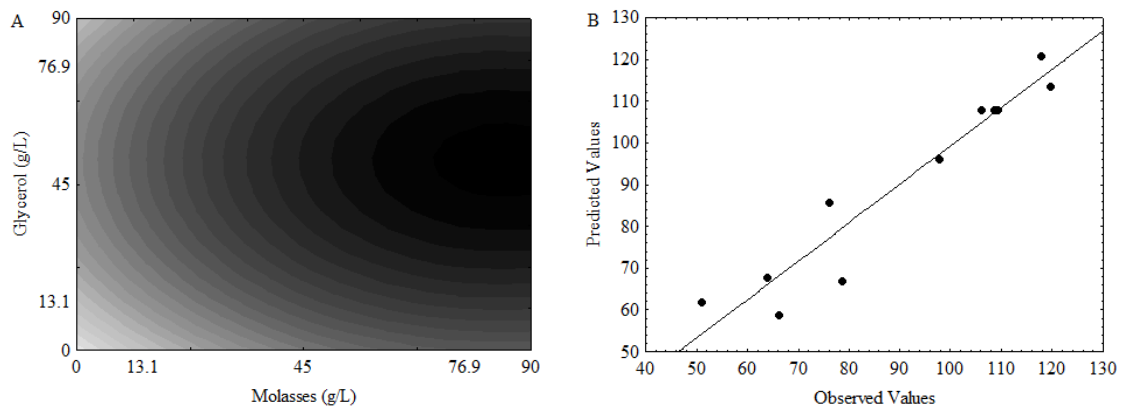
**Table 2** - ANOVA for the regression model for GSH production at 72 h of incubation.

| Source of variation | Sum of square | Degrees of freedom | Mean square | F-value <sup>a</sup> | p-value |
|---------------------|---------------|--------------------|-------------|----------------------|---------|
| Regression          | 5309.41       | 3                  | 1769.80     | 25.89                | 0.0021  |
| Residual            | 478.48        | 7                  | 68.35       |                      |         |
| Total               | 5787.90       | 10                 |             |                      |         |

$$R = 0.9192; {}^aF_{3;7;0.1} = 3.18$$

Through the response surface generated by the model (Fig. 1A), concentrations of the molasses and glycerol were obtained to achieve higher GSH production. GSH concentration increased with the molasses concentration increasing; however, the same was not observed for glycerol, showing that the yeast preferentially consumed molasses as carbon source than glycerol. When the yeast was grown on fermentable substrates such as glucose, the metabolic energy essentially originated from glycolysis, whereas in the presence of a non-fermentable carbon source, such as glycerol, the

mitochondrial oxidative metabolism was fully activated (Magherini et al. 2009). This could explain the results obtained in this study. Through the response surface (Fig. 1A), it was possible to track the optimum GSH production, between 75 and 90 g/L of molasses and between 40 to 60 g/L of glycerol. Figure 1B presents the experimentally observed values versus the predicted values by the model (Eq. 2) for the GSH production, showing good agreement between them, as was expected by the ANOVA result.



**Figure 1** - Contour plot (A) and predicted values versus observed values (B) of GSH production with a CCRD 2<sup>2</sup>.

Comparing the results of biomass and GSH concentrations obtained in the flask culture by other authors using *S. cerevisiae* strains, the present study obtained very satisfactory results. The highest biomass concentration observed (25.3 g/L) was two times higher compared to other authors (Liu et al. 1999; Wen et al. 2004; Rollini and Manzoni 2006; Liang et al. 2008c). Liu et al. (1999) studied GSH production using glucose and peptone with the same *Saccharomyces* strain and obtained a maximum GSH concentration (124.93 mg/L) similar to the present work (119.6 mg/L). As the yeast extract and peptone are expensive, the present study applied yeast extract supplemented with whey protein and corn steep liquor. Also, molasses and glycerol were used as agro-industrial substrates instead glucose. In this way, the costs for nitrogen and carbon sources were substantially reduced. Other studies obtained lower biomass and higher GSH concentrations (Cha et al. 2004; Santos et al. 2007) than in the present study. This difference was due the stimulation on cell growth by the media used, rather the increase in the intracellular content (Wen et al. 2004), which

supplied alternative amino acids and nitrogen sources that increased the GSH content with low biomass concentration.

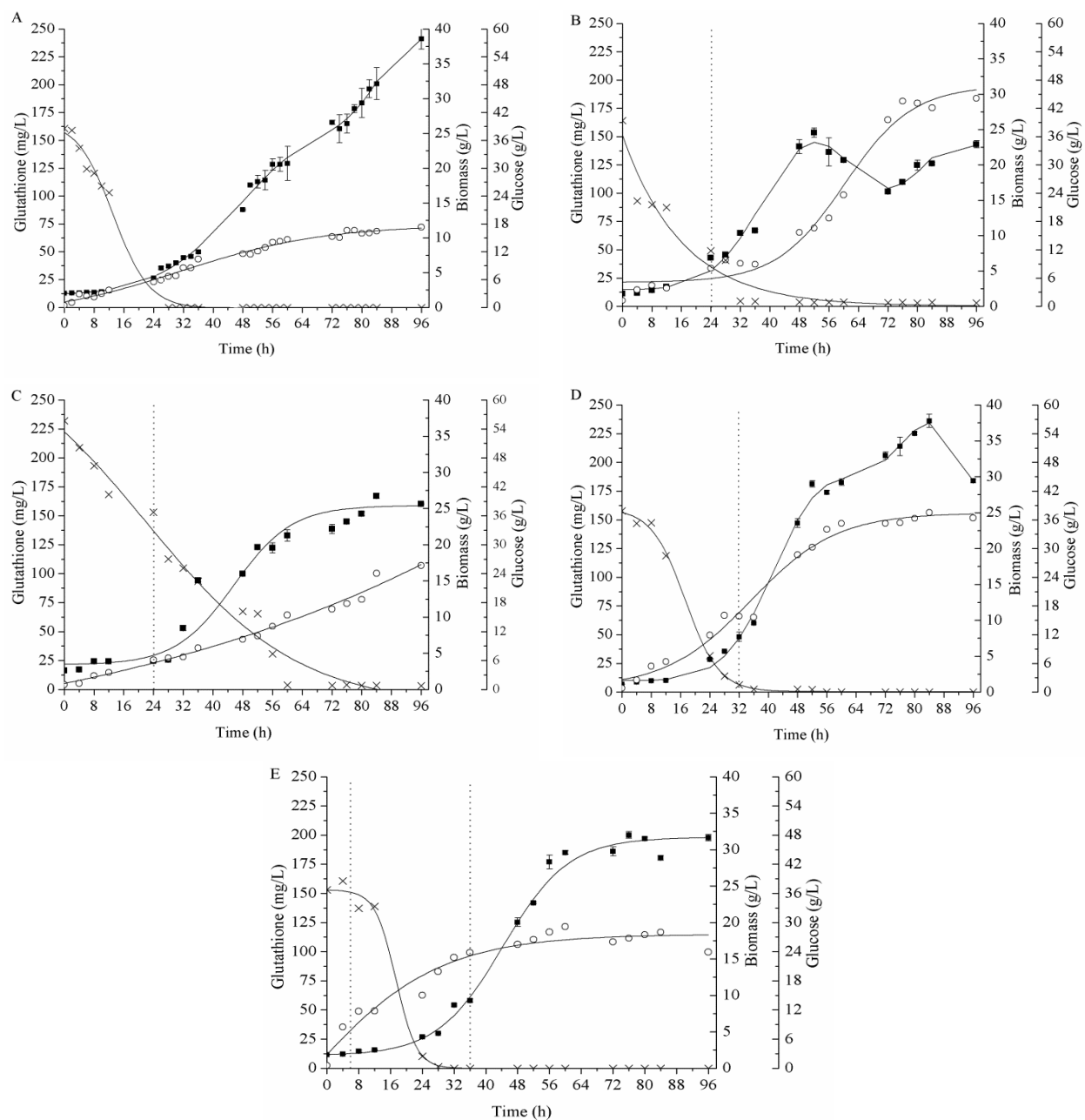
The GSH production was also studied using glucose (70 g/L) and molasses (20 g/L) by *S. cerevisiae* T65 (Zhang et al. 2007) and optimized medium resulted 74.6 mg/L GSH. The carbon and nitrogen sources, inorganic salts, vitamins and others compounds may influence the cell growth and the accumulation of metabolic products in microbial cells. The effects of different carbon (glucose, galactose, fructose, lactose, maltose and sucrose) and nitrogen (yeast extract, peptone, tryptone, casein, soybean meal, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, NH<sub>4</sub>Cl) sources for GSH production were studied using *S. cerevisiae* FF-8 by (Cha et al. 2004). The best result (88.4 mg/L) was found when glucose and yeast extract were used. Cane molasses and glycerol proved to be viable low cost alternatives as carbon sources for GSH production. As glycerol is a byproduct of biodiesel production, it is less costly than other substrates commonly employed such as glucose. This approach could be potentially applicable to a variety of fermentative

production processes for value-added chemicals under aerobic conditions from the viewpoints of efficiency, energy-saving, and cost-performance.

### Batch and Fed Batch Cultivation in Bioreactor

The highest GSH concentration in the bioreactor was obtained at 96 h of batch cultivation (assay 1). Figure 2A shows the results of biomass, GSH and glucose concentration for this assay. The sugars were completely consumed at 28 h of cultivation. In fed-batch experiments (Fig. 2B, Fig. 2C), lower GSH and biomass concentrations were obtained. Figure 2C showed the results of assay 3, which

was used twice the concentration of molasses and glycerol in relation to assay 2. GSH concentration reached 170 mg/L, without any advantage over assay 2. Table 3 summarizes the highest responses obtained for each assay in the bioreactor. Different fed-batch strategies were studied for GSH production using *C. utilis* WSH 02-08 in 7 L bioreactor (Liang et al. 2008b). The highest yield (8.8 mg/L.h) and GSH concentration (981 mg/L) were obtained by feeding at 5.5 g/L.h after 20 h with biomass concentration and productivity of 102.1 g/L and 0.84 g/L.h, respectively.



**Figure 2** - Results of biomass (○), glutathione (■) and glucose (×) for assays 1(A), 2(B), 3(C), 4(D), and 5(E) in bioreactor.

**Table 3** – Maximum responses obtained in bioreactor.

| Mode of cultivation  | Assay          | Biomass conc. (g/L) | GSH conc. (mg/L) | Biomass yield (g/L.h) | GSH yield (mg/L.h) | GSH (%)     |
|----------------------|----------------|---------------------|------------------|-----------------------|--------------------|-------------|
| Bacth                | 1              | 12.1 (78 h)         | 241.3 (96 h)     | 0.41 (4 h)            | 2.38 (96 h)        | 2.09 (96 h) |
| Fed Batch            | 2 <sup>a</sup> | 29.4 (96 h)         | 153.6 (52 h)     | 0.38 (4 h)            | 2.74 (52 h)        | 1.39 (52 h) |
| Fed Batch            | 3 <sup>b</sup> | 17.1 (96 h)         | 166.9 (84 h)     | 0.18 (84 h)           | 2.16 (36 h)        | 1.65 (36 h) |
| Amino acids addition | 4 <sup>c</sup> | 25.0 (84 h)         | 236.1 (84 h)     | 0.40 (56 h)           | 3.35 (52 h)        | 0.94 (84 h) |
| Amino acids addition | 5 <sup>d</sup> | 19.4 (66 h)         | 200.1 (76 h)     | 1.32 (4 h)            | 2.95 (56 h)        | 1.24 (96 h) |

<sup>a</sup> feeding medium with molasses (40 g/L) and glycerol (30 g/L); <sup>b</sup> feeding medium with molasses (80 g/L) and glycerol (60 g/L); <sup>c</sup> addition of 4 mM of cysteine, glycine and glutamic acid at 32 h; <sup>d</sup> addition of 2 mM of cysteine at 6 h and 3.35 mM of cysteine, 18 mM glycine and 10 mM glutamic acid at 36 h.

Even after sugar depletion, biomass and GSH still increased in the bioreactors experiments. This could have occurred because glycerol was not analyzed along the fermentations and might have been consumed as carbon source after the depletion of molasses. *S. cerevisiae* grew very well on glycerol as the main carbon and energy source (Penninckx et al. 1983). The growth on glycerol results in the induction of the GSH-dependent glyoxalase pathway: methylglyoxal + GSH ↔ hemithioacetal (nonenzymic) hemithioacetal → S-D-lactoylglutathione (glyoxalase I: EC 4.4.1.5) S-D-Lactoylglutathione + H<sub>2</sub>O → D-lactic acid + GSH (glyoxalase II: EC 3.1.2.6). The glyoxalase detoxification pathway is possibly not restricted to glycerol catabolism, but may intervene in the detoxification of other oxoaldehydes generated during yeast metabolism, for example, during catabolism of threonine, valine, and isoleucine (Murata et al. 1986).

Molasses consists of water, sucrose, proteins, vitamins, amino acids, organic acids and heavy metals such as iron, zinc, copper, manganese, magnesium, calcium, etc. Heavy metals, when contained in high concentrations in the medium, cause critical problems during the fermentation. They inhibit the microbial growth, influence the pH of the substrate, beside to be involved in the inactivation of the enzymes associated with biosynthesis of the product (Roukas 1998). In the aerobic GSH fermentation process, the growth of *S. cerevisiae* is inhibited by the excess of glucose because of a activity, which is known as the Crabtree effect (Vanurk et al. 1990). This strain (Crabtree-positive) produces ethanol when glucose concentration is not carefully controlled, decreasing the target compound's productivity. The effect of glucose addition by different feeding strategies were compared in the GSH production by *Escherichia coli* and was found that

exponential feeding strategy increased biomass density, productivity and biomass yield (Li et al. 1998). Using this strategy, biomass density and total GSH quantity in broth reached 80 g/L and 880 mg/L, respectively and inhibition of the growth was observed when the initial glucose concentration was above 20 g/L.

The increase of biomass and GSH after the sugar depletion in this study could also be related to the Crabtree effect, by which the respiratory enzymes were inhibited and ethanol was formed when the substrate concentration was above a critical value. In Sakato's report (Sakato and Tanaka 1992), it was shown that the simultaneous utilization of sugar and ethanol has been a key factors in the industrial process to produce GSH and no further increase in GSH was observed after ethanol was consumed in the medium. According to Wen et al. (2006), ethanol concentration in the medium had a significant effect on GSH content in the fed-batch culture. Low ethanol concentration was beneficial for GSH accumulation in the yeast, while high concentration exhibited inhibition on GSH production. GSH content in the fed-batch culture at low ethanol concentration was 2.3 times higher than that obtained with high ethanol concentration. The most important issues affecting the fed-batch fermentation according to Lin et al. (2004) include ethanol accumulation and dissolved oxygen concentration in the fermentation broth. Relative low value of specific growth rate ( $\mu$  below 0.13 h<sup>-1</sup>), rich nutrition supply and high dissolved oxygen (DO) value are beneficial to avoid ethanol accumulation, to reach high biomass density and to synthesize intracellular GSH. When the setting value of  $\mu$  is high (around 0.15 h<sup>-1</sup>), both glucose and ethanol are accumulated in the fermentation broth, which turn the metabolic pathway from aerobic cell growth to anaerobic ethanol fermentation. The concentration of glucose in the

cultivation broth should be controlled precisely at constant low level to reduce ethanol formation and improve the yield of biomass and GSH during the fermentation. To improve GSH in the fed-batch processes it is suggested to keep the specific growth rate at low values, DO at high rates and monitoring the ethanol accumulation in the medium.

Amino acids were added in different concentrations and times of incubation. The higher GSH concentration (236.1 mg/L) was obtained with the addition of the three amino acids (4 mM cysteine, glycine and glutamic acid) at 32 h in assay 4 (Fig 2D), whereas in the experiment where amino acids were added at 6, 24, 48 and 60 h, the maximum GSH obtained was 179.45 mg/L (data not shown). These results indicated that the addition time and concentration of each of the three amino acids were key aspects in GSH increasing and should be further studied to obtain better results. The results of assay 5 are shown in Figure 2E (2 mM of cysteine at 6 h and 3.35 mM of cysteine, 18 mM glycine and 10 mM glutamic acid at 36 h). Higher concentration of GSH was achieved during the stationary growth phase in accordance with other authors (Izawa et al. 1995; Suzuki et al. 1999). The results obtained in this study were lower than those observed by other authors. Wen et al. (2004) studied the addition of the three amino acids (2 mM of cysteine at 6 h and 10 mM glutamic acid, glycine and serine at 35 h) in fed-batch culture using *S. cerevisiae* T65. GSH yield was about 2.67 times higher as without amino acids. Cysteine was the most important amino acids, which increased the intracellular GSH content greatly but inhibited the cell growth at the same time. Due to the high cost and less effect of cysteine, glutamic acid and glycine were used in this study.

In other study, cysteine (2 mM) was added at 6 h and 10 mM glutamic acid, 3.35 mM cysteine and 18 mM glycine at 24, 44 and 56 h. GSH yield reached 2190 mg/L, 26.8% higher than that without any amino acids addition (Wen et al. 2006). One of the best results on GSH production with *S. cerevisiae* was reported controlling the glucose feeding by respiratory quotient and ethanol concentration. Optimized process for this strain with the addition of amino acids resulted in GSH concentration to 2020 mg/L (Wang et al. 2007). GSH metabolism has close relationship with amino acids metabolism, especially the three

constituent amino acids. The addition of amino acids not only affects the cell growth but also affects intracellular GSH synthesis. The production of GSH could be enhanced by increasing the biomass concentration, or intracellular content of GSH in yeast (Wen et al. 2004). Optimal time for cysteine addition is the stationary phase as the cells stop propagating, and single-shot addition of cysteine is better than other addition ways in enhancing GSH production (Vanurk et al. 1990; Liang et al. 2008c). The simultaneous use of sugar and ethanol was shown as a key aspect for the industrial production of GSH and that GSH concentration increased after total consumption of ethanol in the medium (Wang et al. 2007). The adoption of a good method of cysteine addition can increase the rate of GSH with fed-batch in *S. cerevisiae*. However, the addition of these amino acids can cause growth inhibition and the use of glutamic acid and glycine can reduce the inhibitory effect.

## CONCLUSIONS

The results demonstrated that cane molasses and glycerol, cheap substrates widely available in Brazil, have potential for use, but the addition time and concentration of each of the three amino acids would be the key aspects to increase GSH concentration and should be better studied to obtain better results. Thus, the search and/or construction of new and efficient GSH producers as well as optimization of conditions of GSH biosynthesis would assist in its bringing to market for the improvement of quality of food, cosmetic and pharmaceutical products.

## ACKNOWLEDGEMENTS

The authors would like to thank the financial support from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) of Brazil.

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