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Optimization of Protein Production By *Candida Utilis* In Industrial Vinasse With Applicability In Food

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Abstract

Aims: The objective of this study was to optimize the production of cellular proteins by yeast Candida utilis (torula) using vinasse as substrate and to determine the pollutant power of the untreated vinasse and the product obtained by the culture of torula through chemical oxygen demand analysis (COD). **Methods:** Vinasse characterization was based on the following methods: Lowry (1951); Von De Kamer and Van Ginkel (1952); Miller (1959); Silva (1981); AOAC (1990) and IAL (2008). The COD analysis was performed according to the APHA methodology (1998). Yeast extract-malt agar maintenance and uptake were carried out. Construction of the calibration curve of yeast growth was performed by counting cells in the Neubauer chamber and the absorbance at 550 nm in a spectrophotometer. The linear relationship between absorbance and cell concentration was obtained by constructing the calibration curve of cell growth. The analysis was terminated when the yeast presented in the steady state growth phase. **Results:** Cultivation of torula yeast in vinasse resulted in a 70% reduction in COD and an increase in pH from 4.2 to 5.9. The exponential growth of the torula began at zero time. The highest contents of malt extract, ammonium sulfate and ammonium nitrate were related to a higher production of yeast protein biomass. The highest yield was obtained during the growing period of 32 hours. **Conclusions:** The treatment of vinasse with torula reduced the acidity and the polluting power of vinasse. Adequate amounts of yeast inoculant adapted to vinasse conditions lead to a shorter delay phase and an early start of the exponential growth phase. For most treatments, the log phase was more extensive, which increased the yield of the cells. The ideal medium to increase the number of yeast cells contained 100 µL of inoculum, 6 gL-1 peptone, 6 gL^{-1} yeast extract, 0.7 gL-1, 1.3 gL^{-1} malt extract, 0.7 g L^{-1} NH 4 SO 4, 6 g L^{-1} NH_4 NO_3 and 6 g L^{-1} potassium phosphate. The treatment of vinasse with torula offers three benefits. It generates a neutralized residue that can be discarded or used as fertilizer without pollution of the environment. The product obtained is rich in proteins and can be used for animal and human nutrition. The production of torula with the residual vinasse of the bioethanol industry as substrate represents an additional source of income for the producers, besides being a sustainable process.

Key words: *Candida utilis*, torula, protein production, optimization

INTRODUCTION

Brazil is the holder of the world's largest sugar and alcohol production from the cultivation of sugarcane and the production of distilled sugarcane spirits, in which the states of Minas Gerais, São Paulo and the northeastern region have a substantial participation. The generation of waste from these industrial processes, whether in solid or liquid form, is inevitable. The vinasse, the liquid residue from stills, consists of water with a high acidity and high concentration of organic matter and minerals, especially potassium (Waliszewski *et al*., 1997; Cortez & Brossard-Pérez, 1997). Vinasse has been used for fertigation because simultaneous irrigation of crops and fertilization of the soil can be achieved (Satyawali & Balakrishnan, 2008). However, application of this fertilizer indiscriminately results in acidification of the soil and an increase in levels of minerals, especially phosphorus and potassium, which can lead to eutrophication of lakes and rivers. (Tejada *et al*.,2007).

The use of vinasse as a substrate for protein production by yeast has been explored. Among the yeasts used as protein sources, *Candida utilis* (torula) has been studied because of its ability to degrade pentoses, hexoses and other organic substances such as aldehydes, alcohols and acids in`its metabolic processes (Sandrasegarampillai& Arasaratnam,2011). The technology of production of torula yeast from vinasse was developed in Cuba by the Cuban Institute of Investigaciones de los Derivados de la Caña de Azucar -ICIDCA- in the 1970s as an alternative protein source and as a means to contribute to sustainability and

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preservation of the environment because of the fact that vinasse is a poluting industrial waste (Lezcano, 2005), In this process, the organic matter content is attenuated, the pollution potential decreases, and the production of proteins is promoted (Otero *et al*., 2007). After cultivation of the torula in vinasse, the residue is no longer polluting and can be discarded or used as a fertilizer.

Torula has been used for 70 years for the production of single cell protein (SCP) and fine chemicals such as amino acids, xylitol, ethanol, acetaldehyde, acetone, uricase and 2-hydroxybutanoic acid, using a large variety of substrates, and for waste treatment to reduce the biological oxygen demand (BOD) (Hui and Khachatourians, 1995). Torula has been used to treat waste from the paper industry. Torula has been cultivated on cassava peel hydrolysates (Ezekiel *et al*., 2012), rice polishings (Rajoka *et al*., 2004), apple pomace (Villas-Boas *et al*., 2003) and various other substrates (Rajoka *et al*., 2012; Akanni *et al*., 2015; Kasprowicz-Potocka, 2015; Ya-Lei *et al*., 2006).

In addition to its high protein content, torula is rich in polyunsaturated fatty acids (Bicas *et al*., 2016). The fatty acid composition is C16:0, 23.8%; C16:1, 16.5%; C18:1, 19.6%; C18:2, 23.9%; C18:3, 5.2%; polyunsaturated acids, 29.1%. It also contains vitamins B1, B2, B6, pantothenic acid, folic acid, biotin and niacin. The microelements found include P, Ca, Mg, K, Na, Fe, and Al. It also contains the microelements Mn, Cu, B, Zn, Mo, Co, Cd, Cr, Ni, Pb and Si, although, depending on the substrate used, only Mn, Cu and Zn might be found (Halász & Lâsztity, 1991). Yeasts frequently capture concentrations of microelements greater than they normally need (Kieliszek *et al*., 2017). Selenium and magnesium have received special attention (Blazejak, 2006; Wang *et al*., 2012; Wang *et al*., 2016; Kieliszek *et al*., 2017).Yeast preparations enriched with microelements will be used more frequently for medicinal purposes (both in human and in veterinary medicine), and in biopreparations used in feedstock fermentation in agriculture (Weatherholtz & Holsing, 1975; Kuzela *et al*., 1976; Bekatorou *et al*., 2006).

The torula produced from vinasse has been highlighted as a source of protein for animal feed (Akanni *et al*., 2015;Lopez-Perez & Viniegra-Gonzalez, 2016; Overland & Skrede, 2017; Yunus *et al*., 2015).This fact is related to their nutritional characteristics that promote growth, and several studies have reported the positive results of inclusion of torula in swine (Figueroa & Maylin, 1991; Lezcano & Mora, 2008), poultry (Rodriguez *et al*. 2011; Rodrigues *et al*, 2013) and rabbit (Ortiz *et al*. 2013) rations. Buerth *et al*. (2011) identified 37 proteins in the culture medium, 17 of which were exclusively present in the stationary phase and three in the growth phase. With the use of torula protein, the quantity of soy, corn and fish supplements can be reduced (Pezzato *et al*., 1982). The protein from *Candida utilis* has been shown to be rich in lysine (Rajoka *et al*., 2012). With respect to vitamins, the use of torula can dispense with the addition of B vitamins present in commercial premixes except for vitamin B12 (Piloto *et al*., 2008). Torula has also been used in the food industry as an additive in breads and cakes because of its taste and aroma (Ikeda *et al*., 2016; Nishimura *et al*., 2016; Yasumatsu *et al*., 2016).It can be used as a substitute for soy protein, which alters the taste and texture of the food and reduces the palatability (Weatherholtz & Holsing, 1975; Kuzela *et al*., 1976; Halász & Lásztity, 1991).Microbial proteins have advantages (Rywinska *et al*.,2013) over plant-derived and animal-derived proteins. The most important benefits of SCP include the short time required for growth of microorganisms, the high protein content and the ability to shape the amino acid profile of proteins by regulating the substrate composition and cultivation conditions, or through genetic modifications. SCP production can also be a continuous process and does not depend on climatic conditions (Kieliszek *et al*., 2017).

Candida utilis has been explored for the production of several other products, such as lipases and proteases (Moftah *et al*., 2012; Rehman *et al*., 2014), phospholipase B (Fujino *et al*., 2006), beta-fructofuranosidase (Shih, 1996), bioemulsifiers (Campos *et al*., 2014), exogenous amino acids, citric acid, ethanol, xylitol, erythritol, beta-glucans, glucomannans, and mannoproteins that exhibit photoprotective (Ruszova *et al*., 2008), antigenotoxic, antineoplastic (Miadokova *et al*., 2006), and antioxidative (Drábiková *et al*., 2009) properties. These substances are used in the food processing industry, the pharmaceutical industry, and the cosmetic industry (Kieliszek *et al*., 2017).The effects of medium composition on the production of specific substances such as S-adenosylmethionine, glutathione, carotene and lycopene by C. utilis have also been studied by other authors (Shao *et al*., 2010; Suzuki *et al*., 2011; Yang *et al*., 2013; Shimada *et al*., 1998; Miura *et al*., 1998).

The present study sought to contribute new information regarding the applicability of *Candida utilis*in the processing of coproducts obtained in the production chain of biofuels. It also emphasized the social nature of this work proposal because it subsidizes sustainable bioprocesses of food generation, with the potential to provide greater economic security for small producers, large companies and the country in general. The objective of this work was to use the residual vinasse from the production of ethanol as a substrate for the production of proteins by the yeast *Candida utilis*. In addition to the contribution as a protein supplement and the reduction in the polluting character of vinasse, the process can also serve as an alternative source of income for farmers.

MATERIAL AND METHODS

Material:

The vinasse was collected from the distilleries of the Vale do Paracatu Agroenergia Ltda.(DVPA), Paracatu, MG, Brazil and Agropeu – Agroindústria de Pompeu, Ltda. Pompeu, MG, Brazil, filtered through filter paper and packaged aseptically in Peti bottles. The *Candida utilis* yeast sample was provided by Prof. Dr. Carlos Augusto Rosa, Department of Microbiology, Federal University of Minas Gerais, Belo Horizonte, MG, Brazil.

Characterization of vinasse:

Characterization of the vinasse before and after the fermentation with torula was performed in the Laboratório de Tecnologia de Biomassas do Cerrado (LTBC) located on the JK Campus, Federal University of Vales do Jequitinhonha and Mucuri (UFVJM), Diamantina, MG, Brazil. The reagents used in the biochemical analyses were acquired from commercial suppliers. The analysis of the chemical composition, acidity, pH, total soluble solids (°Brix), reducing sugars, and total phenolic compounds described below were performed in triplicate. These analyses were based on the methods of Von Kamer & Van Ginkel (1952); Silva (1981); AOAC (1990); IAL (2008).

pH: The pH of the vinasse was measured with an Instrutherm PHB 2000pH meter.

Total soluble solids (TSS): The amount of soluble solids in the samples was measured using an ATAGO Palette PR -101refractometerafter filtration through cotton. The result was expressed in °Brix.

Reducing sugars (RS): Reducing sugars were determined by the 3.5-dinitrosalicylate (DNS) method (Miller,1959).

Titratable acidity (TTA): Aliquots (20 mL) of the vinasse were titrated with 0.01 M NaOH solution to determine the total titratable acidity

Moisture: Aliquots (20 mL) of the vinasse were dried at 105 °C for a period of three hours and allowed to cool in a dessicator. This procedure was repeated hourly until the weight of the residue was constant.

Lipids: Samples(0.5 g)of dried residue were transferred to paper cartridges and extracted in a QUIMIS Q388-268 Soxhlet extractor for six hours with ethyl ether. After evaporation of the solvent, the sample was heated at 105 °C for 2 hours, cooled in a desiccator for 20 minutes and weighed.

Proteins: The total protein content was determined by the Lowry (1951) colorimetric method.

Crude fiber: About 0.5 g of dried and defatted samplewas added to test tubes, followed by 17.5 mL of 70% acetic acid, 0.5 g of trichloroacetic acid and 1.2 mL of nitric acid. The tubes were heated on a digester block for 30 minutes at the boiling temperature (110 °C). The samples were filtered through a Buckner funnel lined with glass wool, previously calcined at 105 °C, and washed with hot distilled water until the acidity was completely removed. Subsequently, the crucibles were dried at 105 °C to constant weight.

Total Carbohydrates: The carbohydrates were calculated by difference after subtracting the values obtained for moisture, ash, protein, lipids and dietary fiber.

Phenolic compounds: The concentration of phenolic compounds was determined according to the method described by Singleton and Rossi(1965). The vinasse samples were extracted with 80%methanol; 400 µL of appropriately diluted sample was added to a 10-mL test tube, and 400 µL of Folin-Ciocalteu reagent was added. After standing for five minutes, 4 mL of 1.0 M Na_2CO_3 was added, and the volume was completed to 10 mL. After 90 minutes, the absorbance at 750 µm was measured on a SPECTRUM SP 2000 UV spectrophotometer. A calibration curve was prepared with known concentrations of gallic acid, ranging from 0 to 100 mg. L^{-1}, prepared rom a stock solution containing 100 mg L^{-1}. The results were expressed in grams per 100 g.

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Chemical Oxygen Demand (COD):

The COD analysis was performed by refluxing the sample with potassium dichromate in the presence of silver sulphate catalyst and mercuric sulfate as an inhibitor of chlorides (APHA 1998). The excess potassium dichromate was titrated with ferrous ammonium sulfate. The amount of oxygen required to oxidize the organic matter was calculated according to the amount of potassium dichromate consumed.

Production and Maintenance of Torula:

Maintenance and retransfer of the yeast were performed in the Laboratório de Microbiologia do Solo (LMS). The microorganism was kept in YMA (yeast extract, malt extract, agar) medium consisting of yeast extract (3.0 g. L^{-1}), malt extract (3.0 $g.L^{-1}$), peptone (6.0 $g.L^{-1}$), glucose (10.0 $g.L^{-1}$) and agar (20.0 $g.L^{-1}$). The previously purified colony of *C. utilis* was inoculated in liquid YMA medium and incubated at 25 °C for 24 h. After this period, 0.8 mL of the culture was transferred to 2.0-mLEppendorf tubes with the addition of 0.2 mL of sterile glycerol (Silva *et al*., 1999). The culture was retransferred monthly to Petri dishes with YMA medium, incubated at 30 °C for 72 hours and stored at -5 °C.

Adaptation of torula to the vinasse:

With the aid of a platinum loop, a sample of *C. utilis*, grown in YMA medium, was inoculated into 250-mL Erlenmeyer flasks containing 50 mL of vinasse previously autoclaved at 121 °C and 15 psi for 15 min. The pH was adjusted to 5.5. The experiment was performed in triplicate. After 24 hours, 100 µL of each of the inocula was transferred to an Erlenmeyer flask with three replications. This procedure was performed successively three times, each at 24-hour intervals. For each time interval, the flasks were incubated at 30 °C with stirring at 150 rpm in an orbital shaker incubator. After three days of adaptation of the yeast to the vinasse, the construction of the calibration curve for the growth of yeast in the vinasse and optimization of yeast growth were performed.

Construction of yeast growth calibration curve:

Three Erlenmeyer flasks containing 50 mL of vinasse were stirred at 150 rpm in an orbital shaker incubator at 30 °C. Aliquots of 400 µL of vinasse were removed every four hours during a total time interval of 36 hours, cell growth was assessed by counting of cells in a Neubauer chambre, and the absorbance was measured at 550 nm in a SPECTRUM SP 2000 UV spectrophotometer. The counting was accomplished by diluting the samples in peptone water and homogenizingin a vortex tube stirrer. Methylene blue (0.1%) was added to stain non-viable cells, and the count was determined by optical microscopy (Lee *et al*., 1991). The linear relationship existing between the absorbance and the cell concentration was obtained through the construction of a cell-growth calibration curve. The analysis was interrupted when the yeast growth reached the stationary phase.

Optimization of yeast growth:

To optimize the growth of the yeast, a batch wise submerged culture was performed. An experimental design that resulted in 33 treatments was prepared. Each treatment was performed in 250-mL Erlenmeyer flasks containing 50 mL of vinasse at pH 5.5. Inocula of 10 µL and of 100 µL, taken in the exponential growth phase, and two quantities of each of the seven additives -- peptone, yeast extract, malt extract, urea, ammonium sulfate, ammonium nitrate, and potassium phosphate -- were added to the flasks in accordance with Table 1. All the treatments were maintained in the CIENLAB CE-725/Rorbital shaker incubator at 30 °C, with stirring at 150 rpm. Aliquots of 400 µL were removed from each treatment every four hours for a total time interval of 36 hours. Cell growth was assessed by the absorbance at 550 nm in a spectrophotometer. The absorbance of the samples from each treatment was converted to cell numbers using the standard curve equation for yeast growth in pure vinasse.

Table 1: Experimental design utilized for optimization of growth.

Treatment	*Inoculum (µl)*	*Peptone ($g. L^{-1}$)*	*Yeast extract ($g. L^{-1}$)*	*Malt extract ($g. L^{-1}$)*	*Ureia ($g. L^{-1}$)*	*Ammonium sulfate ($g. L^{-1}$)*	*Ammonium nitrate ($g. L^{-1}$)*	*Potassium phosphate ($g. L^{-1}$)*
1	10	14.0	6.0	14.0	3.0	14.0	6.0	0.6
2	100	14.0	14.0	6.0	3.0	14.0	6.0	0.6
3	100	14.0	6.0	14.0	3.0	6.0	14.0	0.6
4	10	14.0	14.0	14.0	1.3	6.0	6.0	0.6
5	10	6.0	6.0	6.0	1.3	6.0	6.0	1.4
6	100	6.0	6.0	14.0	3.0	14.0	6.0	1.4
7	10	6.0	14.0	6.0	3.0	14.0	6.0	1.4
8	100	6.0	14,0	6.0	1.3	6.0	14.0	0.6
9	100	14.0	6.0	6.0	3.0	6.0	6.0	1.4
10	100	14.0	14.0	14.0	1.3	14.0	14.0	0.6
11	10	14.0	6.0	6.0	1.3	14.0	14.0	0.6
12	100	6.0	14.0	14.0	3.0	6.0	6.0	0.6
13	100	6.0	6.0	6.0	1.3	14.0	14.0	1.4
14	100	6.0	6,0	14.0	1.3	14.0	6.0	0.6
15	10	6.0	14.0	14.0	3.0	14.0	14.0	0.6
16	100	6.0	14.0	14.0	1.3	6.0	6.0	1.4
17	10	6.0	14.0	14.0	1.3	14.0	14.0	1.4
18	100	6.0	14.0	6.0	3.0	6.0	14.0	1.4
19	100	6.0	6.0	6.0	3.0	14.0	14.0	0.6
20	10	6.0	14.0	6.0	1.3	14.0	6.0	0.6
21	100	14.0	6.0	14.0	1.3	6.0	14.0	1.4
22	10	6.0	6.0	6.0	3.0	6.0	6.0	0.6
23	10	14.0	14.0	14.0	3.0	6.0	14.0	0.6
24	100	14.0	14.0	6.0	3.0	14.0	14.0	1.4
25	10	14.0	6.0	14.0	1.3	14.0	6.0	1.4
26	100	14.0	14.0	6.0	1.3	14.0	6.0	1.4
27	10	6.0	6.0	14.0	1.3	6.0	14.0	0.6
28	10	6.0	6.0	14.0	3.0	6.0	14.0	1.4
29	10	14.0	14.0	14.0	3.0	6.0	6.0	1.4
30	100	14.0	6.0	6.0	1.3	6.0	6.0	0.6
31	10	14.0	14.0	6.0	1.3	6.0	14.0	1.4
32	10	14.0	6.0	6.0	3.0	14.0	14.0	1.4
33	10	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Statistical analysis:

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The differences between the estimates of the mean number of cells obtained for the treatments were tested statistically by the F test estimated by analysis of variance (ANOVA). When ANOVA showed significant differences at 5%, the Tukey test was applied. The relationship between treatments and time for yeast growth was evaluated by applying the Principal Component Analysis (PCA). For this procedure, the STATISTICA 7.0 2004 software (Statsoft, 2004) was used.

Results:

The vinasse from the ethanol industry is an acidic residue. Its pH ranges from 3.7 to 4.6, and it exhibits a high chemical oxygen demand (COD), factors that contribute to make it highly polluting. After being treated with the torula yeast, the pH of the vinasse increased from 4.2 to 5.9. This change occurred because the yeast promotes the oxidation of organic matter when it decomposes and increases the pH of the medium (Silva*et al*., 1999). An initial COD value of 25.550 mg $O.L^{-1}$ was observed. This demand indicates the amount of oxygen required to mineralize organic matter. The cultivation of the torula yeast in vinasse resulted in a reduction of approximately 70% in the COD. The oxygen concentration of the treated vinasse was 7489 mg $O.L^{-1}$.These results are presented in Table 2.

Table 2: Chemical composition of untreated and treated vinasse

Item	Untreated Vinasse	Treated Vinasse
TTA mL (%)	2.53	1.43
TSS (%)	1.20	1.60
Moisture (%)	97.49	98.40
Dry matter (g.100 g^{-1})	0.040	0.12
RS (g.L^{-1})*	10.61	1.72
Ash (%)*	21.54	34.88
Lipids (%)*	9.51	40.34
Total Protein (g.100 g^{-1})*	16.39	14.87
Phenolic Compounds (g.100 g^{-1})*	2.43	4.97
Total Carbohydrates (%)*	52.56	9.43
VET (kcal.100 g^{-1})*	361.37	332.95
pH	4.02	5.90
COD (mg $O.L^{-1}$)	25.550	7.480

*Dryweight basis

The cultivation of torula in the untreated vinasse substrate yielded a specific growth rate represented by the equation: y = 2E-9x + 0.0215. The increase in absorbance at 550 nm as a function of the number of cells used to construct the standard growth curve for torula can be observed in Figure 1.

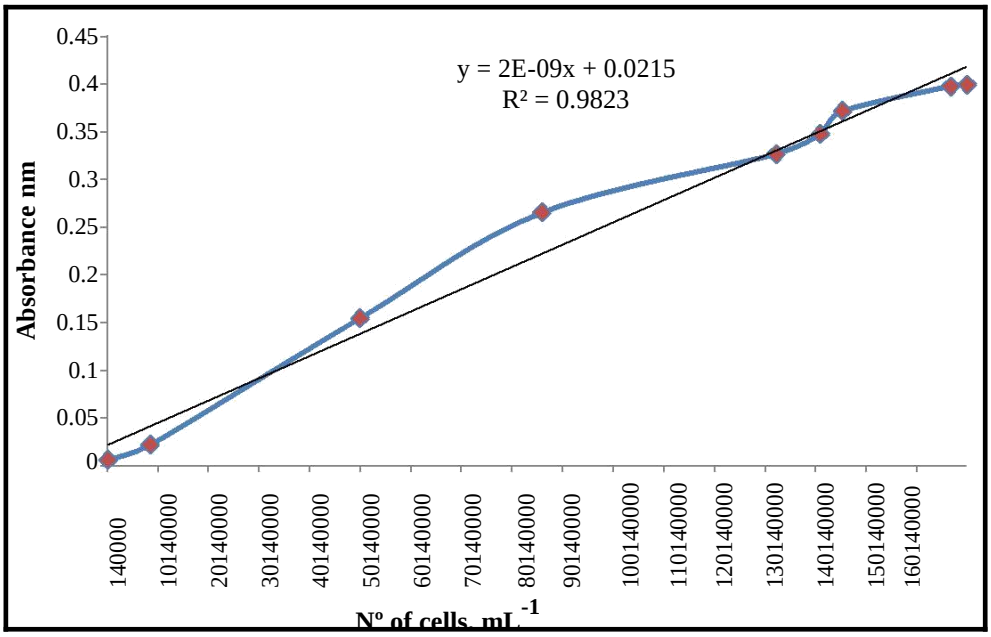

Fig. 1: Growth of *Candida utilis* in untreated vinasse at 30 °C and 150 rpm during 36 hours, measured by counting of cells at intervals of four hours and by measuring the absorbance at 550 nm. Equation: y = 2E-09x + 0.0215; R^2 = 0.9823

According to the representative plot of torula growth in untreated vinasse, the microorganism presented no lag phase, which is the adaptation of the yeast to environmental conditions. The growth was characterized by initiating in the exponential phase, and this phase lasted approximately 16 hours. After 32 hours of growth, the stationary phase began. This phase is characterized by the lack of cell division and the decrease in the population of individuals resulting from the depletion of essential nutrients and the formation of products that inhibit cell metabolism (Gutierrez *et al*., 2002; Betancur, 2005; Chandel *et al*., 2011). The fact that the growth of the yeast began in the exponential phase can be explained by the use of a period of adjustment to the vinasse prior to the beginning of growth (Lezcano,2005). The results obtained for the optimization of the growth of torula yeast in the 33 treatments, the most significant treatments, and the compositions thereof are shown in Tables 3, 4 and 5, respectively.

The factor that contributed most to the average number of the cells in the treatments 14, 21, 26, 8 and 30(in order of decreasing effect) was the amount of inoculum (100 µL). According to the standard growth curve obtained in pure vinasse with 10 µL of inoculum, approximately 105 cells/mL and an absorbance of 0.00625 were observed during the first four hours of growth. Larger inocula, on the order of 107 cells. mL^{-1}, favored the increase in biomass and the quantity of proteins produced by torula (Lezcano, 2005). To increase the fermentation efficiency, the inoculum must contain a suitable number of cells to provide a satisfactory capability for reproduction. Therefore, the use of an inoculum containing cells in the exponential growth phase favors the production of a higher quantity of protein (Carvalho& Sato,2001).

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The treatment with the highest mean cell productivity was number 14. This treatment differed most with respect to the consecutive treatments 16, 9, 33, 7, 25, 15, 17, 11, 3, 32, 20, 31, 23, 24, 5, 4, 10 and 6 in rows and columns (Tables 3 and 4).

Table 3: Mean numbers of cells obtained in each treatment during the period of 36 hours of growth of torula yeast.

Comparison of means by the Tukey test

Treatment		14	21	26	8	30	22	28	27	13	18	1	29	12	19	2	16	9	33	7	25	15	17	11	3	32	20	31	23	24	5	4	10	6
	*Mean number of cells mL^{-1}	113416667	102888889	101472222	98444444	98111111	93138889	90777778	90583333	86805556	86111111	85500000	85055556	83027778	82472222	81083333	80833333	77666667	75940311	75361111	74972222	73916667	64194444	63527778	62583333	62472222	60805556	60500000	59444444	57638889	54263889	52111111	49194444	47638889
14	113416667		ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
21	102888889			ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	*	*	*	*	*	*	*	*	*	*	*	*
26	101472222				ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	*	*	*	*	*	*	*	*	*	*	*	*
8	98444444					ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	*	*	*	*	*	*	*	*	*	*	*	*
30	98111111						ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	*	*	*	*	*	*	*	*	*	*	*	*
22	93138889							ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	*	*	*	*	*	*	*
28	90777778								ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	*	*	*	*	*
27	90583333									ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	*	*	*	*	*
13	86805556										ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	*	*	*	*
18	86111111											ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	*	*	*
1	85500000												ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	*	*	*
29	85055556													ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	*	*	*
12	83027778														ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	*	*
19	82472222															ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	*	*
2	81083333																ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	*
16	80833333																	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	*
9	77666667																		ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
33	75940311																			ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
7	75361111																				ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
25	74972222																					ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
15	73916667																						ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
17	64194444																							ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
11	63527778																								ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
3	62583333																									ns	ns	ns	ns	ns	ns	ns	ns	ns
32	62472222																										ns	ns	ns	ns	ns	ns	ns	ns
20	60805556																											ns	ns	ns	ns	ns	ns	ns
31	60500000																												ns	ns	ns	ns	ns	ns
23	59444444																													ns	ns	ns	ns	ns
24	57638889																														ns	ns	ns	ns
5	54263889																															ns	ns	ns
4	52111111																																ns	ns
10	49194444																																	ns
6	47638889																																	

*Means followed by asterisk in the same row are statistically different according to the Tukey test at a level of 5% of significance.

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Table 4: Treatments and their respective compositions in order of decreasing yield.

Treatment	Composition*	14	21	26	8	30	22	28	27	13	18	1	29	12	19	2	16	9	33	7	25	15	17	11	3	32	20	31	23	24	5	4	10	6
14	I+P-L-M+U -SA+NA- FP-		ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
21	I+P+L-M+U -SA-NA+FP+			ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	*	*	*	*	*	*	*	*	*	*	*	*
26	I+P+L+M-U -SA+NA-FP+				ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	*	*	*	*	*	*	*	*	*	*	*	*
8	I+P-L+M-U -SA-NA+FP-					ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	*	*	*	*	*	*	*	*	*	*	*	*
30	I+P+L-M-U -SA-NA-FP-						ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	*	*	*	*	*	*	*	*	*	*	*	*
22	I-P-L-M-U +SA-NA-FP-							ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	*	*	*	*	*	*	*
28	I-P-L-M+U +SA-NA+FP+								ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	*	*	*	*	*
27	I-P-L-M+U -SA-NA+FP-									ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	*	*	*	*	*
13	I+P-L-M- U -SA+NA+FP+										ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	*	*	*	*
18	I+P-L+M-U +SA-NA+FP+											ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	*	*	*
1	I-P+L-M+U +SA+NA-FP-												ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	*	*	*
29	I-P+L+M+U +SA-NA-FP+													ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	*	*	*
12	I+P-L+M+U +SA-NA-FP-														ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	*	*
19	I+P-L-M-U +SA+NA+FP-															ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	*	*
2	I+P+L+M-U +SA+NA-FP-																ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	*
16	I+P-L+M+U -SA-NA-FP+																	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	*

Table 5: Quantity of each additive in the medium

I+: inoculum 100 µl	U+: urea 3 g.L^{-1}
I-: inoculum 10 µl	U-: urea 1.3 g.L^{-1}
P+: peptone 14 g.L^{-1}	SA+: ammonium sulfate 14 g.L^{-1}
P-: peptone 6 g.L^{-1}	SA-: ammonium sulfate 6 g.L^{-1}
L+: yeast extract 14 g.L^{-1}	NA+: ammonium nitrate 14 g.L^{-1}
L-: yeast extract 6 g.L^{-1}	NA-: ammonium nitrate 6 g.L^{-1}
M+: malt extract 14 g.L^{-1}	FP+: potassium phosphate 1.4 g.L^{-1}
M-: malt extract 6 g.L^{-1}	FP-: potassium phosphate 0.6 g.L^{-1}

A higher yield of cells was obtained in treatment 14 because the medium (Tables 4 and 5) included a larger quantity of inoculum and higher concentrations of malt extract and ammonium sulfate (Table 5). In the culture medium used for storage of *Candida utilis*, the concentration of malt extract was 0.3%. A concentration five times higher was used in treatment 14, and this fact contributed to the greater production of cells in that medium. The addition of malt extract to cell culture media promotes the intake of fermentable sugars such as maltose, fructose, glucose and sucrose, as well as mineral salts, essential amino acids, vitamins, and soluble proteins that enhance yeast production (Hough, 1991; Hickenbottom, 1996). Malt extract is also related to the diastatic power of the system. This factor is a measure of the activity of α-amylase and β-amylase (Steiner *et al*., 2011). The use of an ammonium sulphate concentration at 0.7 g/L resulted in a decrease in the production of yeast cells because it caused conformational changes in the plasma membrane (Alexandre *et al*., 1996; Carmelo *et al*., 1997; Viegas *et al*., 1998; Fernandes *et al*., 1998). The production of succinic acid is suppressed in the presence of the highest concentration of ammonium sulfate because of sidetracking of alpha-ketoglutaric acid, the succinate precursor, for the synthesis of glutamic acid by glutamic dehydrogenase (Lehninger 1988).

Treatments 21, 26, 8 and 30 were equally significant. In these media, the use of a greater quantity of inoculum associated with lower concentrations of urea favored a higher yield of protein. The use of a 0.05% urea concentration promoted the equilibrium of the nitrogen content during fermentation and also prolonged the logarithmic phase (Theerarattananoon, 2011). However, the use of urea, as well as other nitrogen compounds, is related to the formation of ethyl carbamate, a potentially carcinogenic compound (Zimmerli and Schalatter 1991). The inclusion of urea in microorganism culture media for food production requires additional studies. These studies might involve the use of lower concentrations of urea or other harmless nitrogenous compounds to afford the same advantages for the production of yeast cells (Morris *et al*., 1996; Bisson and Butzke, 2000). The inclusion of a peptone concentration of 0.7 g.L^{-1} increased the significance of treatments 21 and 26. When the cell reproduction cycle breaks down because the cell viability reached 50%, a strategy to be followed is to supplement the medium with peptone and yeast extract. As a result, a new cell growth phase occurs, and the vitality of fermentation recovers (Pereira *et al*.,2012).

Considering the results obtained with media 26 and 8, the yeast extract furnished sufficient nitrogen to improve the cell yield. When various sources of organic nitrogen were tested, better results in terms of mycelial growth and a greater production of dry biomass were obtained with yeast extract than with the other conditions tested. This result may be due to the fact that yeast extract contains vitamins and minerals that are required by the torula (Manu-Tawiah & Martin, 1987;Fasidi and Olorunmaiye, 1994; Miranda Jr. *et al*., 2009).

The treatments 22, 28 and 27 had a minor significance for cell growth because of the small quantity of inoculum added. However, the average quantity of cells produced was higher in media 28 and 27 than in the media 13 and 18, even though the first two media began producing cells with 10 µL of inoculum, and the last two media contained 100 µL. The same observation serves for treatments 1 and 29. In these treatments, 10 µL of inoculum was used, whereas 100 µL was used in the treatments 2, 12, 19, and 16. These media contained malt extract at a concentration of 1.4%, and this additive contains sugars, proteins and vitamins essential for the production of proteins. The peptone (14 g.L^{-1}) included in media 1 and 29 also contributed to this production. In experiments in which the flasks were shaken, there was a greater production of biomass and cell viability in the media supplemented with peptone (Cruz *et al*., 2003; Miranda Jr *et al*., 2009)

Treatments 2, 12, 16 and 19 had a minor significance. In larger quantities, yeast and malt extracts and urea associated with the same treatment might have contributed to the higher average production observed in medium 12 than in the other three media. No significant difference in the mean quantities of cells obtained from treatments 9, 33, 7, 17, 25, 15, 11, 3, 32, 20, 31, 23, 24, 5, 4, 10 and 6(Table 4) was observed. Treatment 33 was composed of only untreated vinasse and 10 µL of inoculum. Therefore, the above treatments involve media that resemble pure vinasse or are more deficient than this substrate with respect to the essential nutrients needed for reproduction of the yeast cells. The composition of these media has resulted in a lower average number of cells, which may indicate a lack of nutrients, changes in pH or production of metabolites that are harmful to the bioprocess.

The analysis used to evaluate different amounts of supplements for pure vinasse supported the potentiation of the production of a proteinaceous biomass. Treatments 14, 21, 26, 8 and 30emphasized this potentiation. The interaction between these components and the amounts added, which may be different for each

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treatment could result in various changes in the culture medium. The interactions of microorganisms with the environment and the ways in which the use the compounds available lead to more concrete conclusions about the metabolic pathways utilized and the yield of the cells (Kim *et al*., 2002).

The cell counts per unit time obtained in each treatment with the yeast *Candida utilis* are presented in Figures 2, 3 and 4. The positive effect of greater quantities of inoculum used in the media for the treatments 14, 21, 26, 8 and 30(in decreasing order of growth) is shown in the growth curves (Figure 2). The cultivation periods of 4 to 8 hours were significant because the numbers of cells increased and reached about 6 x 10^7 cells. mL^{-1} in treatments 21 and 30. After this rapid growth, the media were stable throughout most of the period between 8 and 20 hours of cultivation, with the exception of media 14 and 21, which had small variations in the rate of increase in cell numbers. This interval is related to the phase in which yeasts produce enzymes essential to cell metabolism and the adaptation of the microorganism to the culture medium. Therefore, productivity at this stage was not significant (Hiss, 2001). During the period of 20 to 36 hours of cultivation, the growth curves of the media 14 and 26 were exponential, and the curve for medium 8 had the same behavior as those of médium 20 after 32 hours of growth. The exponential phase for the media 30 and 21 was less pronounced. Therefore, the more pronounced growth during the first hours and the longer exponential phase were crucial for the increase in the number of yeast cells. At this stage, the growth velocity was maximal, the production of the enzymes required for the oxidative metabolism was greater so that there was a greater consumption of carbohydrates and a greater energy input to the system (Trivedi *et al*., 1986; Lima *et al*., 2001). The highest average number of cells was observed with treatment 14. This treatment differed at 5% significance by the Tukey test (Tables 4 and 5). This result is explained by the short steady phase and longer exponential phase. During the latter phase, the growth rate was proportional to the cell concentration, and the specific rate of cell proliferation was constant (Stroppa *et al*., 2009).Medium 26 contained higher concentrations of peptone and yeast extract and a lower concentration ammonium nitrate than medium 14. The fact that that rate of cell growth observed in medium 26 was lower than that in medium 14 indicates that the ready availability of nitrogen is more important than the source of carbon or a source of nitrogen the requires metabolization to release the nitrogen (peptone and yeast extract).

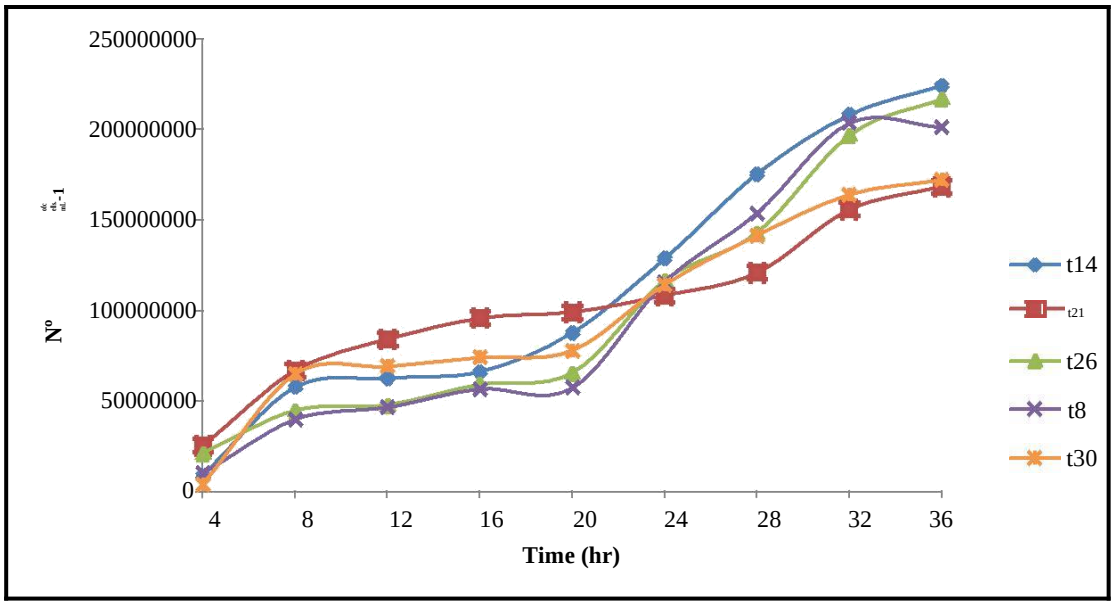

Fig. 2: Graphical representation of *Candida utilis* yeast cell numbers in treatments 14, 21, 26, 8 and 30 per unit of time.

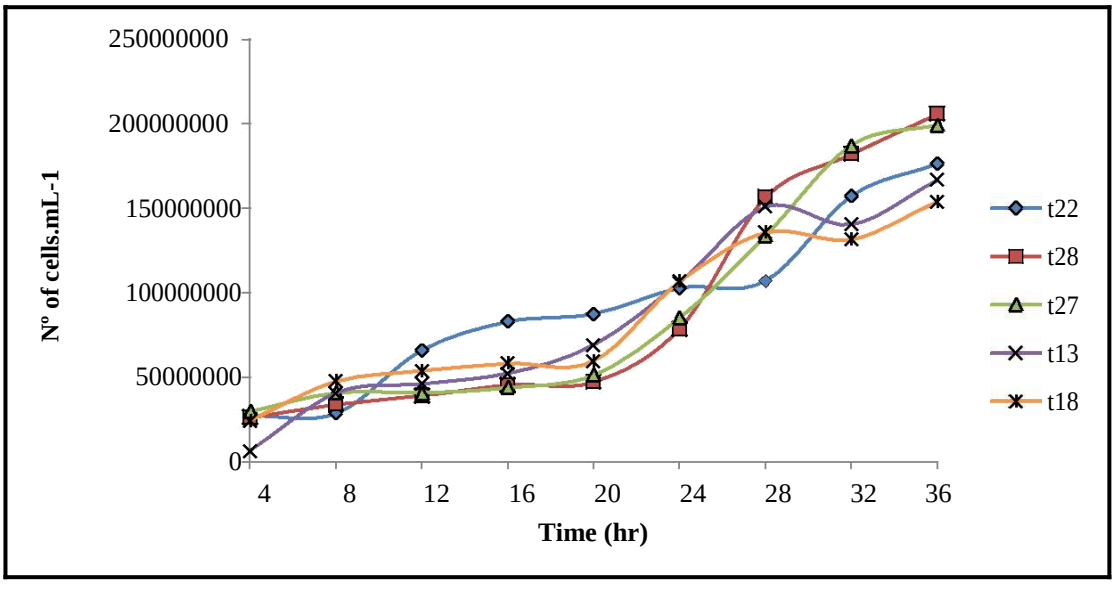

Fig. 3: Graphical representation of *Candida utilis* yeast cell numbers in treatments 22, 28, 27, 13 and 18 per unit of time.

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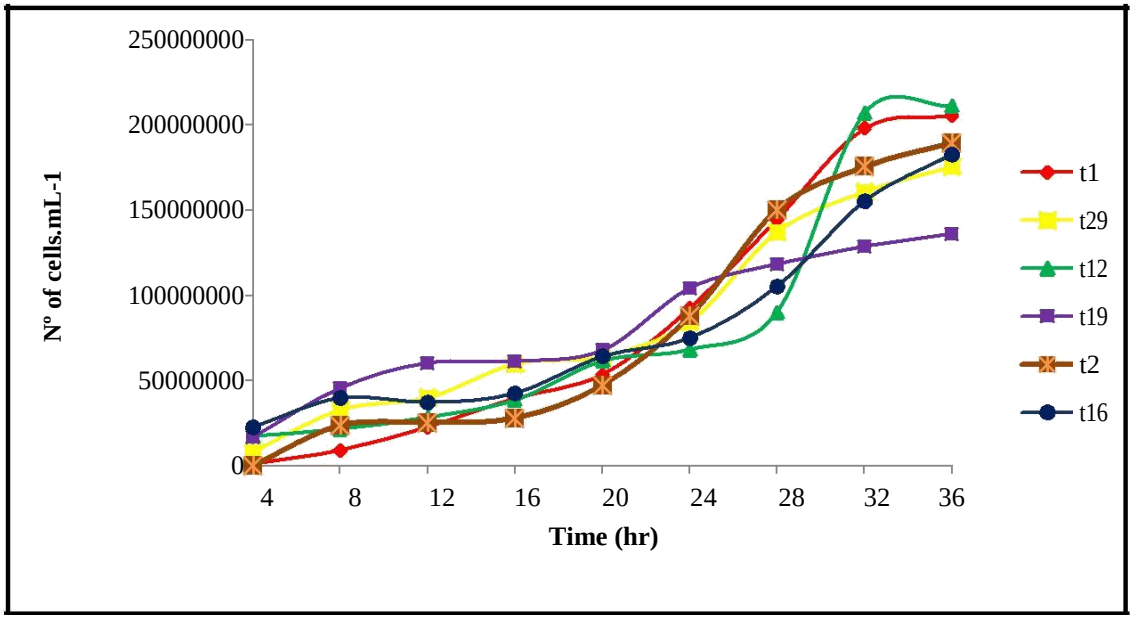

Fig. 4: Graphical representation of the number of *Candida utilis* cells in treatments 1, 29, 12, 19, 2, and 16 per unit of time.

In the second group of treatments —22, 28, 27, 13 and 18 (in decreasing order) — all the treatments had a lag phase lasting 20 hours or more (Figure 3), with the exception of medium 22. The highest yield of cells after 24 hours of growth was obtained with medium 22, which was crucial for the highest average production in this group of treatments, despite the fact that its growth curve was not the most significant. The amount of inoculum influenced the duration of the adaptation phase. Most of the treatments with an inoculum of 10 µL, such as 1, 27, 28, and 29, produced stable growth curves or insignificant growth during 24 hours of cultivation because the use of a smaller number of viable cells in the inoculum diminished the growth curve (Hough 1996). However, from this point on, a decisive exponential phase began for the greatest production of cells (Tables 4 and 5). The growth rate in medium 16 was lower than that in medium 12. The difference in these two media was in the concentration of nitrogen sources, specifically ammonium sulfate and urea. Although medium 12 contained a larger inoculum and a higher concentration of yeast extract, which is a good source of carbon, than medium 1, the growth in medium 12 was slow until after 28 hours of incubation, when a strong exponential phase began. In medium 1 a steady cell growth was observed over the entire period of incubation. Medium 19 contained high concentrations of nitrogen sources, but the concentrations of yeast extract, peptone and malt extract were lower than those of medium 12. The overall growth rate was lower in medium 19 than in medium 12. Although the counting of the number of cells was terminated after 36 hours of cultivation, a cell growth trend is still observed in media curves 13 and 18 (Figure 4). This factor indicates that the nutrients were not depleted and the physical, chemical and biological conditions remained suitable for the production of biomass until the termination of cultivation.

The Principal Component Analysis (PCA) explains 100% of the variation in data with eight major components. However, the first two components are sufficient to show the grouping of the treatments, so the major components PC_1 and PC_2 explain 96.97% of the total variance of the data obtained for treatments according to the cultivation time of the torula (Figure 5). The PC_1, which explains 95.02% of the variance in the positive quadrate, groups all the treatments in the cultivation times 28, 32 and 36 hours. This same component groups treatments with cultivation times of 4 and 8 hours, as well as those of 12, 16 and 20 hours in the negative quadrant. The cultivation time of 24 hours was also part of the group, but it was in the positive PC_1 quadrant. The principal component CP2 separated the groups with initial times of 4 and 8 hours, the intermediate times of 12, 16, 20 and 24 hours, and the times of 28, 32 and 36 hours, which yielded larger numbers of cells in the cultures. The groups were confirmed by cluster analysis (Figure 6), which exhibits distinct colors to form groups by similarity using the Mahalanobis distance for the first two principal components with autoscaling of data and using PCA for data reduction.

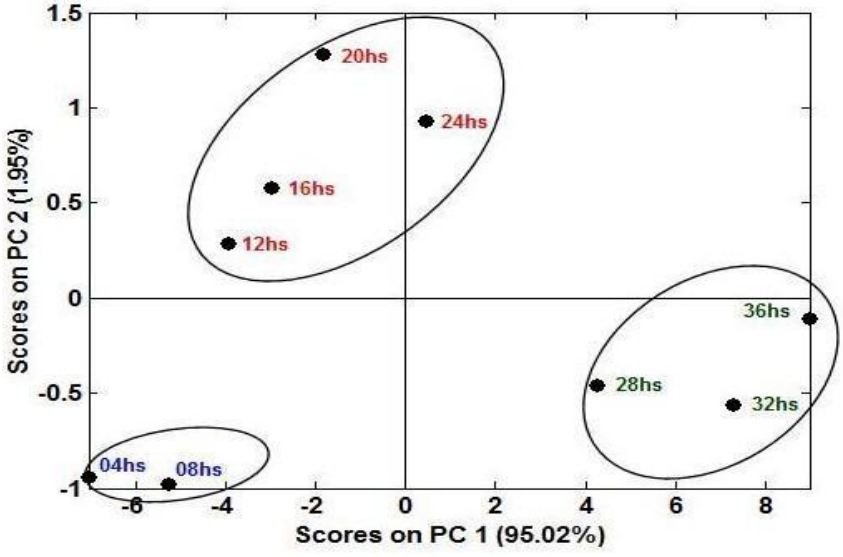

Fig. 5: Analysis of the grouping of the times in which the treatments for the cultivation of the torula cells

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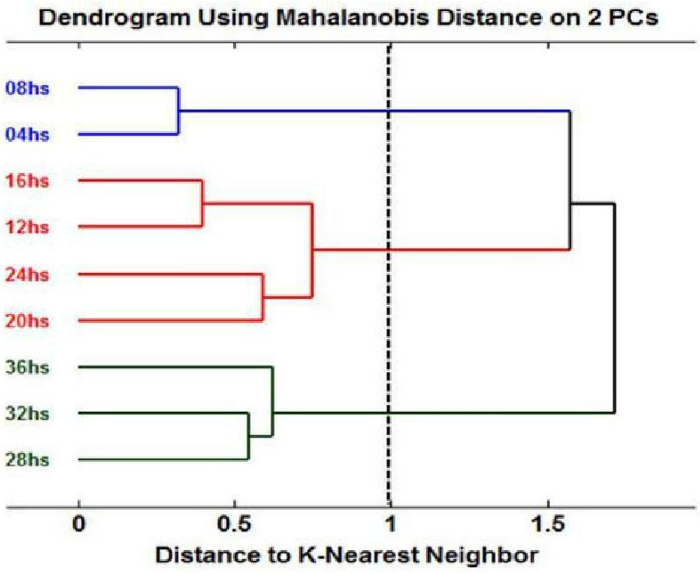

Fig. 6: Cluster analysis of the times in which the treatments for the cultivation of torula were performed.

The duration of 32 hours was identified as optimum for yeast growth even though the concentration of the inoculum suffered variations (Gibbons *et al*., 1984; Manilal *et al*., 1991). When the growth curves of each of the 16 significant treatments per unit time were considered, the interval of 32 hours was found to be the most appropriate (Figure 9).Other studies show that growth periods differ according to the kind of microorganism and substrates used. (Campos, 2003; Suhet, 1999).Cultivation times exceeding the optimum time are directly related to lower productivity of the cells. This result is due to depletion of nutrients from the substrate by the microorganism for the production of biomass (Borzani *et al*., 1983; Lopes *et al*., 2005), the generation of toxic metabolites, such as acetic acid, furfural, hydroxymethylfurfural and phenolic components (Almeida *et al*., 2009; Li *et al*., 2011; Andrade *et al*., 2013) the lowering of the pH because of the high concentration of acids produced (Ribéreau-Gayon and Peynaud, 1996).

Pareto charts relating to the cumulative frequency of the number of cells of each treatment at 24, 28 and 32 hours of cultivation (Figure 7, 8 and 9, respectively) are presented. When the cumulative frequency reached 80% of the cells produced, all the treatments with a significant mean productivity by the Tukey test contributed to this percentage during these three periods. In the time interval of 24 hours, the five most significant treatments in terms of average number of cells produced —14, 26, 8, 30 and 21 (in decreasing order) -— were those the ones that also achieved the highest absolute productivity.

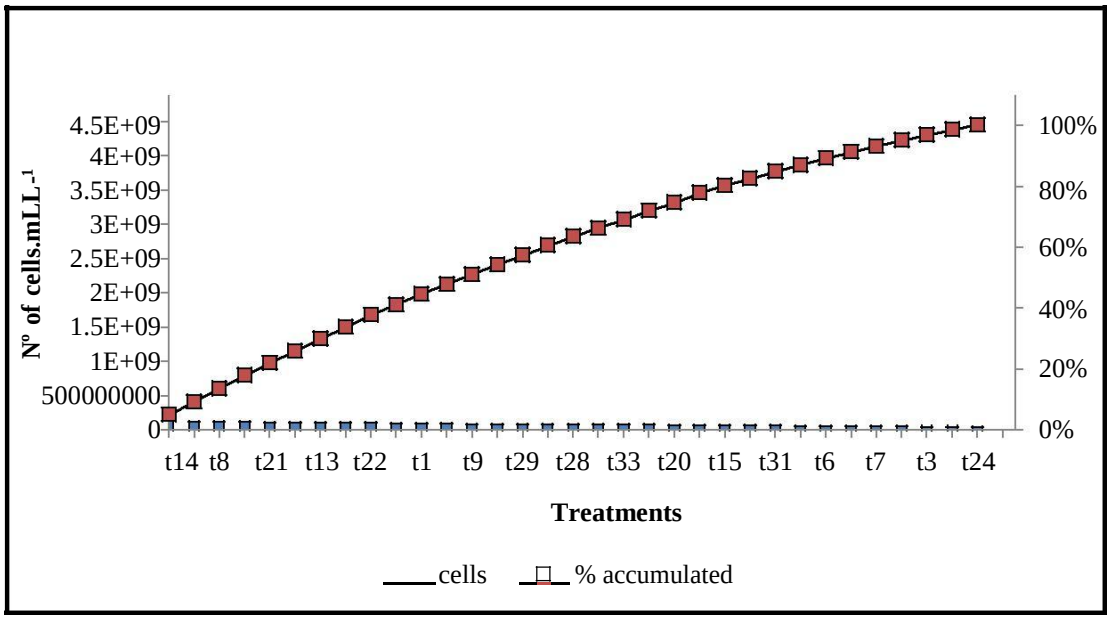

Fig. 7: Pareto graph representing the cumulative frequency of the number of cells of each treatment after 24 hours ofcultivation of *Candida utilis.*

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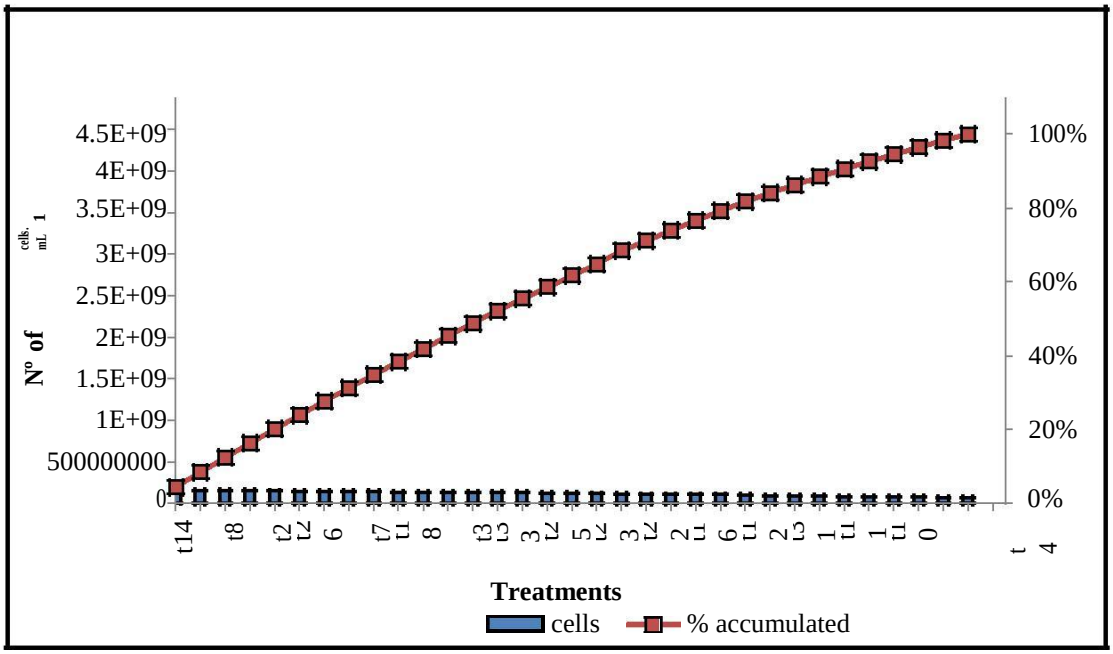

Fig. 8: Pareto graph representing the cumulative frequency of the number of cells of each treatment after 28 hours ofcultivation of *Candida utilis.*

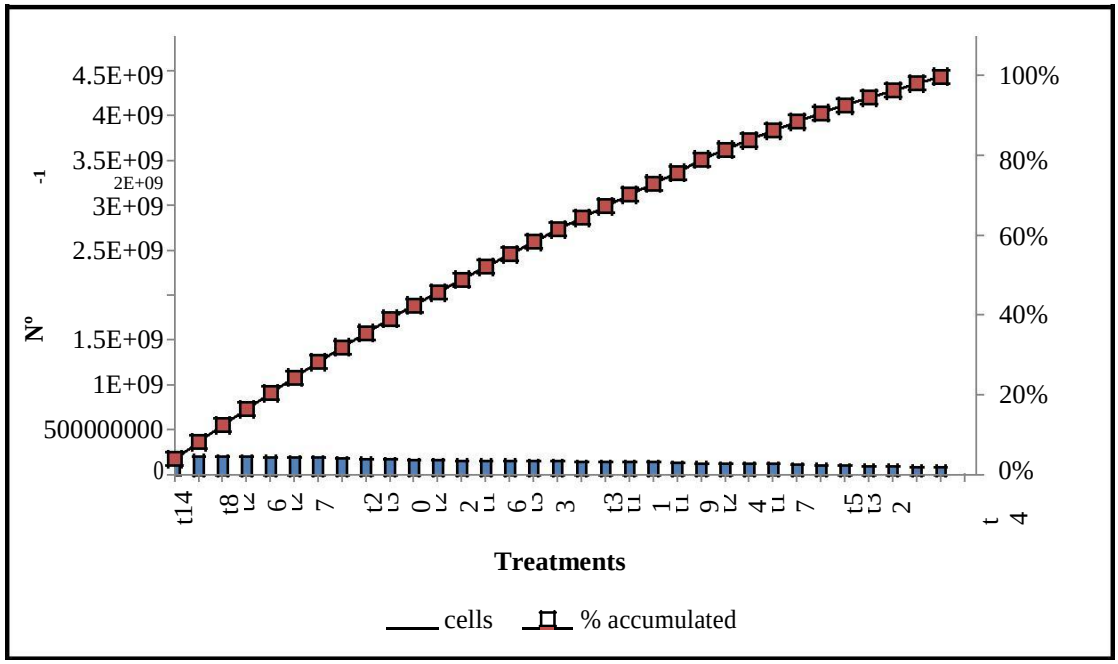

Fig. 9: Pareto graphical representations regarding the cumulative frequency of the number of cells of each treatmentafter 32 hours of cultivation of torula yeast

Discussion:

The composition of vinasse from bioethanol industries is heterogeneous and differs according to the liquid raw material from which it is obtained, the type of soil in which the sugar cane was grown, the active micro-organism employed in the fermentation of the substrate, the method of fermentation adopted, the distillation equipment, and other factors (Silva and Orlando Filho, 1981). In this work, the concentration of total soluble solids (TSS), humidity, reducing sugars, total proteins, and pH encountered in the characterization of untreated vinasse corroborate the results of other authors who developed their research by using this raw material for the production of protein for the purpose of using it in animal feed(Arrigoni *et al*., 1993; Lezcano and Mora, 2008; Giraldo and López, 2008; Ferreira, 2009). With vinasse in its raw form, a greater production of biomass was obtained using *Rhodotorula mucilaginous* (7.05 $g.L^{-1}$), a higher biomass protein yield was furnished using *Saccharomyces cerevisiae* (2.29 $g.L^{-1}$), and this amount corresponded to 50.35% of the total biomass of the yeast (Cazetta and Celligoi, 2005). The decrease in the concentration of reducing sugars and total carbohydrates after culturing the yeast in the vinasse is explained by the consumption of substrate to produce proteins during cultivation of yeast cells. Biomass production was directly proportional to the consumption of sugar by *Candida lipolytica*, *Rhodotorula mucilaginous*, and *Saccharomyces cerevisiae* because these yeasts consumed 50% of the sugars in the médium composed of raw vinasse. The vinasse is characterized as a co-product rich in organic matter, and this fact has aroused interest in the production of lipids because their composition is similar to those of vegetable oils. The carbon present in the vinasse is also incorporated into the lipid molecules (Celligoi, 1993). The results are in agreement with this statement because the incorporation of lipids into the vinasse was observed.

Several studies have been performed to determine the optimum culture conditions for *Candida utilis*(Irfan *et al*., 2011; Mimawar *et al*., 2010; Kieliszek *et al*., 2017; Flores *et al*., 1992;Ouedraogo *et al*., 2017; Ye *et al*., 2014; Wen-wei *et al*., 2010; Jia *et al*., 2017; Dong-Dongand Kun-Sheng,2010). Minawar *et al*. (2010) tested the use of urea, peptone, meat extract, yeast extract and Lab-lamco powder as carbon sources and ammonium nitrate, ammonium sulphate, ammonium

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dihydrogen phosphate, ammonium chloride, ammonium phosphate, and ammonium citrate as nitrogen sources. The maximum yield (49%) was obtained using fruit waste extract and soluble starch as the carbon source and ammonium nitrate as the nitrogen source at pH 6.0. Rosma & Cheong (2007) observed a significant increment in biomass production when a nitrogen supplement (commercial yeast extract, peptone, ammonium dihydrogen phosphate, ammonium sulphate and potassium nitrate) was added to the fermentation medium. Commercial yeast extract was the most suitable among the organic sources selected, whereas ammonium dihydrogen phosphate was the best inorganic nitrogen source, which enhanced the production by 53.7%. These results are somewhat different from those observed in the present study.

The increase in the pH of the treated vinasse and the decrease in COD signifys that this bioprocess reduced the pollution load of this substrate and increased its economic value. It involved the production of protein in a sustainable manner and in a manner less aggressive to the environment (Cabello *et al*., 2009; Rodriguez *et al*., 2011). Fermentation occurs anaerobically. However, fermentation was restricted because the cultivation of yeast was performed with the aeration provided by shaking to provide oxygen under the specific environmental conditions. Aeration induces oxidation of carbohydrates through respiration by yeast. Consequently, the production of alcohol is small, and yeast cell multiplication is favored. (Furletti, 1987).

The higher yields obtained with the treatments 28 and 27 can be explained by the higher concentration of ammonium nitrate associated with 14 g. L^{-1} of malt extract. Similarly, positive results were obtained in treatments 13 and 18, which included ammonium nitrate, 100 µL of inoculum and 14 $g.L^{-1}$ of potassium phosphate. The greater growth of cells in the presence of ammonium nitrate in these treatments reaffirms the necessity of nitrogen for the metabolism of the yeast. It increases the resistance of cells to the toxic action of ethanol and results in a faster bioprocess, higher productivity and higher yield of cells (Cruz *et al*., 2003; Miranda Jr. *et al*., 2009; Batistote *et al*., 2010). The effect of additives that provide inorganic nitrogen for the system can increase the fermentative efficiency up to 77.8% compared to the control media, which do not receive this type of supplement (Puliglunda *et al*., 2011). Potassium phosphate acts as a buffer and furnishes phosphate for the production of yeast protein (Shi *et al*., 2013; Chen *et al*., 2004; Xie *et al*., 2005; Plantz *et al*., 2006). The effect of the addition of potassium phosphate was evaluated. The addition of potassium phosphate to peat extract led to an increase in mycelial growth and biomass concentration (Manu-Tawiah& Martin, 1987).

The fact that no lag phase was observed for the growth curve of the torula yeast in the untreated vinasse is explained by the process of adaptation to this substrate achieved prior to initiating the culture. With regard to the process of optimization of cell production, the most significant treatments were numbers 8, 14, 21, 26, and 30, treatment number 14 being the most significant. The main factor that contributed to the prominence of these media was the larger amount of inoculum used, 100 µl. Suitable inoculants lead to a shorter lag phase, which lead to an early start of the exponential growth phase. For most treatments, the lag phase was more extensive, and it was decisive for significantly increasing the yeast cell yield. Higher malt extract content, ammonium sulfate and ammonium nitrate were also related to a greater yield of protein biomass. Less positive results associated with the 100-µL inoculum were observed when urea was included in the media. The analysis indicated that the periods of 24 and 28 hours were the most suitable for the cultivation of torula, considering all the conditions provided by the media. But for the treatments that furnished the best results, the highest yield was obtained in the period of 32 hours. Treatments 3, 4, 5, 6, 7, 9, 10, 11, 15, 17, 20, 23, 24, 25, 31, 32, and 33, did not differ from untreated vinasse with respect to the promotion of yeast growth. The tests highlighted the importance of determining the quantity of inoculum and media supplementation. The treatments with the greatest potential for the production of the yeast and, consequently, for the production of biomass protein were identified, and the highest productivity of protein biomass was obtained with the following medium composition: 100 µL of inoculum, 6 $g.L^{-1}$ of peptone, 6 $g.L^{-1}$of yeast extract, 14 $g.L^{-1}$ of malt extract, 1.3 $g.L^{-1}$of urea, 14 $g.L^{-1}$ of ammonium sulfate, 6 $g.L^{-1}$of ammonium nitrate and 0.6 $g.L^{-1}$ of potassium phosphate. This result is different from those of Minawar *et al*. (2010) and of Rosma & Cheong (2007), who observed that the best nitrogen sources were ammonium nitrate and ammonium dihydrogen phosphate, respectively.

Conclusions:

The treatments with the greatest potential for the production of the *C. utilis* and, consequently, for the production of biomass protein were identified, and the highest productivity of protein biomass was obtained with the following medium composition: 100 µL of inoculum, 6 $g.L^{-1}$ of peptone, 6 $g.L^{-1}$of yeast extract, 14 $g.L^{-1}$ of malt extract, 1.3 $g.L^{-1}$of urea, 14 $g.L^{-1}$ of ammonium sulfate, 6 $g.L^{-1}$ of ammonium nitrate and 0.6 $g.L^{-1}$ of potassium phosphate. Urea was not found to be very important for cell growth, although the ammonium salts were more important than the carbon sources utilized. Previous adaptation of the torula to the vinasse lead to an immediate exponential phase without the presence of a lag period. Industrial vinasse was observed to be a good medium for the production of torula. Contribution: This work furnished additional information regarding the cultivation of *C. utilis* in vinasse obtained as a coproduct of the industrial production of bioethanol. Future work: The additives that appeared to be most important for cell growth will be tested individually, the possible toxicity of the product will be assessed, and sensorial assays to determine the extent to which torula and its protein fractions can be added to food in terms of acceptance will be performed.

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