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ENHANCEMENT OF LACTIC ACID FERMENTATION BY *LACTOBACILLUS DELBRUECKII* ATCC 6949 USING SUGARCANE MOLASSES

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ABSTRACT

Experiments for lactic acid production from sugarcane molasses, an agro-industrial residue, were performed using *Lactobacillus delbrueckii* ATCC-6949. The effects of medium agitation, inoculum age, inoculum size, and height-diameter ratio (H/D) of the bioreactor on the organic acid production were investigated. The tests employed a mechanically agitated reactor equipped with temperature, agitation and pH controllers. The best reaction yield obtained was 96%. For that, the following bioprocess conditions were employed: inoculum age of 24 hours, 5% inoculum size, bioreactor height-diameter ratio of 1.5, and agitation of 200 rpm.

Keywords: Lactic acid, *Lactobacillus delbrueckii*, sugarcane molasses, lactic fermentation.

INTRODUCTION

Lactic acid is a substance widely distributed in nature that can be found, for example, in fruits, agro industrial residues, and some animal tissues. Although it can be synthesized through chemical processes, Gao *et al*. (2005) and Ding and Tan (2006) reported that the majority of the commercialized lactic acid is produced through bioprocesses using renewable raw materials. The lactic acid and its derivatives can be applied in various industries of chemical processes, as such as paper and cellulose, pharmaceutical, and cosmetic industries. In addition, the applications of this organic acid in the synthesis of biodegradable polymers have prompted recent studies for its biosynthesis optimization (Ding and Tan, 2006). The presence of a chiral carbon in the lactic acid molecule provides the existence of *L* and/or *D* isomers. Chemical processes and some microorganisms are able to synthesize racemic mixtures of this organic acid. However, the biodegradable lactic acid polymers can only be obtained from L isomers, and consequently the importance of choosing the appropriate microbial agent to be employed is due to productivity and economic issues. Kadam *et al*. (2006) reported that only strains of *Lactobacillus brevis*, *Lactobacillus helveticus*, and *Lactobacillus delbrüeckii* are able to produce pure isomers of lactic acid.

In bioprocesses, a significant part of the production costs is associated with the propagation and maintenance of the employed strain as well as energy demand and employed carbon source. Brazil is one of the world's major producers of alcohol and sugar derived from sugarcane. Annually, the country produces more than 18 million tons of molasses, one of the major sugar industry residues from processing sugarcane that has a high content of sucrose and inorganic salts. The quantity of this residue and its chemical characteristics makes its disposal unfeasible in effluent treatment stations (ETS) with biological steps. The increase in biomass proliferation since sucrose is metabolized for a variety of microorganisms increases the ETS sludge volume and, consequently, the costs of its disposal. Another problem is the increase in the effluent's biochemical oxygen demand and the preferential biodegradation of sucrose in detriment to recalcitrant compounds, such as phenols. These factors contribute to the decrease in the ETS efficiency and increase the environment costs of sugar manufacture.

Aiming to reduce bioproducts production costs and considering aspects of environment sustainability, some researchers are developing works that employ cheap and renewable raw materials. In this context, some carbon sources from agro-industrial residues and other cheap materials are being investigated for lactic acid synthesis (Adsul *et al*., 2007; Oh *et al*., 2005; Bustos *et al*., 2005; Téllez-Luis *et al*., 2003; Kotzamanidis *et al*., 2002).

Recent studies are demonstrating that physical and physicochemical parameters such as temperature and pH, as well as parameters related to the fermentation medium, influence lactic acid production by microbial strains, including *Lactobacillus delbrüecki* (Nagarjum *et al*., 2005; Nancid *et al*., 2005; Akeberg *et al*., 1998). In the present work, lactic acid production from molasses, an agro-industrial residue from the sugarcane sugar producing process, was investigated employing a

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Lactobacillus delbrüecki ATCC 6949 strain and studying the agitation speed, inoculum age, inoculum size, and height/diameter ratio of the bioreactor.

MATERIALS AND METHODS

The *Lactobacillus delbrüecki* ATCC 6949 microorganism was used in the experiments because of its homofermentative metabolism and its ability to ferment sucrose.

The cells cultivated in nutrient medium (Difco Laboratories 0001, Illinois, USA) were centrifuged at 8000*g* and suspended in sterile skimmed milk containing 5% of sodium glutamate. Samples of 0.3 mL from these cell suspensions were placed in sterile glass tubes and frozen in a chamber in the interior of the lyophilization apparatus containing liquid N_2. After that, the cells were lyophilized for 6 hours and stored.

For each essay, the content of an ampoule containing the lyophilized culture was placed in 10 mL of the culture medium and was incubated at 45 ± 1˚C for 24 hours. This time was chosen based on the growth curve that showed that at this time the cells were in exponential growth phase with 109 x 10^7 viable cells/mL. The culture medium employed showed the following composition in g/L: tryptone 10; yeast extract 10; KH_2PO_4 5, sucrose 50, tap water 1L. The medium, after pH adjustment to 6.8, was autoclaved at 110˚C for 20 minutes.

The determination of the microbial growth curve was performed at 45 ± 1˚C. Samples from the fermentation medium were taken in intervals of 1h and centrifuged at 8000 g for 5 minutes. The cells were washed 3 times with sterile saline solution, NaCl 9.0 g/L, and the absorbance was measured by spectrophotometry at 570 nm in a Hach spectrophotometer, Odssey model. The absorbance was related to the dry weight at 90˚C; absorbance of 0.3 corresponded to 50mg/L of cells. For the viable cell count, the samples, after successive dilutions, were plated in nutrient agar (Merck, Darmstadt, Germany). Colony forming units counts were performed after 24 hours of incubation at 45 ± 1˚C.

In all experiments the sugarcane molasses, an agro industrial residue, was used. The molasses was provided by Companhia Usinas Nacionais, Brazil. The characterization of the molasses employed in the essays is shown in Table 1. Due to the high sucrose content and the reduced content of nitrogenous substances and ashes, the molasses employed was characterized as a molasses of the first crystallization of the sugar present in sugarcane syrup. The experiments were conducted in a Virtis bioreactor with 4L of capacity and equipped with temperature, pH and agitation controllers. The bioreactor showed a diameter, D, of 13.3 cm and height, H, of 26.6 cm. The fermentation medium was mixed through two turbine stirrers (1.4 cm x 0.9 cm) and three bafflers. The distance between the impellers was kept in 9.0 cm and the distance between the inferior impeller and the bioreactor base was 5 cm. The production medium was prepared by diluting the molasses in tap water in order to obtain 100g/L of sucrose. After this, the medium was enriched with (g/L): yeast extract, 0.6; $MgSO_4.7H_2O$, 0.15; $MnSO_4.H_2O$, 0.007, and $Na_2HPO_4.7H_2O$, 0.25. The medium was autoclaved at 110°C for 30 minutes. The pH control was performed using a 50% (w/v) NH_4OH solution, with an electrode connected to a pH controller system.

Table 1. Molasses physicochemical characterization

Parameter	Content (%)
Specific weight	1.43
Sucrose	66.40
Reducing sugars	1.15
Nitrogenous substances	2.80

Experiments with agitation speeds of 100, 200, 250, and 300 rpm were performed in order to verify the effect of agitation in the lactic acid production by *Lactobacillus delbruecki* ATCC 6949 bacteria. The fermentation medium volume was 2.58 L with 5% (v/v) of inoculum and pH of 6.8 ± 0.2. Cells harvested at the exponential growth phase in different time intervals of growth (20h or 24h) were employed in order to verify the effect of inoculum age in the process. In these tests, the agitation speed previously selected was employed.

Experiments with inoculum volumes from 2.5 to 15% (v/v) were performed to investigate the influence of the inoculum size on the organic acid biosynthesis. These experiments sought process cost reduction through the employment of adequate inoculum size. In all cases, the other selected experimental conditions were maintained.

Considering the previously selected conditions, experiments to investigate the influence of the bioreactor H/D ratio were performed. The ratios were: 0.80, 1.00, 1.24, 1.50, and 2.00.

The lactic acid concentrations were determined by high performance liquid chromatography (HPLC). A Waters system (Milford, USA) equipped with a Spherisorb Octyl 250 mm x 4.6 mm column, a UV detector at 210nm, and a 0.6 mM H_2SO_4 mobile phase at a 0.5 mL/min$^-$flow. Sucrose was quantified during the experiments by the method described by Dubois *et al*. (1956).

The results presented are the mean value of four independent experiments.

RESULTS AND DISCUSSION

Figure 1 shows the microbial growth curves, relating dry weight and viable cells. The growth curves showed exponential phase in the period between 12 to 24 hours of incubation. It is worth highlighting that at the exponential growth phase, expressed in dry weight, dead and viable cells can be detected. As a consequence, the weight remains constant, unlike that observed on the curve expressed in terms of viable cells, which decreases due to the absence of detection of dead cells. The biomass propagation steps are very important for bioprocesses conducted in batch. Apart from contributing to the increase in energy consumption of the production process, the steps of biomass propagation require inputs such as water, carbon and nutrient source, energy, and workforce for their development. The optimization of the time for inoculum propagation involves the manipulation of the variable inoculum age in order to reduce production costs. In addition, inoculum age is relevant for the establishment of the incubation time in which cells may reach the maximum fermentation activity, which is expected to correspond to an intense cell multiplication such as the one reached in the exponential growth phase.

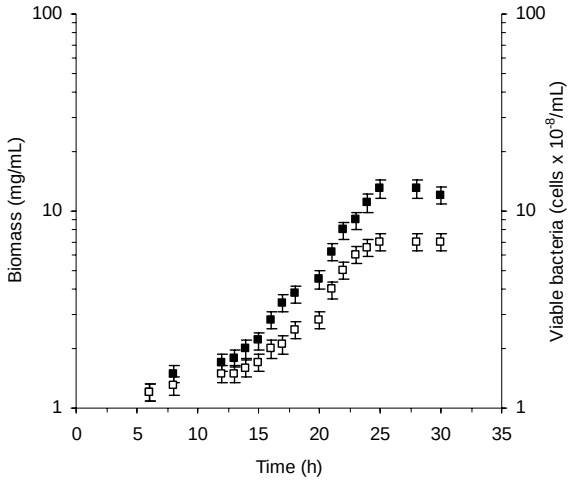

Fig. 1. *Lactobacillus delbruecki* ATCC 6949 growth curve expressed by dry weight and number of viable cells.

The data that relate the effect of inoculum age on the acid production are shown in Table 2. From this table one can observe that the fermentation yield coefficient showed a small variation in relation to the inoculum age. This can be explained by the fact that the 24 h inoculum has shown a higher number of cells. However, the difference between the Y_s values was not significant, showing that in this case it is important that the cells are in the exponential growth phase, where it is known that the fermentative activity is constant.

The steps necessary for bioprocess optimization may also involve other steps for the establishment of the appropriate inoculum size. This variable is very relevant in the performance of biochemical reactions due to its relation to obtaining adequate rates of bioproduct synthesis in the shortest time possible. In addition, the initial biomass constitutes an important fraction of the process costs, and, therefore, it is prudent not to disregard it in optimization and bioprocess economic feasibility studies. The ideal is to work with an initial number of cells that is able of generating the desired bioproduct amount. Table 3 shows the results of the effect of the inoculum size on the yield obtained from lactic acid formation. The results from Table 3 indicate that from 5% of inoculum size, the yield coefficients, Y_s, decreased with the increase in the quantity of inoculated cells initially added to the bioreactor.

Under this condition, the reaction yield was approximately 96%. Kanwar *et al.* (1995) reported maximum lactic acid production of 24.2 g/L and yield coefficient of 0.79 achieved using 3% (v/v) inoculum of a 36 h old culture in molasses medium containing sugars (5%; w/v) supplemented with peptone (2.5 g/L) and $(NH_4)_2SO_4$ (7.5 g/L), pH 6.5 at 40ºC after 72 h of fermentation. These distinct production yield results may be related to age and inoculum size differences. Göksungur and Güvenc (1997) obtained a yield of 95.4% of conversion of beet molasses employing *Lactobacillus delbrueckii* IFO 3202 at 45ºC, pH 6.0, and 78.2 g/L of sugar concentration with 10 g/L of yeast extract. In the present work, using a different row material and a lower yeast extract concentration, a similar reaction yield was obtained for the experiments conducted with 5% of inoculum size. This suggests that the use of this substrate and process conditions is very promising. It is worth highlighting that apart from the number of initial cells

Table 2. Yield coefficient (Y_s) on lactic acid production as a function of inoculum age.

Inoculum Age (h)	Cells (g/L)	Final Sucrose Concentration (g/L)	Lactic Acid (g/L)	Yield coefficient (Y_s) [a]
20	4.7±0.5	12.0 ± 0.9	99.0 ± 8.0	3.79
24	7.0±0.7	1.0 ± 0.1	101.0 ± 10.0	3.86

[a] Y_s = Mol of lactic acid produced by mol of initial sucrose

Table 3. Yield coefficient (Ys) from lactic acid production as a function of the inoculum volume added to the medium.

Inoculum volume (%)	Sucrose (g/L)		Lactic acid (g/L)	Yield coefficient (Y_s)[a]
	Initial	Final		
2.5	100	11.8 ± 0.9	85.0 ± 7.0	3.25
5.0	100	1.0 ± 0.1	101.0 ± 9.0	3.86
7.5	100	12.9 ± 0.6	81.6 ± 6.0	3.13
10.0	100	13.8 ± 1.0	73.0 ± 7.2	2.79
12.5	100	14.4 ± 1.2	70.4 ± 6.5	2.69
15.0	100	19.0 ± 1.9	54.0 ± 4.5	2.07

[a]Y_s = Mol of lactic acid produced by mol of initial sucrose

Table 4. Yield coefficient (Ys) from the production of lactic acid, related to the agitation.

Agitation (rpm)	Sucrose (g/L)		Lactic acid (g/L)	Yield coefficient (Y_s)[a]
	Initial	Final		
100	100	9.3 ± 0.2	76.0 ± 2.0	2.91
200	100	1.0 ± 0.01	101.0 ± 2.5	3.86
250	100	12.5 ± 0.3	58.0 ± 1.2	2.11
300	100	45.0 ± 1.5	35.2 ± 0.8	1.28

[a]Y_s = Ratio between mols of produced lactic acid per mols of initial sucrose (100 g/L)

Table 5. Yield coefficient (Ys) from the production of lactic acid, related to the height of broth and bioreactor diameter ratio (H/D).

Height (cm)	Diameter (cm)	H/D[a]	Sucrose (g/L)		Lactic acid (g/L)	Yield coefficient (Y_s) [a]
			Initial	Final		
10.6	13.3	0.80	100	11.5 ± 1.0	21.0 ± 0.1	0.80
13.3	13.3	1.00	100	12.0 ± 1.2	59.5 ± 0.3	2.27
16.5	13.3	1.24	100	5.0 ± 0.6	82.6 ± 0.6	3.16
19.9	13.3	1.50	100	1.0 ± 0.06	101.0 ± 1.2	3.86
26.6	13.3	2.00	100	10.0 ± 1.6	42.5 ± 0.3	1.62

[a]H/D is ratio between height of broth and bioreactor diameter; Y_s = Ratio between mols of produced lactic acid per mols of initial sucrose (100 g/L).

employed in the culture, cell multiplication occurred, reaching the same number of cells at the final growth phase. Therefore, the inoculum which replicated a greater number of times needed a larger energy quantity for its growth and, consequently, produced a larger quantity of acid.

In the mechanically agitated bioreactors, the importance of the agitation speed is related to economic and process performance factors. Comparing fluids of the same viscosity, highest agitation speeds foster a better level of fluid mixture, contact between the phases, and, therefore, higher coefficients of mass transfer. However, for these conditions a higher energy intake is required for the movement of the engine axes, which significantly contributes to the increase in bioprocess costs. Another important factor is medium agitation. It promotes contact between the microbial agent and the substrate for synthesis of the bioproduct of interest and its excretion (Christi, 1989). In this case, the need to find agitation speeds with a better cost-benefit ratio is also evident. In the specific case of fermentation with acid production a natural reduction in the medium pH occurs, which can reach values incompatible with the good performance of the microbial strains. The control of fermentation medium pH by the addition of alkaline solutions increases even further the need for adequate mixture in the bioreactors to promote contact and, consequently, neutralization of the acids formed.

Figure 2 shows the results obtained for the microbial specific growth rate and lactic acid formation as a function of the medium agitation speed. One can observe that the maximum specific growth rate (μ_m) obtained at 100 rpm agitation speed was 1.62 x 10^{-1} (h^{-1}). For the agitation speeds of 200, 250, and 300 rpm, the μ_m values decreased to 1.0 x 10^{-1} (h^{-1}), in a process time between 15 and 25 h. A plateau in the μ_m curve between 15 and 25 hours was observed, which corresponds to the microorganism exponential growth phase. In relation to

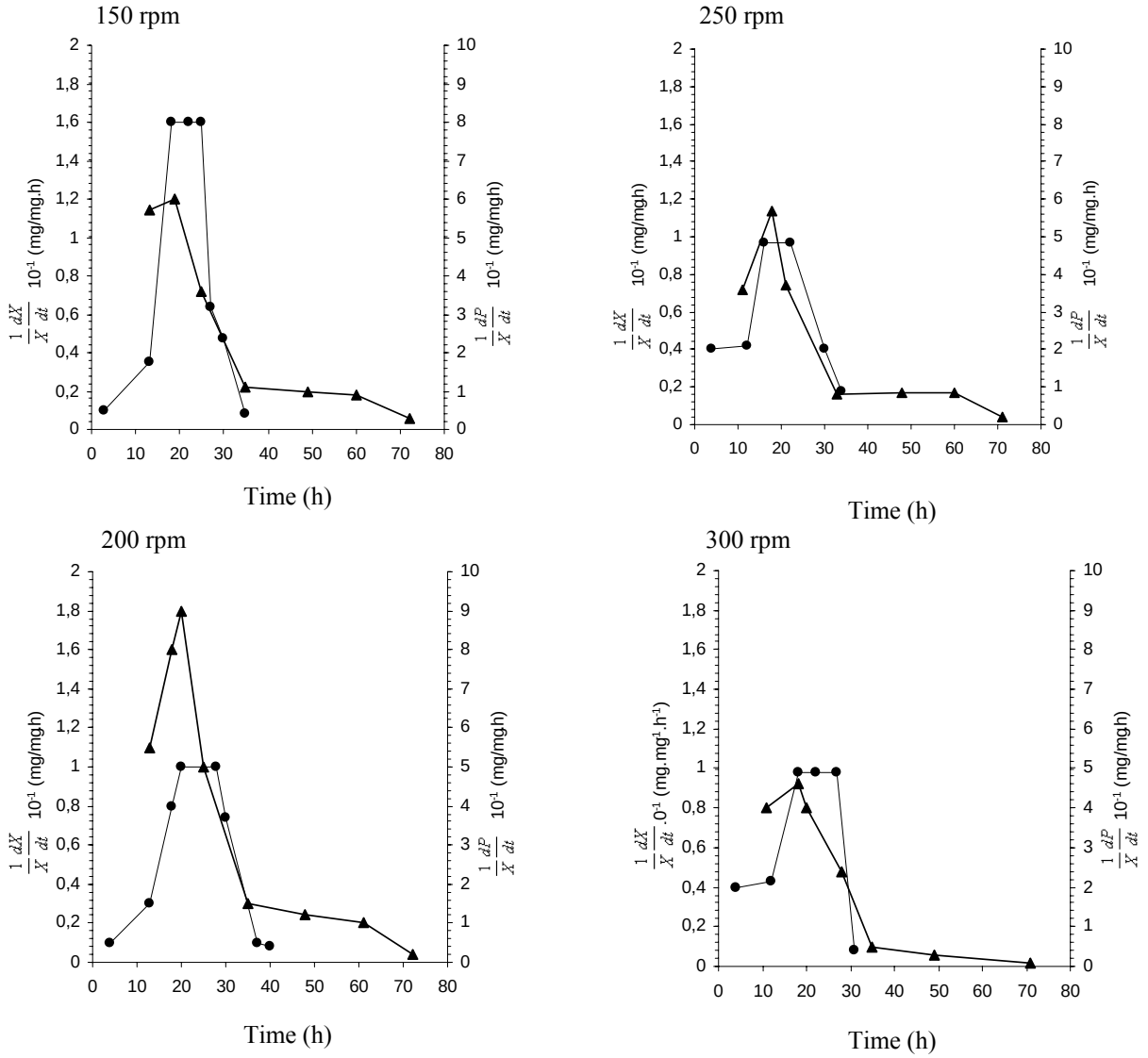

Fig. 2. Specific lactic acid production as a function of agitation speed. (- ● - *X* = cells, - ▲ - *P* = product, t = time)

specific rate of product formation maximum values of 6.1 x 10^{-1} (h^{-1}), 9.1 x 10^{-1} (h^{-1}), 5.8 x 10^{-1} (h^{-1}), and 4.8 x 10^{-1} (h^{-1}) were obtained, respectively, for 100, 200, 250, and 300 rpm after 20 hours of process, which corresponded to the beginning of the exponential phase of microbial growth. The agitation speed of 200 rpm fostered lactic acid production probably as a consequence of better homogenization of the fermentation medium, which allowed the adequate product neutralization. The highest agitation speeds were unfavorable to the process due to the higher oxygen transfer to the cells, not favoring, therefore anaerobic metabolism.

In Table 4 one can observe that the agitation speed of 200 rpm was favorable to microbial growth and to the lactic acid formation with a lower concentration of residual sucrose, 1.0 g/L, which favors the recuperation and purification of the product.

It is fitting to emphasize that studying the ratio of medium height and bioreactor diameter is relevant for optimization studies as it aids in determining the medium maximum quantity for production medium in a determined kind of bioreactor and, therefore, increases the bioprocess yield. The data from table 5 show that the 1.5 H/D ratio showed the higher lactic acid yield. In these tests, agitation speed was kept constant at 200rpm. Under this condition considering the distance between the impellers and the distance from the inferior impeller to bioreactor bottom, it was noted that fermentation media was 5cm above the superior impeller. This fact positively contributed to the medium mixture and, consequently, to the neutralization of the acid formed and the better performance of the microbial strain. In this case, a yield of lactic acid production of approximately 96% was obtained, which is very satisfactory. Under the other H/D tested conditions, a decrease of the lactic acid production yield was observed.

In the case of H/D of 1.24, the second best reaction yield of 79% was obtained. This percentage of conversion is considered low; besides that, a smaller quantity of fermentation medium is used. Therefore, one can conclude that the H/D ratio of 1.5 was the most adequate for the bioprocess.

CONCLUSIONS

The variables inoculum size and age, agitation speed, and ratio of fermentation medium height to bioreactor diameter influenced the lactic acid production from sugarcane molasses by *Lactobacillus delbrueckii* ATCC-6949. Among the tested conditions, a lactic acid production yield of 96% was obtained, which corresponds to the concentration of 101 g/L. This condition was reached employing agitation speed of 200rpm, 1.5 of H/D, 5% (v/v) of inoculum, and inoculum age of 24h. These parameters fostered the lower concentration of residual sucrose (1.0 g/L), which favors the recuperation and purification of the product.

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