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Comparison of Citric Acid Production by Solid-State Fermentation in Flask, Column, Tray, and Drum Bioreactors

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Abstract

Studies were conducted to evaluate citric acid production by solid-state fermentation (SSF) using cassava bagasse as substrate employing a fungal culture of *Aspergillus niger* LPB 21 at laboratory and semipilot scale. Optimization of the process parameters temperature, pH, initial humidity, aeration, and nutritive composition was conducted in flasks and column fermentors. The results showed that thermal treatment of cassava bagasse enhanced fungal fermentation efficacy, resulting in 220 g of citric acid/kg of dry cassava bagasse with only treated cassava bagasse as substrate. The results obtained from the factorial experimental design in a column bioreactor showed that an aeration rate of 60 mL/min (3 mL/[g·min]) and 60% initial humidity were optimum, resulting in 265.7 g/kg of dry cassava bagasse citric acid production. This was almost 1.6 times higher than the quantities produced under unoptimized conditions (167.4 g of citric acid/kg of dry cassava bagasse). The defined parameters were transferred to semipilot scale, which showed high promise for large-scale citric acid production by SSF with cassava bagasse. Respirometry assays were carried out in order to follow indirectly the biomass evolution of the process. Citric acid production reached 220, 309, 263, and 269 g/kg of dry cassava bagasse in Erlenmeyer flasks, column fermentors, a tray bioreactor, and a horizontal drum bioreactor, respectively.

Index Entries: Citric acid; solid-state fermentation; respirometry; column fermentors; horizontal drum.

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Introduction

The global annual production of citric acid is estimated at about 1 million metric t. The food industry consumes about 70% of total citric acid production, pharmaceutical industries consume about 12%, and the remaining 18% is consumed by other industries *(1,2)*. There is an estimated 3.5–4.0% annual growth in demand of citric acid *(1,3)*. In general, citric acid is commercially produced by submerged fermentation of molasses, and *Aspergillus niger* is the main source worldwide. In recent years, a considerable interest has been shown in using agricultural products and their wastes as alternative sources of carbon for citric acid production by *A. niger*.

Solid-state fermentation (SSF), often referred to as the *Koji* process, is characterized by the development of a microorganism in a low-water-activity environment on a nonsoluble material acting as both nutrient source and physical support *(4–6)*. It is the simplest method for citric acid production and has been an alternative to the use of agroindustrial residues *(7–13)*. A variety of agroindustrial residues and byproducts have been investigated with the SSF technique for citric acid production *(3)*. A cost reduction in citric acid production can be achieved by using less expensive substrates, such as apple and grape pomace, carrot waste, carob pod, orange and pineapple waste, cassava bagasse, coffee husk, corncob, kiwifruit peel, mussel-processing wastes, okara (soy residue), rice, and wheat bran *(1,2,4–6,9,14–24)*. These residues are very well adapted to solid-state cultures owing to their cellulosic and starchy nature *(25)*.

Brazil is one of the main producers of cassava, with 23 million t of cassava annually *(26)*. Cassava bagasse is a solid residue generated in the starch extraction process during the separation stage *(3,12)*. Cassava bagasse is disposed of in the environment, causing serious concerns owing to its high organic material content and biodegradability *(4)*. Utilization of cassava bagasse by fermentation has shown promise.

The aim of the present work was to compare the production of citric acid by SSF in flasks using different proportions of gelatinized starch. Optimal conditions of temperature, pH, initial humidity, and nutritive composition were previously defined in experiments carried out in Erlenmeyer flasks *(24)*. In the present work, four different bioreactors were tested—Erlenmeyer flasks (as control), column fermentor, horizontal drum bioreactor, and tray-type bioreactor—using optimal conditions.

Materials and Methods

Microorganism

A strain of *A. niger* LPB 21 was used. It was grown on potato-dextrose-agar (PDA) medium by incubating the slants at 30°C for 6 d. Slants were preserved at 4°C and subcultured monthly.

Substrate and Its Thermal Treatment

Cassava bagasse was supplied by Agroindustrial Paranaense de Polvilho (Paranavaí-PR, Brazil) and used as substrate. It was ground in a mill and sieved to obtain a particle size between 0.8 and 2.0 mm *(6)*. Thermal treatment for gelatinizing the starch present in cassava bagasse was carried out by adding 110 mL of distilled water/100 g of dry cassava bagasse and heating at 121°C for 20 min *(24)*. Different ratios of treated and nontreated cassava bagasse (0:100, 20:80, 40:60, 60:40, 80:20, 100:0 [w/w]) were used for fermentation.

Cassava bagasse was supplemented with a salt solution containing 2.93 g/L of urea, 1.86 g/L of KH_2PO_4, and 0.0105 g/L of $FeSO_4 \cdot 7H_2O$, which was sterilized at 121°C for 15 min. After cooling, methanol (4% [v/v]) was added under sterile conditions *(24)*. Different quantities of dry substrate were used for the different bioreactors: 10 g for Erlenmeyer flasks; 20 g for column bioreactors; 150, 300, and 450 g for trays with 2, 4, and 6 cm of substrate thickness, respectively; and 2 kg for horizontal drum.

Inoculum

The spores of *A. niger* were produced in 250-mL Erlenmeyer flasks containing 40 mL of PDA medium, inoculated with the spores from the stock culture, and incubated at 28°C for 6 d. The spores were recovered in an aqueous solution of Tween-80 (0.01%). The obtained suspension contained 10^8 spores/mL and was stored at 4°C for 6 d.

Solid-State Fermentation

SSF was carried out by placing 10 g of dry cassava bagasse in 250-mL Erlenmeyer flasks, and the initial moisture was adjusted to 70% with nutrient solution and distilled water. Substrate was inoculated with the spore suspension at a rate of 10^7 spores/g of dry cassava bagasse. Flasks were incubated at 28°C for 144 h *(24)*.

SSF in column fermentors was carried out in glass columns with a 4-cm id and 20-cm height (volume of 250 mL). Column fermentors were used to study the influence of aeration rate and humidity on citric acid production. A 2^{2-0} factorial experimental design was used. Aeration rate was varied from 60 to 100 mL/min or 3 to 5 mL/g of dry matter/min. The initial humidity of the substrate was also varied from 60 to 70%.

The third step of the experiment included scale-up studies in trays with an initial humidity of 70% and inoculum rate of 10^7 spores/g of dry cassava bagasse. The moist and preinoculated substrate was distributed in trays (0.0045 m^3) in a way to obtain 2, 4, and 6 cm of bed thickness (0.15, 0.30, and 0.45 kg of dry substrate, respectively), and the trays were placed in a room with controlled temperature (28°C) and a humidity of about 97%. Fermentation was carried out for 120 h.

SSF was also conducted in a horizontal drum with 2 kg of dry substrate with an initial moisture of 60%. Inoculated substrate was placed inside the

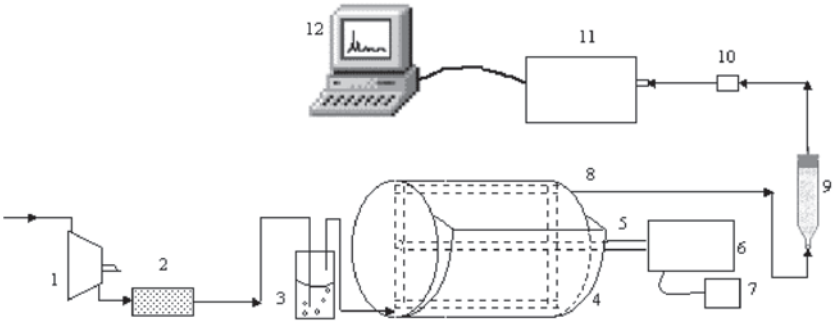

Fig. 1. Diagram of horizontal drum bioreactor and auxiliary equipment: 1, compressor; 2, air filter; 3, humidifier; 4, horizontal drum bioreactor; 5, axis; 6, motor; 7, speed controller; 8, air discharge; 9, silica gel column; 10, automatic injector; 11, gaseous chromatograph; 12, computer.

drum (Fig. 1). The drum was made using steel 360, with a 32-cm diameter and 30-cm length (internal volume of 0.024 m^3) and consisted of a shovel coupled to a motor axis that rotated with a controlled speed. The material was revolved three to four times per day. After 20 h of fermentation, saturated air was passed continually into the drum in order to control substrate temperature and moisture. The airflow was maintained at 5 L/min. Fermentation was carried out for 144 h.

All the experiments were carried out in duplicate.

Analytical Methods

Citric acid extraction was done by mixing 5 g of fermented sample with 50 mL of distilled water on a magnetic stirrer for 10 min and filtering through filter paper. The filtrate obtained was centrifuged at 10,000*g* for 10 min at 15°C, and the supernatant was subjected to high-performance liquid chromatography analysis using a BioRad Aminex HPX-87H (300 × 7.8 m*M*) column. A temperature of 60°C and 5 m*M* H_2SO_4 as the mobile phase at a flow rate of 0.6 mL/min were used. Citric acid was detected in the column eluate by differential refractometer (Shimadzu RID-10A). Each analysis was conducted in triplicate. Other parameters—pH and moisture—were determined as described by Soccol *(6)*. Starch content was determined using the method proposed by the National Starch Chemical Corporation described by Soccol et al. *(23)*.

Estimation of Growth

The respiratory metabolism of the microorganism was evaluated by determining the O_2 consumption and CO_2 production. This was utilized to estimate the biomass biosynthesis by the fungal culture *(23,27)*. The exit air from the bioreactors was passed through a silica gel column and then analyzed by gas chromatography in order to determine the oxygen uptake rate and the CO_2 evolved during the process *(23)*. The gas chromatograph

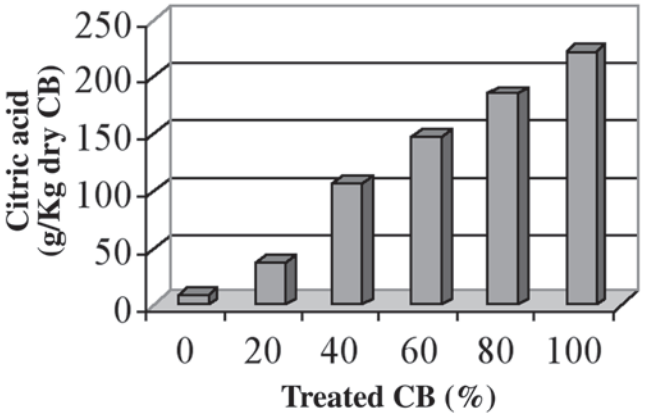

Fig. 2. Citric acid production in Erlenmeyer flasks with 0, 20, 40, 60, 80, and 100% treated cassava bagasse (CB).

(Shimadzu GC-8A) had an automatic injector and a thermal conductivity detector (Model IGC 11; Delsi, France) equipped with a gas-separating concentric column (CTRI; Alltech) connected to a data acquisition program (Chroma Biosystèmes, France), which controlled the whole monitoring system.

Results and Discussion

Effect of Thermal Pretreatment of Cassava Bagasse on Citric Acid Production

The influence of thermal treatment of cassava bagasse on citric acid production was clearly observed in Erlenmeyer flasks. Figure 2 shows citric acid production behavior when variable percentages of treated and untreated cassava bagasse were used. The best observed condition was determined as 100% of treated cassava bagasse with 220 g of citric acid/kg of dry cassava bagasse. Thermal treatment resulted in the hydrolysis of the starch, which facilitated its consumption by the fungus *(24)*.

Effect of Aeration and Initial Humidity on Citric Acid Production

One of the most important factors of SSF with filamentous fungus, at both the laboratory and industrial scale, is the estimation of biomass. Current methods used in liquid fermentation cannot be applied in SSF. This is because of the intense adhesion of filamentous fungal mycelium to the solid substrate/support and because of the SSF system's heterogeneity. O_2 consumption and CO_2 production are the result of metabolic activity of microorganisms from which they obtain the necessary energy for growth and maintenance. In addition, the metabolic activity is associated with the growth, and it can be employed for estimation of biomass biosynthesis. Several researchers used respirometry to follow the gas effluents from the

Table 1
Effect of Initial Humidity and Aeration Rate on Citric Acid Production and Starch Consumption on Cassava Bagasse by *A. niger* LPB 21

Essay	Humidity (%)	Aeration (mL/min)	Citric acid (g/kg of dry cassava bagasse)	Consumed starch (g/kg of dry cassava bagasse)
HAR1	70	100	169.9	512.0
HAR2	70	60	243.4	508.0
HAR3	60	100	235.1	433.4
HAR4	60	60	265.7	419.7
HAR5	65	80	171.9	515.5

bioreactor (CO_2 and O_2) in order to control fermentation and to evaluate microbial activities *(28–30)*. Vandenberghe and colleagues *(24,30)* showed that an environment with high concentrations of CO_2 has a positive effect on citric acid synthesis. In fact, a low oxygen environment is directly involved in growth limitation, which is crucial for citric acid production.

In a column bioreactor, the substrate humidity changes during fermentation owing to the saturated air passing through the medium. Consequently, it was very important to define optimal conditions for the parameters humidity and aeration rate in order to attain high citric acid synthesis. The results obtained from the factorial experimental design showed that an aeration rate of 60 mL/min (3 mL/[g·min]) and a 60% initial humidity were optimum, resulting in 265.7 g/kg of dry cassava bagasse citric acid production (Table 1). This was almost 1.6 times higher than the quantities produced under unoptimized conditions (167.4 g of citric acid/kg of dry cassava bagasse in column bioreactors).

Figure 3 shows the evolution of CO_2 rate (%) when *A. niger* was cultivated with low aeration rates; it produced less CO_2 and more citric acid (see Table 1). Total CO_2 production was 1.2 times more with an aeration rate of 100 mL/min or 5 mL/g of dry cassava bagasse/min. Low aeration rates (60 mL/min) are considered to limit the respiration activity of *A. niger* and, consequently, to turn the metabolism to citric acid synthesis. It was also observed (qualitatively) that strongly aerated cultures (5 mL/[g of dry cassava bagasse·min]) resulted in higher sporulation. Results were submitted to analysis of variance ($R^2 = 0.9994$). The criteria used for validation of significance of the parameters was based on p value (<5%).

Based on these results, it was concluded that a high partial pressure of CO_2 probably retards spore liberation of the filamentous fungus, limits its growth, and favors citric acid synthesis. In a normal culture of *A. niger*, with aeration starting at 0 h of incubation, germination occurs when CO_2 begins to be detected. Consequently, another experiment was carried out to optimize citric acid accumulation by delaying the start of aeration. Aeration rate was fixed at 60 mL/min and started after 12, 16, 20, 24, 28, or 32 h of incubation. The results obtained on the evolution of CO_2 are shown in Fig. 4.

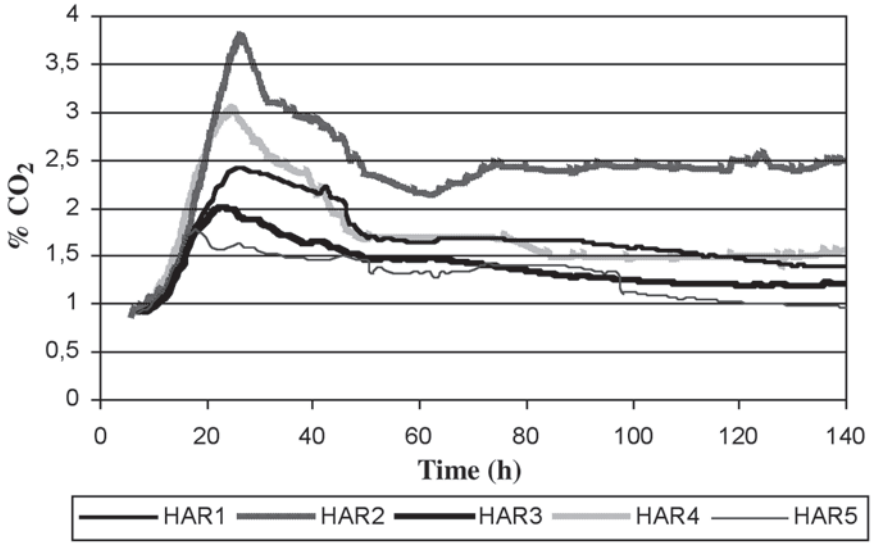

Fig. 3. Evolution of CO_2 rate (%) during culture of *A. niger* LPB 21 on cassava bagasse in SSF with different aeration rates (mL/min) and initial humidity (%), respectively: 100 and 70 (HAR1); 60 and 70 (HAR2); 100 and 60 (HAR3); 60 and 60 (HAR4); 80 and 65 (HAR5).

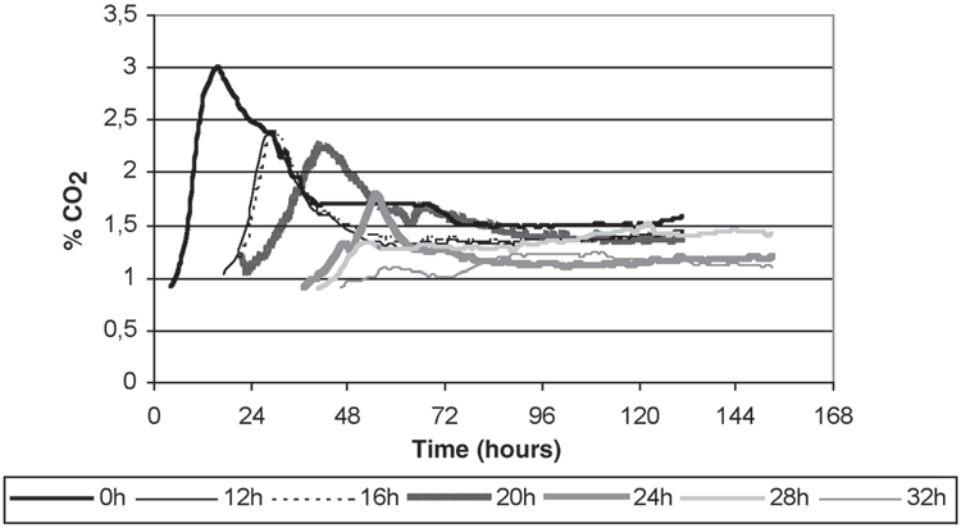

Fig. 4. Evolution of CO_2 rate (%) during culture of *A. niger* LPB 21 on cassava bagasse in SSF with aeration starting after 0 h, 12, 16, 20, 24, 28, and 32 h of fermentation.

When aeration started after 20 h of culture, the fungus produced 150.8 mL/g of dry cassava bagasse. This was 1.7 times less than that obtained with aeration beginning at 0 h. Table 2 shows that the effect of delayed aeration on citric acid production was very positive. In fact, with the start of air

Table 2
Influence of Forced Aeration Retardation on Citric Acid Production and Starch Consumption of Cassava bagasse by *A. niger* LPB 21

Aeration retardation (h)	Citric acid (g/kg DCB)	Consumed starch (g/kg DCB)	Yield* %
0	267.3	365.0	73.2
12	291.5	379.0	76.9
16	303.2	387.0	78.3
20	309.0	363.0	85.0
24	172.1	250.0	83.0
28	90.1	236.0	38.2
32	15.0	215.0	7.0

*Based on starch consumption; aeration rate: 60 mL/min/column or 3 mL/g dry CB/min.

supply after 20 h, citric acid production reached 309 g/kg of dry cassava bagasse. The corresponding value for starch consumption was 363 g of starch/kg of dry cassava bagasse. However, with aeration starting at 0 h, the production was only 267.3 g/kg of dry cassava bagasse with a corresponding value for consumed starch of 365 g/kg of dry cassava bagasse.

When aeration was started after 24 h of inoculation, cellular growth was limited and there was not enough biomass to produce the same levels of citric acid (172.1 g of citric acid/kg of dry cassava bagasse and 250 g of consumed starch/g of dry cassava bagasse). In addition, other operational factors also probably affected the fungal growth and citric acid synthesis.

Scale-Up Studies

Table 3 shows a comparison of citric acid production in a tray-type bioreactor and a horizontal drum bioreactor with different percentages of treated and nontreated cassava bagasse. In the horizontal drum bioreactor, citric acid production reached 269 g/kg of dry cassava bagasse with 100% thermal treated cassava bagasse (Table 3). On the other hand, in the tray-type bioreactor, it was not necessary to gelatinize cassava bagasse starch at high levels. This fact could be explained by the material compaction, owing to starch gelatinization, which limited air insertion throughout the medium. The best result for the tray bioreactor was 263 g of citric acid/kg of dry cassava bagasse with 80% treated cassava bagasse in a 4-cm bed thickness. Citric acid values for 100% raw cassava bagasse were negligible. These results showed the importance of starch gelatinization, which made the starch structure more accessible to *A. niger* attacks.

The high partial pressure in CO_2 probably delays the liberation of spores by filamentous fungus, favoring citric acid synthesis. In more compact layers of substrate, there was more CO_2 accumulation and, consequently, more citric acid production. The values for 80% treated cassava bagasse did not follow this trend, but in this case, citric acid produced in a bed thickness of 4 and 6 cm did not present significant differences (Table 3).

Table 3
Comparison of Citric Acid Produced
in Horizontal Drum and Tray-Type Bioreactors

Nontreated cassava bagasse (%)	Treated cassava bagasse (%)	Horizontal drum bioreactor (g citric acid/kg dry cassava bagasse)	Tray-type bioreactor (g citric acid/kg dry cassava bagasse)		
			Substrate thickness		
			2 cm	4 cm	6 cm
0	100	269	144	181	219
20	80	245	184	263	260
40	60	231	168	224	240
60	40	194	98	161	214
100	0	27	7	8	7

Citric acid production in the horizontal drum bioreactor (semipilot scale) with the same conditions applied to cassava bagasse reached 268.94 g/kg of dry cassava bagasse, corresponding to a yield of 69% based on starch consumption (387 g of starch consumed/g of dry cassava bagasse; data not shown). The production was higher in the horizontal drum bioreactor when compared with that in the tray-type bioreactor with a 2-cm bed thickness for all degrees of gelatinized starch present in cassava bagasse (Table 3). Citric acid production was also higher in the horizontal drum bioreactor for 0, 40, 60, and 100% treated cassava bagasse in comparison with trays with a 4-cm bed thickness. Only for 0 and 100% treated cassava bagasse was citric acid production in the horizontal drum better than in the tray with a 6-cm bed thickness. Another important fact to be considered was the fermentation time, which for trays (120 h) was shorter than for the horizontal drum (144 h).

Conclusion

Our study showed the feasibility of using *A. niger* LPB 21 in SSF of cassava bagasse for citric acid production. Cassava bagasse was pretreated (gelatinization) in order to facilitate starch consumption and, consequently, citric acid synthesis in laboratory (flasks) and semipilot scale. From the evolution of kinetic parameters of fermentation, such as CO_2 production and O_2 uptake, it was observed that low respiration rates resulted in the production of high concentrations of citric acid. Online monitoring of fermentation allowed determination of the relationship between CO_2 evolution, biomass, and citric acid production by *A. niger*. Experiments in packed-bed column bioreactors made it possible to analyze the influence of forced aeration, which was responsible for higher growth and sporulation of *A. niger*. Consequently, the control of its metabolism prior to citric acid accumulation was possible with low aeration rates and by delaying air supply to the fermentation medium after 20 h of inoculation. In column

bioreactors, citric acid production reached a maximum of 309 g/kg of dry cassava bagasse, corresponding to a yield of 85% based on consumed starch.

It is very important to understand how all process parameters act on the metabolism of fungus and the synthesis of some metabolites. When working in larger scales, a great number of factors can affect process behavior, such as oxygen and heat transfer, particle size, layer thickness, and type and shape of bioreactor. Consequently, a detailed scale-up study must be conducted in order to test every factor and its influence on a specific process. It is no different for citric acid production in SSF by *A. niger*.

Acknowledgments

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