

Citric acid production by *Aspergillus niger* from sugarcane bagasse with vinasse at different bed heights in packed-bed column

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Abstract

Solid-state cultivation (SSC) is the growth of microorganisms on solid supports in the absence or near absence of free water. Sugarcane bagasse and vinasse are generated abundantly in Brazil as byproducts from the ethanol industry, and bagasse has been used as solid matrix for SSC to produce a variety of bioproducts. The present study aimed to evaluate the SSC of *Aspergillus niger* on sugarcane bagasse impregnated with vinasse, with airflow rate and column bed height as scale-up criteria. Airflow rate of 0.8 L min⁻¹ caused a citric acid productivity of 3.77 mg (L.h)⁻¹ and a yield of 0.73 g g⁻¹ at 100 mm bed height. On the other hand, an airflow rate of 3 L min⁻¹ resulted in a productivity of 8.46 mg (L.h)⁻¹ with 2.26 g g⁻¹ of citric acid at 200 mm, and an estimated specific oxygen consumption rate of 0.008 mg per mg of biomass per hour.

Keywords: citric acid, bagasse, vinasse, solid-state cultivation, scale-up.

1 Introduction

Citric acid (2-hydroxy-propane-1, 2, 3-tricarboxylic acid), an intermediate of the tricarboxylic acid (TCA) is an organic acid of huge commercial importance, due to its applications as a preservative, sequestrant, flavorant, acidulant, emulsifier and buffering agent, being used in food, beverages, pharmaceutical, cosmetics and nutraceutical products (Verhoff, 2005; Yin et al., 2015; Wang et al., 2017). Currently, citric acid is considered to be the most extensively utilized organic acid [4], and it is the world's second largest product obtained by fermentation processes (Dhillon et al., 2011; Ciriminna et al., 2017). Citric acid can also be obtained through synthetic chemical reactions, but currently fermentation is the main technology for its production, which relies on the use of a diversity of renewable feedstocks such as silage, syrups, molasses and grains (Sun et al., 2017).

The processing of agricultural products generates an extensive amount of lignocellulosic by-products such as bagasse, fruit peel, husk and straw, among others (Yazid et al., 2017). Many of those solid residues have been reported in studies on the solid-state fermentation for citric acid production (Adeoye et al., 2015; Bastos et al., 2015; Ali et al., 2016; Bastos et al., 2016; Biz et al., 2016; Campanhol et al., 2019). Brazil is one of the main ethanol producers globally, obtaining over 25 billion liters of ethanol in the 2016/2017 sugarcane harvesting period (UNICA, 2018). Sugarcane is a key agricultural commodity, being one the world's largest crops grown (Reddy and Yang, 2015). The canes are squeezed to obtain its juice, and the remaining materials are usually called bagasse – a by-product. About 30-32% of sugarcane (in weight) is produced as

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Recebido para publicação em 23/05/2019 e aceito em 24/10/2019

by-products (*Lee and Mariatti, 2008*). The bagasse consists of about 45-55% cellulose, 20-25% hemicelluloses and 18-24% of lignin. (*Reddy and Yang, 2015*). Sugarcane processing also generates an important effluent called by vinasse. Vinasse is the main liquid stream generated from the ethanol distillation process, and has been considered as a potently pollutant effluent, due to its high levels of nutrients (mainly potassium and lower concentrations of nitrogen and phosphorous) and organic compounds (*Moraes et al., 2015*). In Brazil, each liter of ethanol produced generates between 10 to 15 liters of vinasse (*Souza et al., 1992; Bonomi et al., 2011; Cavalett et al., 2012; Moraes et al., 2015*). Considering that the total ethanol production in Brazil in 2016/2017 was near 25 billion liters, the volume of vinasse generated is considerable.

Solid-state cultivation (SSC) could be defined as a process in which microbial growth occurs near or in the absence of free-water, on a natural or inert solid material, usually impregnated with a nutrient solution (*Pandey, 2003; Bhargav et al., 2008; Kumar and Kanwar, 2012; Ramos-Sánchez et al., 2015; Bastos et al., 2016*). The literature regarding the combination of two agro industrial by-products for SSC is scarce, with a wastewater as a moistening medium and nutrients source impregnating the solid material. Therefore, the use of two agro industrial byproducts for the production of a biotechnological metabolite is innovative.

The scale-up of biochemical processes is very complex due to many different scientific and technological challenges such as engineering, fluid mechanics, chemistry and biochemistry (*Tiso, 2013*). Limitations in oxygen transfer can affect solid-state cultivations greatly, due to its importance as a substrate for fungal growth (*Onken and Liefke, 1989; Oostra et al., 2001*). The reduction of substrate bed due to mycelial growth modifies the bed porosity, as well as the diffusivity. The CO₂ moving in the opposite direction hampers the oxygen transport into the bed in the bioreactor, indicating that proper oxygen diffusion rates depend upon the transport properties of the bed. It is important to study oxygen transfer and bed heights because they are strongly related, since there could be a formation of oxygen gradients at some locations in the bioreactor, especially deeper into the bed, where the oxygen concentrations approach a value near zero (*Muniswaran et al., 2002*).

In this context, the present study aimed to extend the findings of previous research by focusing on airflow rate and column bed height as scale-up criteria for the solid-state cultivation of *Aspergillus niger* on sugarcane bagasse impregnated with vinasse.

2 Material and Methods

2.1 Inoculum

Aspergillus niger CCT 4355 was maintained on 50 mL of potato dextrose agar (PDA) slants at 4°C. Previously to every experiment, the inoculum was propagated on synthetic media composed of 15% sucrose, 0.25% ammonium nitrate (NH₄NO₃), 0.1% potassium phosphate (KH₂PO₄), 0.025% magnesium sulfate (MgSO₄), 0.004% copper sulfate (CuSO₄), and was then sterilized at 121°C for 20 min. *Aspergillus niger* was kept under constant agitation (150 rpm) and at pH 4.0.

2.2 Solid Support

The vinasse and sugarcane bagasse were obtained from sugar and ethanol industries in the region of Araras/SP, Brazil, and stored at the Laboratory of Applied Microbiology (LABMAC/

CCA/UFSCar). Bagasse particles were classified and selected with Tyler sieves in the range of diameters between 0.59 and 1.77 mm. Vinasse was acid pretreated for the availability of simple sugars and adjusted to a pH of 4.0 (Oliveira et al., 2012; Campahol et al., 2019).

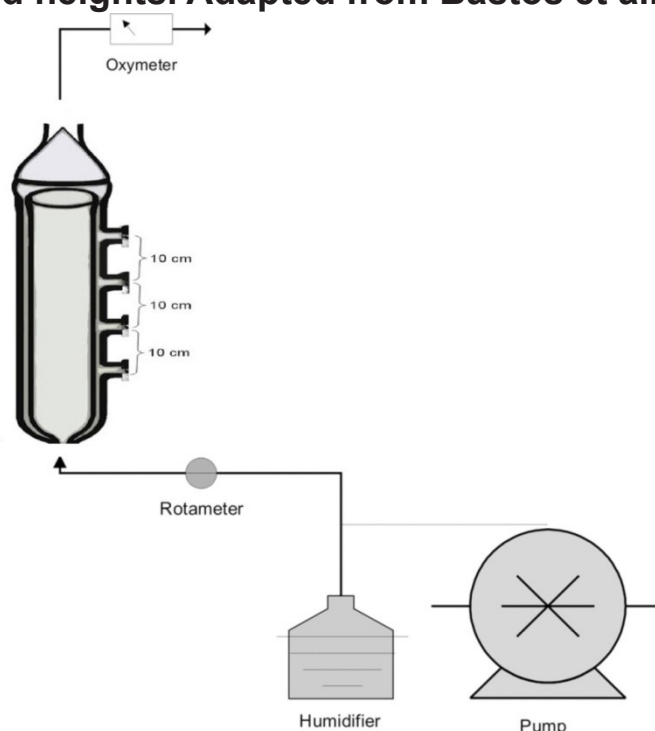
The bagasse and vinasse were both sterilized at 121°C for 20 minutes. Solid material was added to the inoculum suspension and vinasse at 70% initial moisture.

2.3 Solid-state Cultivation

Solid-state cultivation (SSC) of *Aspergillus niger* on sugarcane bagasse impregnated with vinasse was performed in a packed-bed bioreactor with 500 mm height and 50 mm diameter. The bioreactor has a tubular heat exchanger and axial samplers that allow sampling of solids and for dissolved oxygen measurements at every 100 mm of bed height (Fig.1). The experiments were planned according to previous studies performed by our research group, where Raimbault columns were studied for the solid state cultivation, but the solid medium volume was then ten times lower (Oliveira et al., 2012; Bastos et al., 2015; Campahol et al., 2019). The temperature and airflow selected from previous experiments were 25°C and 0.8 L.min⁻¹.

A new airflow rate (3 L min⁻¹) was calculated in order to maintain the oxygen transfer rate (the relation between air volume per bed height per minute) for the overall process due to bigger dimensions of the packed-bed bioreactor.

Figure 1. Solid-State Cultivation of *Aspergillus niger* in sugarcane bagasse impregnated with vinasse at different airflow rates and bed heights. Adapted from Bastos et al. (2015).



2.4 ANALYSIS

Samples were collected daily from the 100 and 200 mm heights. A crude extract was obtained from solid medium by adding water and acetone 50% for 45 minutes at 28°C in an orbital shaker at 150 rpm (Khosravi-Darani and Zoghi, 2008). The crude extract was then characterized by its pH through potentiometry. Glucose was quantified by the glucose oxidase-peroxidase

method using a kit (LABORLAB®, São Paulo, Brazil), according to the manual provided by the manufacturer (Oliveira et al., 2012). Citric acid was determined by reaction with pyridine and acetic anhydride (Khosravi-Darani and Zoghi, 2008). Dissolved oxygen was measured with an oximeter model YSI® 55-D, which was attached to the system.

3 Results and Discussion

Figure 2 shows the axial profiles of citric acid in the packed-bed column with an airflow rate of 0.8 L min^{-1} . The results indicate similar citric acid production behavior until day three on both axial positions, and then the 100 mm curve tends to a maximum value while the 200 mm curve decelerates, suggesting limited oxygen conditions. That limitation results in a citric acid productivity of $3.77 \text{ mg (L.h)}^{-1}$ at 100 mm and $1.51 \text{ mg (L.h)}^{-1}$ at 200 mm. Also, the yields in glucose and citric acid were 0.73 g g^{-1} at 100 mm and 0.33 g g^{-1} at 200 mm.

The maximum citric acid concentration obtained was very close to values from the literature, even with a lower productivity (Khosravi-Darani and Zoghi, 2008). However, the results cited in the literature describe experiments conducted with synthetic nutrient medium impregnating the solid particles and in optimized sugarcane bagasse pretreatment conditions. Since our studies report vinasse as a moistening medium, our results are very promising. Vinasse does not have high concentrations of glucose, which results in the hydrolysis of sugarcane bagasse particles by *Aspergillus niger*, slowing its metabolism and thus achieving lower productivities (Oliveira et al., 2012; Bastos et al., 2015; Campahol et al., 2019). On the other hand, Figure 3 indicates that the 3 L min^{-1} airflow rate resulted in maximum citric acid productivity of $2.29 \text{ mg (L.h)}^{-1}$ at 100 mm and $8.46 \text{ mg (L.h)}^{-1}$ at 200 mm, with yields of citric acid from glucose of 0.16 and 2.26 g g^{-1} , respectively. Yields above 1.0 suggest that not only the glucose in the vinasse was being consumed to produce biomass and metabolites, but *Aspergillus niger* was also hydrolyzing structural polysaccharides in the solid support and this substrate was not considered for yield calculations. Since the glucose analysis is an enzymatic assay, only monosaccharides were being quantified. This phenomenon occurs especially in the first hours of cultivation in aerobiosis, shown by Figure 4. The difference between values at 100 and 200 mm in the first hours reflects the bigger volume of solids and, consequently, more cells of *Aspergillus niger* and twice the demand of oxygen. After this period, there is a tendency to reach equilibrium due to greater difficulty in oxygen transfer through the extent of the packed bed reactor.

Considering Ikasari and Mitchell (1998), the SSC of *Aspergillus niger* in sugarcane bagasse impregnated with vinasse has an estimated specific oxygen consumption rate of 0.008 mg per mg of biomass per hour, which suggests low demand and non limiting oxygen conditions.

Vandenbergh et al. (2000) reported citric acid productions of 88g per kg of dry mass for cassava bagasse, 48.7 g kg^{-1} for sugarcane bagasse and 12.7 g kg^{-1} for coffee husk. The highest citric acid concentration, at 200 mm with 3 L min^{-1} , was 27.1 g kg^{-1} , a value that even though is inferior than those reported in the literature, it is very promising since only vinasse was used to impregnate the solid support.

4 CONCLUSIONS

Considering the production in terms of citric acid obtained from two byproducts from the sugar-cane industry combined with the required oxygen demand, which is usually a limitation

Figure 2. Axial profiles of citric acid in the crude extract obtained from the SSC of *Aspergillus niger* on sugarcane bagasse and vinasse, with an airflow rate of 0.8 L min⁻¹ at 100 (□) e 200mm (○) bed heights.

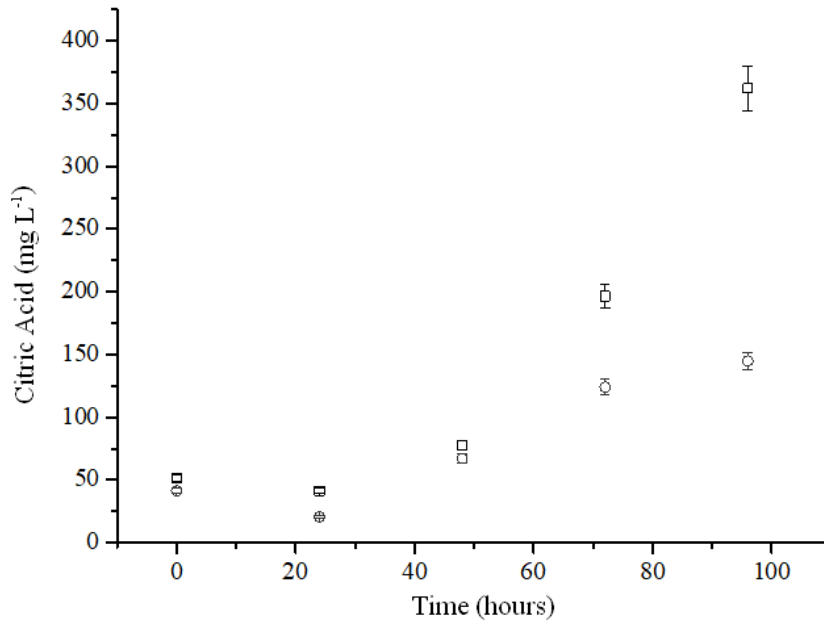


Figure 3. Axial profiles of citric acid in the crude extract obtained from the SSC of *Aspergillus niger* on sugarcane bagasse and vinasse, with an airflow rate of 3 L min⁻¹ at 100 (□) e 200 mm (○) bed heights.

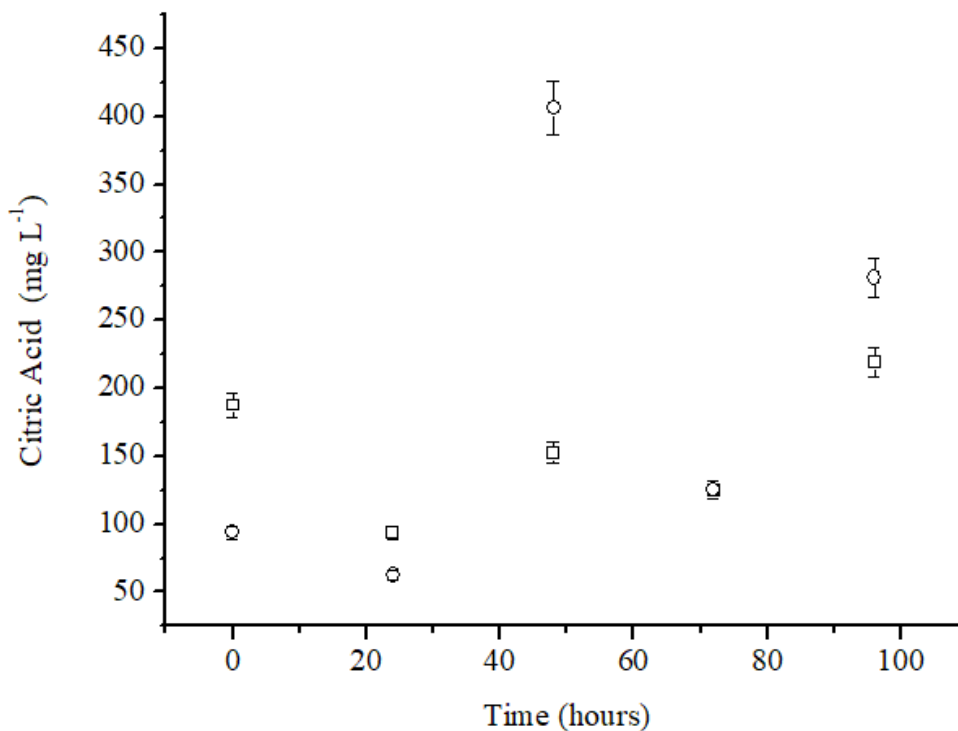
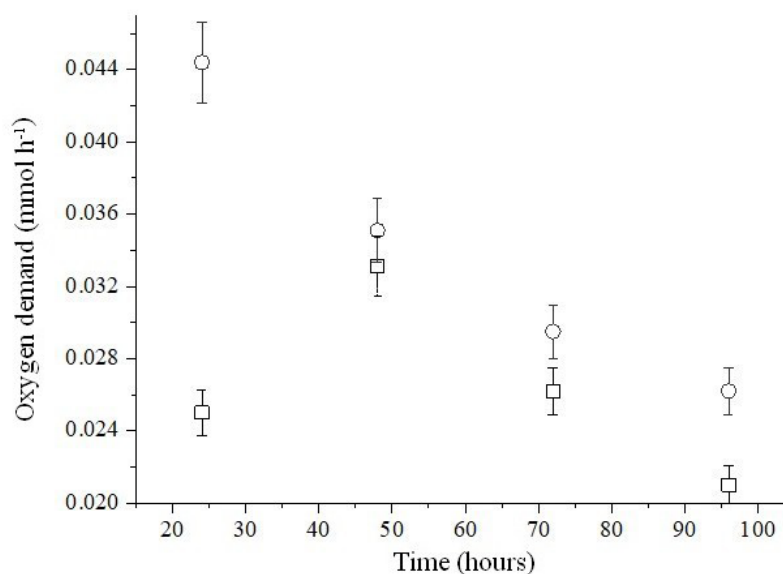


Figure 4. Oxygen demand of the SSC of *Aspergillus niger* on sugarcane bagasse and vinasse, with an airflow rate of 3 L min⁻¹ at 100 (□) e 200 mm (○) bed heights.



in solid-state cultivations, the results indicate successful scale-up of this process from air-flow rate and bed height as scale up criteria.

5 Acknowledgements

We gratefully acknowledge the financial support from FAPESP (Process Number 2011/07802-0).

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