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INFLUENCE OF SOLID MOISTURE AND BED HEIGHT ON CULTIVATION OF *Aspergillus niger* FROM SUGARCANE BAGASSE WITH VINASSE

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Abstract - Solid-state cultivation (SSC) may be defined as growth of microorganisms on a solid support impregnated or not with a nutrient solution in near absence of free-water conditions. The use of sugarcane bagasse as a support for SSC usually demands that the particles are impregnated and moistened with nutrient solution. Vinasse is the main wastewater of ethanol fermentation-distillation. As there are no reports of the use of wastewater for moistening solid supports in SSC, the proposal is the development of an innovative process, with valuation of these by-products. Thus, the aim of this research was to evaluate SSC of *Aspergillus niger* using sugarcane bagasse and vinasse for citric acid production. The results indicate that citric acid production and glucose consumption are dependent on oxygen availability, which can be modulated by selection of bed height and air-flow in packed-bed bioreactors.
Keywords: Solid-state cultivation; Citric acid; *Aspergillus niger*; Sugarcane bagasse; Vinasse.

INTRODUCTION

Solid-state cultivation (SSC) has been defined as microbial growth occurring in the absence or near absence of free-water, employing a natural or an inert support impregnated with nutrient solution (Mitchell *et al.,* 1999; Pandey *et al*., 2000; Gamarra *et al.,* 2010; Singhania *et al.,* 2009). In recent years, SSC has shown much promise for the development of several bioprocess due to low investment and energy consumption and the possibility of using agro-industrial wastes as a solid medium (Prado *et al*., 2005; Couto and Sanromán, 2006; Singhania *et al*., 2009; Wu *et al*., 2010; Angumeenal and Venkappayya, 2013). Nevertheless, there are no literature reports of the combination of two agroindustrial by-products for SSC, with the use of wastewater as the nutrient and moistening medium impregnating the solid supports. In this sense, the proposal to use two by-products generated by the same industrial platform for obtaining biotechnological metabolites is innovative.

Brazil is the one of the largest producers of sugar and ethanol from sugarcane in the world. Bagasse is a by-product resulting from juice extraction, being generally used as fuel for electricity generation (Lopes Silva *et al*., 2014). Another important waste of ethanol production, which is generated from distillation processes, is generally known as vinasse. This wastewater is one of the most recalcitrant residues since it contains the remaining non-volatile organic matter after the fermentation-distillation of molasses or sugarcane juice. As a consequence, vinasse will contaminate the environment if discharged directly into the environment, and the production of 1 liter of ethanol generates 8 - 15 liters of vinasse (Sumardiono *et al.*, 2013). The reported vinasse treatments include mainly anaerobic digestion and concentration, corresponding to the waste of nutrients such as potassium,

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nitrogen and organic molecules (Freire and Cortez, 2000; Navarro *et al.*, 2000; Baez-Smith, 2006; Syaichurrozi and Sumardiono, 2013).

SSC with sugarcane bagasse and vinasse would allow an even greater utilization of the main residues from sugarcane processing. Moreover, there are no studies of the utilization of vinasse as moistening liquid of supports for SSC. Nutrient solutions profoundly affect yields in SSC and moistening agents generally consist of salt solutions and tap water (Pal and Khanum, 2010).

Many microorganisms are able to grow on solid substrates, but only filamentous fungi can grow to a significant extent in the absence of free water because SSC resembles the natural habitat of these microorganisms (Singhania *et al.,* 2009). The filamentous fungus *Aspergillus niger* has been used for production of several enzymes and is an alternative for citric acid production using agro-industrial residues, such as sugarcane bagasse (Kumar *et al*., 2003; Khosravi-Darani and Zoghi, 2008; Kumar and Jain, 2008). Citric acid has been widely used as an acidifying agent and antioxidant in food, beverages and the pharmaceutical industries, usually produced by submerged processes (Kuforiji *et al*., 2010; Angumeetal and Venkappayya, 2013).

According to Mitchell *et al*. (2002), critical bed height is the value usually estimated for a tray bioreactor. At bed heights greater than the critical height, O_2 limitation occurs in the deeper regions of the bed during the fermentation. However, in wide bioreactors without internal heat transfer plates, the axial temperature gradient imposes a maximum height on the bed if it is desired to maintain the temperature rise over the bioreactor within predefined limits. This maximum height depends on the maximum growth rate of the microorganism and the superficial air velocity used (Mitchell *et al*., 1999). Therefore, in the scaling up of such packed beds, the bed height can be increased only up to a certain point, after which increases in scale should be achieved with an increase in diameter of the bed without further increase in height. Given typical growth kinetics, critical bed heights would be around 1 m.

As important as selecting the ideal bed height is the solid moisture, which impacts the availability of oxygen to the microorganisms. Low or high moisture decreased the enzyme production for *Aspergillus niger* in a medium that contained wheat bran and soybean cake (Pal and Khanum, 2010). According to these authors, the reason is that low moisture substrates reduce mass transfer processes and high moisture substrates reduce the medium porosity. Thus, it is essential to determine the optimal height of the bed in packed-bed SSC for a given process in order to establish criteria for scale-up of these bioreactors.

In this context, the aim of this study was to evaluate cultivation of *Aspergillus niger* on sugarcane bagasse moistened with vinasse, improving this solid-state cultivation and the management of wastes from sugarcane processing. Our results prove the viability of SSC without added nutrient for citric acid production, with the consumption of glucose dependent on bed height for a given air flow in a packed bed bioreactor.

MATERIAL AND METHODS

Inoculum

The inoculum of *Aspergillus niger* CCT 4355 was maintained on 50 mL of synthetic media in the Laboratory of Applied Microbiology (LABMAC/CCA/UFSCar), according to Kumar *et al*. (2003), i.e., on potato dextrose agar (PDA) slants and stored at 4 °C. Before each SSC experiment on sugarcane bagasse, the inoculum was propagated using synthetic media with 15% sucrose, 0.25% ammonium nitrate (NH_4NO_3), 0.1% potassium phosphate (KH_2PO_4), 0.025% magnesium sulfate ($MgSO_4$) and 0.004% copper sulfate ($CuSO_4$), sterilized at 121 °C for 20 min, pH 4.0 for seven days under constant agitation (150 rpm).

Solid Medium

Bagasse and vinasse were obtained from sugarcane industries in the region of Araras/SP, Brazil. Bagasse was classified with Tyler sieves in the range of diameters between 0.59 and 1.17 mm. Vinasse was pretreated with hydrochloric acid for the availability of simple sugars and adjusted to a pH of 5.0. (Bastos *et al*., 2009; Oliveira *et al*., 2012). Bagasse particles and vinasse were sterilized at 121 °C for 20 minutes. Solid material was added to the inoculum suspension and vinasse at different initial moistures.

Crude Extract

Crude extract from the solid medium with microorganisms at different batch times was evaluated by experimental design (Table 1), varying the time of each stage of extraction and temperature. The experiments were conducted in an orbital shaker at 150 rpm using 1 g of bagasse for 20 mL of solvent, in a first stage of extraction with distilled water, followed stage of extraction with distilled water, followed by a second stage with 50% acetone.

Table 1: Experimental design for evaluation of crude extract.

Parameter/Level	-1.41	-1	0	1	1.41
Time of each stage (min)	6	15	30	45	54
Temperature (°C)	25	28	33	38	41

Solid-State Cultivation

Experiments were performed in batch with a continuous inflow of 0.4 L min^{-1} of air saturated in a humidifier. Packed-bed columns 200 mm in height and 30 mm in diameter (Figure 1) were filled with sugarcane bagasse, vinasse as moistening agent and inoculum suspension (10% volume/volume) to initial solid medium moistures of 80, 70, 60 and 50 g 100 g^{-1} (wet basis). At each selected moisture, experiments were set up with different bed heights (60, 90, 120 and 150 mm). The temperature of all experiments was 25 °C.

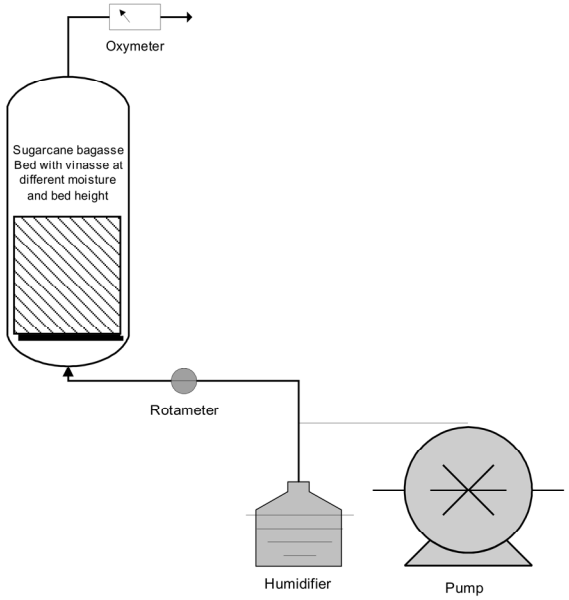


Figure 1: SSC of *Aspergillus niger* in sugarcane bagasse with vinasse at different moisture and bed heights.

Analysis

Packed-bed bioreactors were collected daily for evaluations, with separation of solid material. The moisture content of the sugarcane bagasse was determined by drying the solid medium at approximately 130 °C until a constant weight (Shojaosadati and Babaeipour, 2002).

The crude extract was obtained as above, while the samples were quantified as glucose by the glucose oxidase-peroxidase method using a kit (LABORLAB®, São Paulo, Brazil), according to the manufacturer's manual (Oliveira *et al*, 2012) and protein content was determined using the Bradford method (Bradford, 1976). Citric acid was determined by reaction with pyridine and acetic anhydride (Khosravi-Darani and Zoghi, 2008), using the reagent kit IN VITRO®, Itabira, Brazil.

The overall oxygen transfer rate (N) can be measured by using the oxygen balance of the columns, stated mathematically as Equation (1) (Thibault *et al*. 2000):

$$N = \frac{1}{V_S}[(FC_G)_{in} - (FC_G)_{out}] \tag{1}$$

The oxygen mass balance permits calculation of the average rate of oxygen consumption in the liquid film Eq. (2)):

$$R_{O_2} = \frac{((FC_G)_{in} - (FC_G)_{out})}{n_p(3/4)\pi(R_2^3 - R_1^3)} \tag{2}$$

where V_S is the volume of the solid substrate, F is the gas flow rate, C_G is the oxygen concentration in the gas phase, n_p is the number of solid particles in a specific bed (calculated directly using the relation of bed volume to particle volume, and considering bed porosity), R_{O_2} is the average oxygen consumption rate per biofilm volume, R_1 is the particle radius and R_2 is the radius of particle with its biofilm. To calculate the dissolved oxygen concentration in the liquid film, a radial profile of oxygen can be calculated using Eq. (3), with the assumption of a zero-order consumption reaction:

$$\frac{C_L(r)}{C^*} = 1 + \frac{1}{6}\left(\frac{R_{O_2}R_2}{D_{O_2,L}C^*}\right)\left[\left(\left(\frac{r}{R_2}\right)^2 - 1\right) + 2\left(\frac{R_1}{R_2}\right)^3\left(\frac{R_2}{r} - 1\right)\right] \tag{3}$$

where C^* is the equilibrium concentration at the gas-liquid interface on the liquid side, r is the radial position coordinate and $D_{O_2,L}$ is the oxygen diffusivity in the biofilm. The values of the parameters assumed for the simulation were 5 to 45 μm for the biofilm thickness, $D_{O_2,L}$ of 2.5 x 10^{-9} m/s^2 (assuming oxygen in water at 25 °C) and the gas-liquid phase oxygen transfer coefficient (K_La) was calculated using Equation (4) (Gowthaman *et al*., 1995):

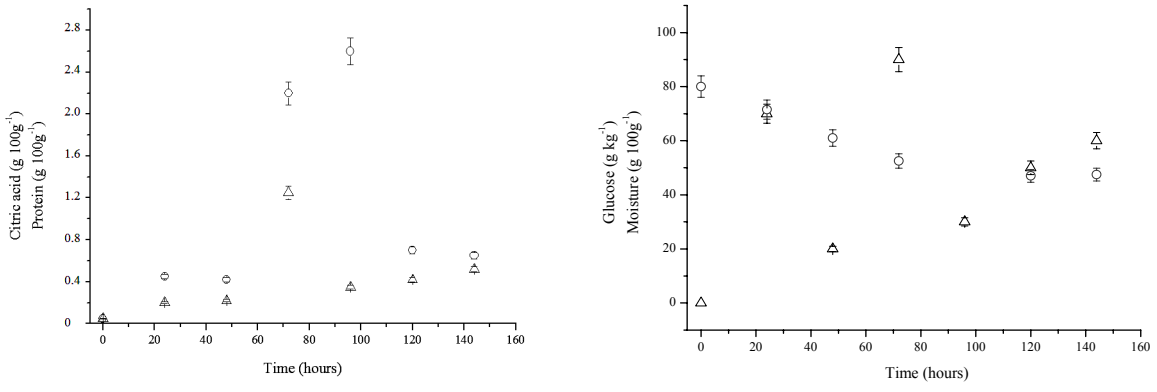
$$N = K_L a(C^* - C_L) \quad (4)$$

RESULTS AND DISCUSSION

Figure 2 shows profiles of citric acid, protein and glucose in crude extract obtained from SSC of *Aspergillus niger* in sugarcane bagasse with vinasse. These results were obtained in experiments using a packed-bed bioreactor that was almost filled, i.e., 150 mm of bed height. Maximum citric acid productivity at 72 hours was 17.4 mg per 100 g of solid medium per hour. This value is about five times lower than that obtained by Kumar and Jain (2008). However, it is important to note that these authors used solid medium containing sucrose, unlike this study where there was only impregnating vinasse.

Angumeenal and Venkappayya (2013) report that *Aspergillus niger* under certain growth conditions accumulates citric acid due to a failure of the Tricarboxylic Acids (TCA)Cycle. The TCA Cycle is an intermediate step involving the final conversion of carbohydrates, proteins and lipids into CO_2 and water, with simultaneous energy generation. Since production is purely enzymatic, this depends on the regulation of the enzymes involved in the TCA Cycle as regards the presence or absence of certain co-factors. Fungi and yeasts require trace amounts of metal ions for their growth. Thus, the production of citric acid by *Aspergillus niger* on sugarcane bagasse could be induced by the availability of metal ions, such as for example manganese, in the vinasse, which is used as the moistening agent of SSC. Vinasse presents non-volatile organic molecules (high molecular weight) that could induce the hydrolysis of structural polysaccharides of bagasse due to the low availability of simple sugars. It is difficult to estimate the yield of citric acid from glucose since, according to Figure 2b, the glucose molecules were initially generated by the hydrolysis of sugarcane bagasse structural polysaccharides (until 72 hours), leading to release and consumption by the fungi, featuring a "*quasi steady-state*" after 120 hours.

The solid medium shows a reduction of moisture during the experimental period, leading to variations of the overall oxygen transfer rate due to changes in the liquid film. Reducing the amount of water in the solid medium tends to reduce the thickness of the liquid film on the surface of the sugarcane bagasse particles. Thus, this effect reduces resistance and improves oxygen transfer, according to Figure 3. From these results, is only possible to estimate the overall oxygen transfer coefficient ($K_L a$) considering a constant gradient of oxygen of 10% in the liquid film (Gowthaman *et al*., 1995). Thus, $K_L a$ was 0.0164 s^{-1}, a lower value that suggest inefficient oxygen availability in this process. However, as it was not possible to calculate the thickness of the liquid film, the calculation of dissolved oxygen concentration in the biofilm from Eq. (3) must necessarily be performed for constant thickness (Gowthaman *et al*., 1995). For a maximum thickness of 25 μm, the variation of oxygen in the film was estimated to be around 4%, which indicates non-limiting oxygen conditions in our moisture range. Nevertheless, according to these results, it can be considered that the variation of moisture is a parameter that indirectly affects microbial growth. Glucose profiles are more complex because there is not only substrate consumption, but also generation of this monosaccharide.



(a) (b)

Figure 2: Profiles of citric acid (Δ), protein (○), glucose (Δ) and moisture (○) during SSC of *Aspergillus niger* in sugarcane bagasse and vinasse using a column with 150 mm packed bed height.

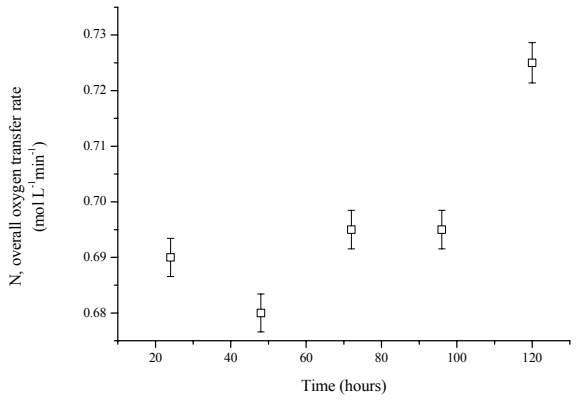
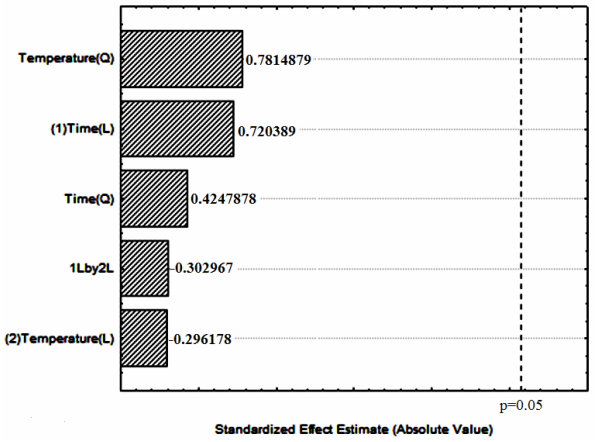


Figure 3: Overall oxygen transfer rate during SSC of *Aspergillus niger* in sugarcane bagasse and vinasse using a column with 150 mm packed bed height.

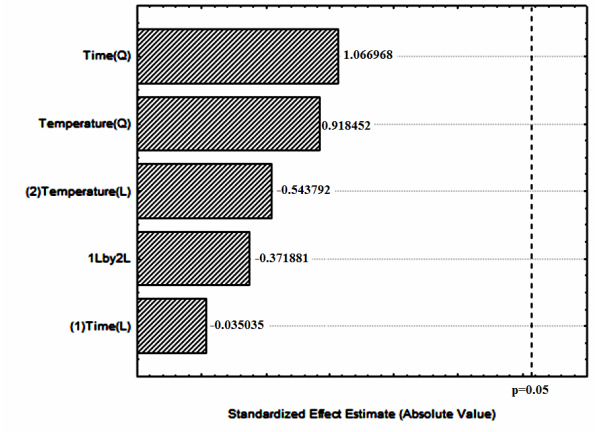
In order to select the optimal conditions for obtaining the crude extract, assays were set up according to an experimental design with variable times of extraction (with water and acetone 50% of the mass) and the incubation temperature. Considering submerged microbial processes, carboxylic acids such as citric, lactic, succinic and itaconic acids, are often recovered after cell removal by using liquid–liquid extraction, adsorption, precipitation or conventional electrodialysis (Pinacci & Radaelli, 2002; López-Garzón & Straathof, 2014). In industrial processes, citric acid is produced using low pH fermentation. After filtering off the cell mass, calcium hydroxide is added to precipitate calcium citrate and thus remove water and impurities. Citric acid is crystallized from the solution. On other hand, liquid–liquid extraction processes using amine extractants have been shown to be a prospective alternative to the conventional calcium salt precipitation process for the recovery of organic acids from aqueous streams (Juang and Chen, 2000). Except for the research of Khoshavi-Darani and Zhogi (2008), the literature is poor about downstream processes in SSC, since the studies follow the recovery steps by crystallization used in conventional citric acid production (Kumar and Jain, 2008). Aiming at future applications, in our experiments it is important to assess how much citric acid and glucose it was possible to extract following the methodology proposed by Khoshavi-Darani and Zhogi (2008). Thus, according to the results of Table 2, it can be seen that both optimal citric acid (40.32 mg L^{-1}) and glucose (12.55 mg L^{-1}) are obtained under the assay conditions 2, or is, 45 min of time for each stage and 28 °C. Higher temperatures and longer times possibly led to degradation of glucose, whereas citric acid is more thermostable (Wyrzykowski *et al.* 2010). However, from the statistical analysis, it is apparent that time and temperature were not significant for extraction yield of citric acid and glucose at the 5% significance level (Figure 4). Regarding the recovery steps (downstream), the conditions tested did not show significant differences, which facilitates the scale-up process, while suggesting that other factors may interfere with the extraction.

Table 2: Results of experimental design for obtaining crude extract in terms of glucose and citric acid (coded values).

Assay	Time of each stage	Temperature	Glucose (mg L^{-1})	Citric acid (mg L^{-1})
1	-1	-1	10.46	28.23
2	1	-1	12.55	40.32
3	-1	1	7.32	32.26
4	1	1	6.28	36.29
5	-1.41	0	4.18	8.06
6	1.41	0	3.14	16.13
7	0	-1.41	2.09	20.16
8	0	1.41	4.18	12.10
9	0	0	2.09	12.10
10	0	0	4.18	16.13
11	0	0	2.09	24.19



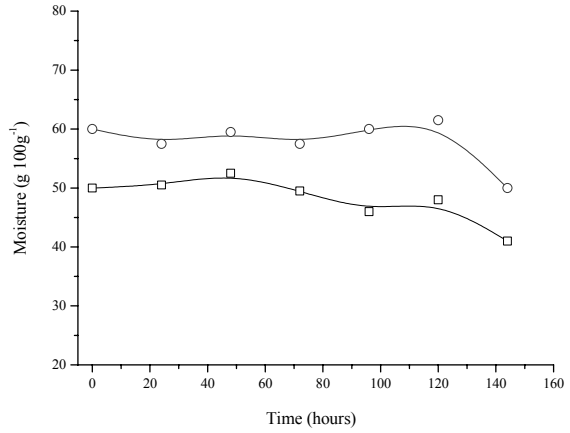
(a)



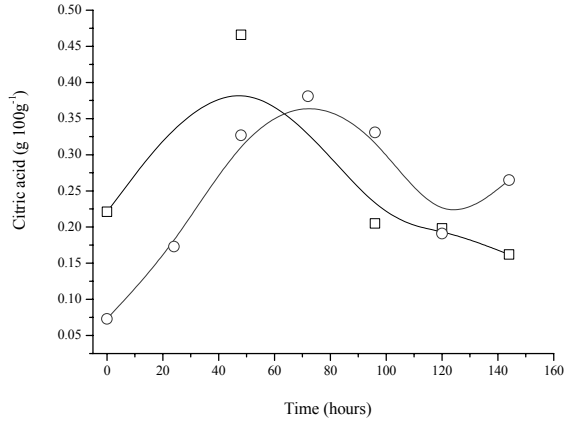
(b)

Figure 4: Pareto Chart of standardized effects on citric acid (a) and glucose (b).

Despite the lower productivity compared to SSC using sugarcane bagasse impregnated with standard medium, assays using vinasse as nutrient solution and moistening agent of the solid medium demonstrate the feasibility of the combined use of these two residues of sugarcane processing. Thus, experiments were conducted at different initial moisture conditions to evaluate the effect of this parameter using a column with 150 mm packed bed height, according to Figure 5. Because the maximum levels of citric acid with 80% initial moisture content were obtained at about 60% moisture, experiments were performed starting with 60 and 50% initial moisture. In fact, the moisture of the solid remained practically constant during the experimental period, i.e., close to the initial value. This indicates that the experimental conditions allowed for the maintenance of the physical characteristics of the SSC, i.e., absence of free water, maintaining the moisture and porosity of the solid bed. This is an interesting differential of this system, considering that in most SSC it is difficult to evaluate the effect of moisture.



(a)



(b)

Figure 5: Profiles of moisture (a) and citric acid (b) at 50 and 60 g 100g^{-1} initial moisture.

Normally, the SSC studies involving selection of initial moisture conditions neglect the unsteady-state of the process. According to our results, the bed height and air-flow ratio used in the packed-bed columns keep the solid moisture practically constant, with no water loss. This suggests maintaining the characteristics of the SSC sampling periods without a limiting effect of oxygen or considerable variation in porosity and thickness of the liquid film.

Figure 5b shows the profiles of citric acid obtained per mass of sugarcane bagasse. For all tested moisture conditions, there are higher yields between 2 and 3 days. Despite this, the maximum citric acid productivity was higher at 80 than 50 or 60 g 100 g^{-1}, which may suggest that the availability of water promotes hydrolysis of polysaccharides of bagasse.

Kumar *et al*. (2003) reported maximum sugar consumption in SSC of *Aspergillus niger* in SSC with fruits waste at 70% moisture level. At 80% moisture level, both citric acid production and sugar consumption were less and these authors suggest that this occurs due to reduced porosity, with poor heat and mass transfer.

According to Lekanda and Pérez-Correa (2004), the mass balance of water in the SSC when the solid substrate is sugarcane bagasse, without addition of water and when evaporation losses are negligible, can be described by Equation (5), where X_W is the solid moisture, R_W is the rate of water production and M_S is the mass of solid medium:

$$\frac{dX_W}{dt} = R_W - \frac{X_W}{M_S}\frac{dM_S}{dt} \tag{5}$$

Considering the mass balance of water for experiments with 80% initial moisture, until the 3rd day of the experiment there was a drop in moisture due to degradation of the bagasse to glucose. Thus, the reaction term of Eq. (5) is lower than the production term, since the microbial growth was not strong and little metabolic water was generated. After this period, the rates of water production and degradation of the solid medium had values that were close, which keeps moisture constant. According to Delabona *et al*. (2013), the highest solid medium moistures lead to a reduction in bed porosity and consequently a limitation of oxygen transfer. However, these authors report that it is difficult to compare trials considering only the effect of initial moisture, because it is a dynamic process. Moreover, SSC is by definition microbial growth on solid matrices in conditions of the absence of free water (Pandey, 2003). Thus, evaluation considering only the initial moisture is erroneous since there is a tendency to accumulate

water from the environment at different batch times, changing the characteristics of the SSC, generating a hybrid system between solid state and submerged cultivations and limiting the mass transfer. Thus, in these experiments the moisture range guaranteed a proper SSC and the characteristics of the absence of free water and, consequently, solid porosity.

Scale-up of batch processes in bioreactor type columns inevitably involves the increase of volume. In the case of aerobic cultures, it is important to assess the optimal height of the bed, since oxygen availability is affected by this parameter and the gas residence time in the column. In this sense, Figure 6 shows the concentrations of citric acid and glucose crude extract for experiments with different bed heights at 3 days of cultivation. The results show a higher citric acid production and glucose consumption in 120 mm of particle bed. This suggests that there is an optimal residence time of air in the bed of particles in this condition. Thus, for low bed heights (low volume of solids), there is a low residence time, limiting fungal growth and production of metabolites. On the other hand, at high bed heights (high volume of bagasse), there is a longer residence time, with possible oxygen-limiting zones. As the production of citric acid by *Aspergillus niger* is extremely dependent on the available oxygen, it can be predicted that 120 mm is the ideal bed height at a 0.4 L min^{-1} airflow rate.

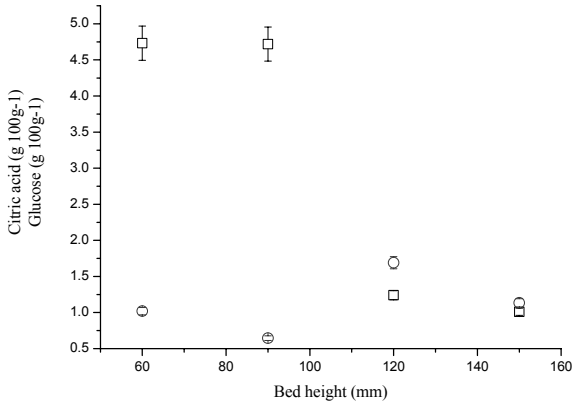


Figure 6: Citric acid (○) and glucose (□) at 3 days-cultivation of *Aspergillus niger* in sugarcane bagasse with vinasse at different bed heights.

CONCLUSIONS

The results show that it is possible to obtain citric acid from the by-products of sugarcane processing bagasse and vinasse. Citric acid production and glucose consumption by *Aspergillus niger* depends of oxygen availability. Moreover, the selection of bed height and air flow is very important to maintain the solid moisture and improve the oxygen transfer in packed-bed bioreactors.

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