Aspects of the Cell Growth of *Candida guilliermondii* in Sugar Cane Bagasse Hydrolysate

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In this work the behavior of the growth of *Candida guilliermondii* FTI 20037 in sugar cane bagasse hemicellulosic hydrolysate on various oxygen transfer rates was investigated. The yeast was able to grow and produced xylitol at different performence levels. At 1.0 vvm (volume of air per volume of medium per minute) the highest growth with 24.4 g/l was observed, but no xylitol was produced. At aeration rate of 0.5 vvm the growth was lower, but therefore slight amounts of xylitol (xylitol yield factor – $Y_{p/s}$ = 0.15 g/g) were observed. The lowest cell concentration (10.7 g/l) and the highest xylitol yield ($Y_{p/s}$ = 0.46 g/g) was observed when aeration was changed from 0.5 vvm to 0.05 vvm after 14 h.

Introduction

Lignocellulosic biomass from agriculture and forestry residues like rice husk, eucalyptus and sugar cane bagasse, are the most abundants organic compounds in the biosphere, with an annual production of approximately 50×10^9 tons (Lutzen *et al.*, 1981). Every year large amounts of waste biomass is accumulated in Nature, which causes great environmental pollution problems and a loss of potential valuable resources. Based on these facts it becomes necessary to find new technologies to use this renewable feedstock in different processes to produce economically valuable products. The biotechnological approach is one way to make this biomass an important substrate for microorganisms for production of several useful feedstocks such as ethanol, xylitol and 2,3-butanediol. Xylitol is a sugar-alcohol of high economical value that can be produced from lignocellulosic biomass by biotechnological means, and presents some important chemical properties such as sweetening power comparable to that of sucrose as well as anticariogenic effects (Aguirre-Zero *et al.*, 1993). Furthermore, the human metabolism of xylitol is insulin independent; therefore it is well suited as a substitute of sucrose in cases of diabetes (Bär, 1986). The use of lignocellulosic residues as substrate in fermentative processes for xylitol production consists initially in releasing sugars from the hemicellulose portion of this biomass through a mild acid hydrolysis process. This process is accompanied by the formation of appreciable amounts of hemicellulose decomposition products, such as furfural, hydroxymethylfurfural, acetic acid and other products derived from lignin degradation (Haying, 1981). These chemicals interfere negatively on the yeast cell growth and in additional xylitol fermentation. Thus, the use of this biomass hydrolysate as a fermentation medium for microorganism growth is critical and several treatments are necessary for removing these products. The performance of the cell growth on this hydrolysate and the xylitol formation depends on the treatment employed and the fermentation conditions used. In this communication we present a simple method of treatment of sugar cane bagasse hemicellulosic hydrolysate and some aspects of *Candida guillermondii* FTI 20037 growth in this biomass under different O_2 conditions, since the oxygen supply is the most important parameter affecting xylose fermentation.

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Material and Methods

Microorganism

The yeast strain *Candida guilliermondii* FTI 20037 was obtained from the Biotechnology Department of the Faculty of Chemical Engineering of Lorena, FAENQUIL, Lorena, S.P. – Brazil. The culture was maintained on yeast agar slants at 4 °C.

Preparation of the sugar cane bagasse hemicellulose hydrolysate

The hemicellulose hydrolysate was obtained by acid hydrolysis of the sugar cane bagasse in a 250 L stainless steal reactor. The sugar cane bagasse was percolated with 10 % H_2SO_4 per dry weight of the bagasse for 20 min at 120 °C with an initial 6:1 liquid:solid ratio. To reach a higher sugar concentration the hydrolysate was concentrated under reduced pressure in a lab-scale evaporator at 70 °C. To remove the toxic components formed by acid hydrolysis, the hydrolysate was treated according to Felipe *et al.* (1993) by increasing the pH of the medium up to 10 using CaO and stirring at room temperature. The precipitate formed was removed by centrifugation at 1000×*g* for 20 min. The pH was then readjusted to 5.5 with concentrated H_2SO_4 .The precipitate was also removed through centrifugation at 1000×*g* for 20 min. The treated hydrolysate was then autoclaved with steam at 100 °C for 20 min, and aseptically supplemented with yeast extract (5 g/l) and $(NH_4)_2SO_4$ (2 g/l). This treated hydrolysate was then used as a fermentation medium for evaluating the performance of *C. guilliermondii*.

Inoculum preparation

The inoculum was grown in synthetic medium containing: 40 g/l D – xylose, 10 g/l D-glucose, 0.1 g/l $CaCl_2$, 2 g/l $(NH_4)_2SO_4$ and 5 g/l yeast extract. A loopful of cells from stock culture was inoculated in 50 ml of this medium in 125 ml Erlenmeyer flasks and incubated at 30 °C for 24 h at 200 rpm in an ETICA Mod. 500 rotary shaker. The cells were harvested by centrifugation at 1000×*g* for 20 min., washed twice with destilled water and resuspended in 10 ml destilled water. The cell concentration was determined and a volume sufficient to give 0.5 g/l cell dry weight was used to inoculate the bioreactor.

Fermentation conditions

Batch fermentation runs were carried out in a 2 l fermenter (New Brunswick Scientific Co, USA) with 4 chicanes and 2 set of disk Rushton turbines with six flat-blade, containing a working volume of 1.0 l of medium prepared as described above. The fermentation system was equipped with temperature and aeration rate controllers and electrodes for pH and dissolved oxygen monitoring were set into the reactor. The temperature were maintained at 30 °C and the agitation rate was set at 300 rpm. The oxygen supply was varied from 0.05 to 1.0 vvm (volume of air per volume of medium per minute) and the oxygen volumetric transfer coefficient (k_La) in all conditions was determined.

Analytical methods

The cell growth was monitored by measuring the culture turbidity at 600 nm with a Micronal digital spectrophotometer Model B 34211. The cell mass was estimated by a correlation between optical density (600 nm) and cell dry weight (1.0 O.D = 1.44 g/l).

Xylose, glucose, arabinose, acetic acid and xylitol were analyzed in a Shimadzu high performance liquid chromatograph (HPLC), using a Bio-Rad Aminex HPX-87 H column at 45 °C and 0.02 N H_2SO_4 as the eluent at a flow rate of 0.6 ml/min.

The volumetric oxygen transfer coefficient (k_La) was determined in the standard fermentation conditions by the gassing-out method as described by Pirt (1975). The dissolved oxygen concentration in the fermentor was first lowered to zero by gassing the medium with nitrogen. The deoxygenated liquid was aerated and agitated and the increase in dissolved oxygen concentration was monitored as a function of the time. From these data the mass transfer coefficient was calculated.

Results and Discussion

Fermentation of hemicellulosic hydrolysate is complex and critical since it contains several compounds toxic to the microorganism. With the treatment employed the toxicity of the sugar cane ba-

gasse hydrolysate was reduced and cell growth, substrate uptake and product formation (xylitol) was observed (Fig.1A-1C). By increasing the pH of the hydrolysate from 0.5 to 10, the Ca^{2+} ions may bind and precipitate toxic factors present in the hydrolysate and improve the further fermentation (Gong and Tsao, 1982). The improvement of the fermentation can also be attributed to the removal of furfural or its conversion to furfural alcohol or reaction of furfural and other hydrolysate compounds like phenolics as suggested by Strickland and Beck (1984). These results are in agreement with those found by Van Zyl (1988) using the xylose-fermenting yeast *Pichia stiptis.*

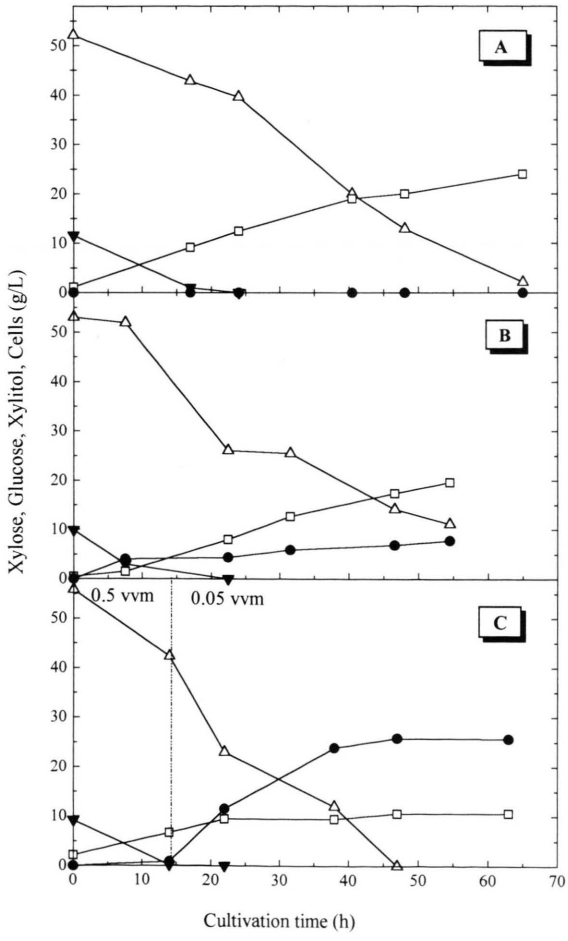

Fig. 1. Effect of aeration rate on cell growth (-□-), sugar consumptions (xylose -△-, glucose -▼-,) and xylitol formation (-●-), by *C. guilliermondii* FTI 20037 grown in sugar cane bagasse hemicellulose hydrolysate. A: K_La = 115 h^{-1}, B: K_La = 43 h^{-1}, C: K_La = 43 → 4 h^{-1}.

According to Fig.1A-1C *Candida guilliermondii* was able to grow, consume the sugars present in the hydrolysate and excrete xylitol with different performances depending on the O_2 conditions employed. The highest final biomass (24.4 g/l) was formed at highest O_2 levels, whereas the lowest growth occurred at conditions where the aeration level was reduced, after 14 h, from 0.5 vvm to 0.05 vvm (Fig. 1C). This fact reflects the importance of oxygen in the utilization of xylose (the major sugar present in the hemicellulosic hydrolysate) by microorganisms, specially by yeasts. Initially xylose was regarded to be nonfermentable to xylitol by yeasts (Barnett, 1976), due to the effect of oxygen on the initial steps of the xylose metabolism. Later the first xylose fermenting yeasts were discovered by Schneider *et. al.*(1981). The yeast strain used in the present work is considered a good xylose fermenting yeast (Barbosa *et al.*, 1988) since this strain presents the major enzymes, xylose reductase and xylitol dehydrogenase, which are the key enzymes for xylose metabolism. From Fig. 2 it can be seen, that the growth of the yeast depends on the formation of xylulose in the initial steps of the xylose metabolism. Xylose is first, through a NADPH depending xylose reductase, reduced to xylitol. Then xylitol is oxidized, through a NAD dependent xylulose dehydrogenase, to xylulose, which is then followed by formation of xylulose-5-phosphate through an ATP dependent xylulose kinase and the further entrance of xylulose-5-phosphate into the pentose phosphate pathway. The formation of ATP and the oxidation of NADH are carried out at the respiratory chain in the mitochondria and it is strongly dependent on oxygen availability. The oxygen transfer rate is fundamental for the regeneration of cofactors, which are essential for xylose metabolism and consequent formation of biomass from this sugar.

In Fig. 1A-1C it can be seen that at an aeration rate of 1.0 vvm (initial k_La =115 h^{-1}) the highest cell growth has been observed, with a final cell concentration of 24.4 g/l. As expected the cell growth was lower at the aeration rate of 0.5 vvm (initial k_La = 43 h^{-1}) with a cell concentration of 19.6 g/l. The lowest growth (cell concentration of 10.6 g/l) was observed for the fermentation where the aeration rate after 14 h was changed from 0.5 vvm (initial k_La = 43 h^{-1}) to 0.05 vvm (representing an initial k_La = 4.0 h^{-1}). In this case growth almost

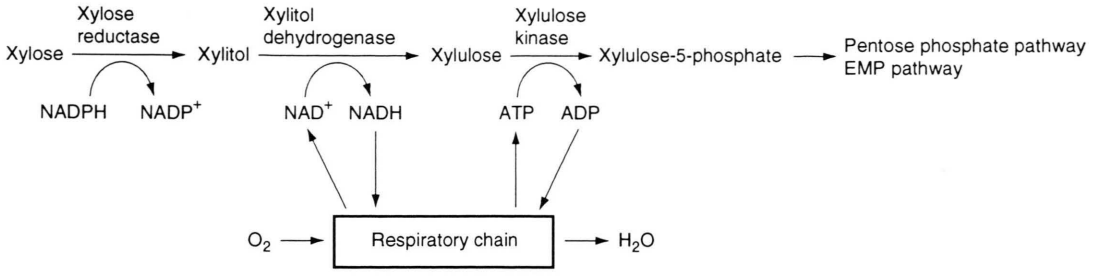

Fig. 2. Scheme of D-xylose fermentation by xylose-fermenting yeasts (Barbosa *et al.*, 1988).

stopped after change. In contrast the production of xylitol increased. While at 1.0 vvm no xylitol production occurred a slight xylitol production was observed at 0.5 vvm ($Y_{p/s}$ = 0.18 g/g). The highest xylitol yield has been achieved in the fermentation were the aeration rate had changed after 14 h. At this fermentation run the xylitol yield was 0.46 g/g which is 50.5 % of the theoretical yield (Barbosa *et al.*, 1988). Grootjen *et al.*, (1991) suggested that at a certain stage in oxygen limited batch fermentation, all available oxygen is used for maintenance processes. This fact may explain the results obtained in the present work. Slinniger *et al.*, (1987) reported that due to the need for mitochondrial function, the rate and extent of biomass formation is dependent on the available oxygen. According to Shook and Hahn-Hägerdahl (1988) the available oxygen has a great influence on xylose fermentation. Under aerobic conditions the organism produces mainly cell mass and under semiaerobic or anaerobic conditions mainly by-products are formed. In our case, xylitol was the major product formed in the sugar cane bagasse hemicellulose hydrolysate fermentation by *Candida guilliermondii* (Fig. 1A-1C) and the xylitol formation was strongly dependent on the oxygen supply.

It can be seen in Fig.3 that the fermentation pH was also affected by the aeration rate. At an aeration rate of 1.0 vvm the pH increased from 5.5 to 7.7 within the first 18 h of fermentation. At the aeration rate of 0.5 vvm the pH almost increased up to the same level, but only after 30 h of fermentation. Under an oxygen-limited condition the pH increased to a pH 6.8 and needed 38 h to reach

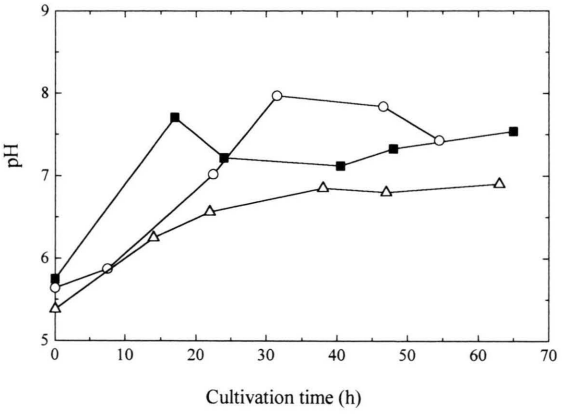

Fig. 3. Change of the pH value during batch fermentation of sugar cane bagasse hemicellulose hydrolysate under different aeration rates. (1.0 vvm -■-; -○- 0.5 vvm; -△- 0.5 → 0.05 vvm).

Table I. Kinetic parameters of sugar cane bagasse hydrolysate fermentation of *Candida guilliermondii* under O_2 conditions.

Aeration rate [vvm]	K_La [h^{-1}]	ΔS [%]	XOH [g/l]	X [g/l]	Yp/s [g/g]	Yx/s [g/g]	δ [g/l·h]	T_F [h]
1.0	115	99.03	–	24.4	–	0.38	–	72
0.5	43	78.96	7.8	19.64	0.15	0.31	0.14	55
*0.5 → 0.05	**43 → 4	100	25.7	10.65	0.46	0.16	0.41	63

ΔS, substrate consumption; XOH, xylitol produced; x, cell dry weight; δ, xylitol productivity; Yp/s, xylitol produced/substrate consumed; Yx/s, biomass produced/substrate consumed; T_F, final fermentation time. * Change on aeration condition after 14 hours of batch fermentation. ** Reduction of K_La after change on aeration condition.

this value. These results are in agreement with those found by van Zyl *et al.*, (1991). According to these authors under aerobic conditions xylose and acetic acid (which are also present in sugar cane bagasse hydrolysate) were consumed simultaneously, but under anaerobic conditions no consumption of this acid was observed. The consumption of acetic acid as a carbon source by *Candida guilliermondii* is a fact observed in previous work (Felipe *et al.*, 1995) and the effect of the acid on cell growth is highly dependent on the fermentation pH. It is known that the toxicity of acetic acid depends on its concentration and strongly interferes with the energy metabolism of yeasts by reducing the H^+ gradient across the mitochondrial membrane used for ATP generation.

According to the results obtained in the present work the pretreated sugar cane bagasse hemicellulosic hydrolysate is a valuable substrate for xylitol fermentation by *Candida guilliermondii* FTI 20037. However, the fermentative parameters of this bioprocess must be optimized in order to achieve higher product concentration and productivity to develop a efficient fermentation process for industrial applications.

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