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Evaluation of sugar cane hemicellulose hydrolyzate for cultivation of yeasts and filamentous fungi

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Sugar cane bagasse hemicellulosic fraction submitted to hydrolytic treatment with 100 mg of sulfuric acid per gram of dry mass, at 140°C for 20 min, was employed as a substrate for microbial protein production. Among the 22 species of microorganisms evaluated, *Candida tropicalis* IZ 1824 showed TRS consumption rate of 89.8%, net cell mass of 11.8 g L^{-1} and yield coefficient ($Y_{x/s}$) of 0.50 g g^{-1}. The hydrolyzate supplemented with rice bran (20.0 g L^{-1}), P_2O_5 (2.0 g L^{-1}) and urea (2.0 g L^{-1}) provided a TRS consumption rate of 86.3% and a cell mass of 8.4 g L^{-1}. At pH 4.0 cellular metabolism was inhibited, whereas at pH 6.0 the highest yield was obtained. The presence of furfural (2.0 g L^{-1}) hydroxymethylfurfural (0.08 g L^{-1}) and acetic acid (3.7 g L^{-1}) in the hydrolyzate did not interfere with cultivation at pH 6.0.

Keywords: xylose; hemicellulosic hydrolyzate; sugar-cane-bagasse; microbial protein

Introduction

The hemicellulosic fraction of sugar cane bagasse can be easily removed by acid treatment and the resulting hydrolyzate is rich in fermentable sugars, mainly xylose. This lignocellulosic material includes about 35% of hemicellulose [6,11]. Hemicelluloses consist of polymeric substances such as xylans and glucomannans, which differ from cellulose in having shorter molecular chains, a homo- or heteropolymeric backbone structure and branch molecules like acetic acid and a variety of pentoses and hexoses [5]. Many of these materials serve as substrates for the production of microbial protein [9].

Brazil is the greatest sugar cane producer in the world [12]. However, technology concerning the utilization of by-products of sugar cane processing still needs to be developed. Each ton of milled sugar cane originates 180–280 kg of bagasse residues, which represents an annual surplus of 5–12 million tons [2]. These residues can be effectively used for generation of energy [2], furfural [4], agglomerate plates [4], fertilizers [13] and xylitol [8]. In order to produce microbial protein from hemicellulosic materials, different types of microorganisms were used, namely *Paecilomyces variotii* [15], *Candida tropicalis* [9] and *Pichia stipitis* [16]. The present study evaluates the sugar cane bagasse hydrolyzate as a cultivation medium for the production of this kind of protein. The growth capacity of 22 different species of microorganisms, including fungi and yeasts, was tested. After selecting the microorganism with the best performance regarding cell mass production and sugar consumption, other variables, such as pH and nutrients, were also studied.

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Materials and methods

Preparation and characterization of the hydrolyzate

Sugar cane bagasse was obtained from Usina Nova América S/A (Assis/SP, Brazil). Acid hydrolysis was performed at 140°C for 20 min after mixing an oven-dried chopped bagasse (solid part) with a sulfuric acid solution (liquid part). The solid-liquid ratio was 1 : 10. The final concentration of sulfuric acid in the hydrolysis suspension was 100 mg per gram of dry mass of sugar cane bagasse. After heating it, the solid residue was separated by filtration and the hydrolyzate was neutralized with commercial calcium oxide and then centrifuged ($1500 \times g$) to remove the solids. Total reducing sugars (TRS), xylose, glucose, acetic acid, furfural and hydroxymethyl-furfural of the resulting hydrolyzate were determined (Table 1). Sugar cane bagasse 'in natura' was also characterized (Table 2).

Chemicals

All chemicals were purchased from Merck (Darmstadt, Germany) and were of analytical grade.

Analytical methods

Total reducing sugars (TRS) were determined as glucose using the Somogyi–Nelson method [14]. Glucose, xylose

Table 1 Partial composition of the sugar cane bagasse hydrolyzate

Parameter	Value
pH	1.0
TRS (g L^{-1})	25.4
Xylose (g L^{-1})	18.5
Glucose (g L^{-1})	5.1
Furfural (g L^{-1})	2.0
Hydroxymethyl-furfural (g L^{-1})	0.08
Acetic Acid (g L^{-1})	3.7

Table 2 Partial composition of the sugar cane bagasse 'in natura' (% w/w of the dry matter)

Component	%
TRS	70.9
Xylose	25.2
Glucose	41.0
Lignin	23.0
Ash	1.1
Moisture[a]	47.8

[a]% w/w of the wet matter.

and acetic acid concentrations were determined by HPLC (HPX-87H Bio-Rad column with a RI 16× detector). Aliquots of 20 μl were analyzed at 45°C with 0.01 N sulfuric acid as the eluent (flow rate of 0.6 ml min^{-1}). Furfural and hydroxymethyl-furfural were analyzed by HPLC (20 μl of sample injected) under the following conditions: RP18HP column, acetic acid : acetonitrile : water solution (1 : 10 : 80 volume ratio) as the eluent with a flow rate of 0.8 ml min^{-1}, temperature of 25°C, UV detector.

Cell mass determination

For determination of the dry weight of cells in the medium, 5-ml samples were used. The yeasts were centrifuged at 1600 × *g* for 10 min, washed twice with 5–10 ml distilled water and then dried to a constant weight at 80°C. Fungi samples from fungal cultures were vacuum-filtered through cellulose acetate membrane filters with 0.45-μm pores (Millipore Corp, Nilford, USA). The mycelial mats were washed twice with 5–10 ml deionized water, placed with the filter membrane in an oven, dried at 80°C and weighed.

Microorganisms and growth conditions

All 18 species of yeasts and three species of filamentous fungi were obtained on malt extract agar slants from Faenquil/Debiq (Lorena, Brazil), Instituto Zimotécnico (Esalq, Brazil) and Instituto Oswaldo MA Cruz (Rio de Janeiro, Brazil). The malt extract agar slants, on which the microorganisms have been kept at 4°C, are renewed every 3 months. The microorganisms were transferred to 125-ml Erlenmeyer flasks containing 25 ml of medium and cultivated, in triplicate, under the following conditions: xylose (20.0 g L^{-1}), peptone (2.0 g L^{-1}), yeast extract (2.0 g L^{-1}), KH_2PO_4 (2.0 g L^{-1}), $(NH_4)_2HPO_4$ (2.0 g L^{-1}) and $MgSO_4 \cdot 7H_2O$ (1.0 g L^{-1}); 30°C; pH 5.5; 200 revolutions min^{-1} of agitation in a rotary shaker. Cultivation of yeasts and fungi was carried out for 48 and 98 h, respectively. The yeasts were separated by centrifugation at 1600 × *g* for 20 min and the fungi were filtered and used for preparing a suspension in sterile water. The volume of inoculum for each strain was determined in order to obtain an initial cell concentration around 1.0 g L^{-1} (dry weight). A selection of the microorganisms was made in two steps. In the first step xylose was replaced by sugar cane hemicellulose hydrolyzate (Table 3). In the second step, the yeast extract and peptone were replaced by rice bran (20.0 g L^{-1}) as follows: rice bran produced in the region of Lorena, Brazil was added to the hydrolyzate, autoclaved at 112°C for 15 min and centrifuged (1600 × *g*) for 10 min. The supernatant phase after the addition of salts, was placed in 250-ml Erlenmeyer flasks and used as the cultivation medium. The flasks, containing 55 ml of medium and inoculum, were incubated in a rotary shaker at 30°C and 200 revolutions min^{-1} of agitation. After 48 h, yeast samples were collected and centrifuged (1600 × *g*) for 10 min and fungi samples were filtered. Samples of the supernatant medium or filtrate were used for determining the TRS remaining, and pellet or retentate samples for determining cell mass.

Three pH values (4.0, 5.0 and 6.0) were tested under the following conditions: sugar cane hemicellulose hydrolyzate, rice bran (20.0 g L^{-1}); KH_2PO_4 (2.0 g L^{-1}), $(NH_4)_2HPO_4$ (2.0 g L^{-1}) and $MgSO_4 \cdot 7H_2O$ (1.0 g L^{-1}); 30°C; 200 revolutions min^{-1} on a rotary shaker. A nutritional supplementation of the hydrolyzate was made by varying the concentration of the medium components and by using pH 6.0 (Table 4).

Results and discussion

In the first step of the selection of microorganisms TRS consumption rate was used as an efficiency criterion. The microorganisms consuming more than 80% of the TRS were selected for the second step. *Candida tropicalis* IZ 1824 and *C. tropicalis* IZ 1958 consumed 93.5% of the TRS and showed the highest efficiency. *Kluyveromyces marxianus* FTI 20015, *C. guilliermondii* FTI 20037 and *K. marxianus* FTI 20014 consumed 85.2, 84.9 and 80.8%, respectively. Some microorganisms herein studied, for instance *Paecilomyces variotii* IZ 1556, showed low sugar consumption. The differences in behaviour concerning sugar consumption and cell growth indicate that the microorganisms cultivated in hemicellulosic hydrolyzate exhibit different degrees of tolerance for toxic components (furfural, hydroxymethyl-furfural and acetic acid) generated during the acid hydrolysis (Table 1). The degrees of toxicity of these compounds depend on the microorganisms. Some yeasts are able to reduce the amount of furfural and, consequently, its inhibitory effect [7].

In the second step, yield and cell mass production were also adopted as criteria. Two of the nutrients (yeast extract and peptone) used in the first step of the cultivation medium were replaced by rice bran, which is a source of amino acids and vitamins. The microorganisms with the best performances were the same as in the first step (Table 3). The performances of *C. tropicalis* IZ 1824, *C. tropicalis* IZ 1958 and *K. marxianus* FTI 20015 decreased by less than 5%, whereas the performances of *C. guilliermondii* 20037 and *K. marxianus* FTI 20014 decreased by 13.7 and 16.8%, respectively. The yields ($Y_{x/s}$) obtained were higher than those obtained by Barbosa *et al* [1] when they cultivated these microorganisms in synthetic medium with xylose as a carbon source.

C. tropicalis IZ1824 was selected to continue the experiments. The influence of the initial pHs (4.0, 5.0 and 6.0) on cell growth and total reducing sugar consumption was studied. The results are shown in Figure 1. There was neither growth nor substrate consumption at pH 4.0. This observation is not in accordance with the results of some authors [7] who observed that yeasts are able to grow at pHs between 3.2 and 6.0. On the other hand, Tauk and

Table 3 Remaining TRS (%), dry cell mass (g L^{-1}) and yield ($Y_{x/s}$) after growing the microorganisms in sugar cane hemicellulosic hydrolyzate: first and second steps of screening

Microorganism	1st step	2nd step		
	TRS (%)	TRS (%)	Cell mass (g L^{-1})	$Y_{x/s}$[b] (g g^{-1})
Candida tropicalis IZ 1824	6.5 A[a]	10.2 A[a]	11.8 A[a]	0.50
Candida tropicalis IZ 1958	6.5 A	10.8 A	11.5 A	0.49
K. marxianus FTI 20015	14.8 B	17.6 A	9.8 A	0.42
Candida guilliermondii FTI 20037	15.1 B	28.8 B	7.4 B	0.31
K. marxianus FTI 20014	19.2 C	36.0 B	4.9 C	0.21
Pachysolen tannophilus FTI 20067	22.9 D			
Candida krusei FTI 20043	72.4 E			
Paecilomyces variotii OC 3764	76.2 F			
Torula utilis FTI 20040	76.7 F			
K. marxianus FTI 20115	76.9 F			
Candida guilliermondii IZ 1422	82.8 G			
Candida tropicalis FTI 1004	84.2 G			
Candida guilliermondii IZ 1231	87.2 H			
Hansenula anomala FTI 20104	90.0 I			
Candida intermedia FTI 20103	91.0 I J L			
Aspergillus niger 10V10	92.0 I J L			
Candida utilis FTI 20109	92.5 I J L			
Candida guilliermondii IZ 1823	93.5 J L M			
Candida tropicalis FTI 20106	94.6 L M			
Candida guilliermondii IZ 1322	95.6 M N			
Pichia stipitis FTI 20110	97.5 M N			
Paecilomyces variotii IZ 1556	0 O			

[a]Averages of three analyses represented by the same letter do not differ on a 5% probability level.
[b]$Y_{x/s}$ was calculated as grams of dry cell mass produced per gram of TRS (glucose + xylose) used.

Table 4 Remaining TRS (%) and cell mass (g L^{-1}) after culturing *Candida tropicalis* IZ 1824 in sugar cane hemicellulosic hydrolyzate supplemented with other nutrients under the conditions: initial pH 6.0; temperature 30°C and agitation 200 revolutions min^{-1}

Medium	H	A	B	C	D	E	F	G	Remaining TRS (%)	Cell mass (g L^{-1})
1	+								54.6	5.5 D[a]
2	+	+							20.5	7.0 C
3	+				+				10.1	7.7 BC
4	+	+			+				2.9	7.4 BC
5	+		+	+					45.2	5.9 D
6	+	+	+	+					52.5	5.9 D
7	+	+	+		+				7.4	7.1 C
8	+	+		+	+				6.4	7.1 C
9	+	+	+	+					12.5	8.6 A
10	+	+	+	+	+				6.8	8.7 A
11	+					+			13.5	7.6 BC
12	+						+		27.6	7.4 BC
13	+							+	16.7	7.8 B
14	+					+	+		13.7	8.4 A

[a]There are no differences among the average of three values represented by the same letters on a 5% probability level.
H – Hemicellulosic hydrolyzate
A – Rice bran (20 g L^{-1})
B – $MgSO_4$ (1.0 g L^{-1})
C – KH_2PO_4 (2.0 g L^{-1})
D – $(NH_4)_2HPO_4$ (2.0 g L^{-1})
E – P_2O_5 (2.0 g L^{-1})
F – Urea (2.0 g L^{-1})
G – $(NH_4)_2SO_4$ (2.0 g L^{-1})

Gambale [17] affirm that the optimum cultivation pH depends on the medium. The hydrolyzate employed in our experiments contains some toxic compounds and their negative effects on *C. tropicalis* increase at pH 4.0 [17]. The level of toxicity of acetic acid depends on the pH. At a low pH this acid is not dissociated and freely diffuses through the yeast cytoplasm. As a consequence the inner cell pH decreases [10]. At higher pHs (5.0 and 6.0) the cell membrane is impermeable to the dissociated acetic acid. The amounts of dry cell mass produced were 8.5 at pH 5.0 and 9.4 g L^{-1} at pH 6.0, while TRS consumption rates were 93.4 at pH 5.0 and 93.2% at pH 6.0. Similar results were reported by Chahal *et al* [3] who found that around pH 6.0 the inhibitory effects of the toxic compounds are minimized.

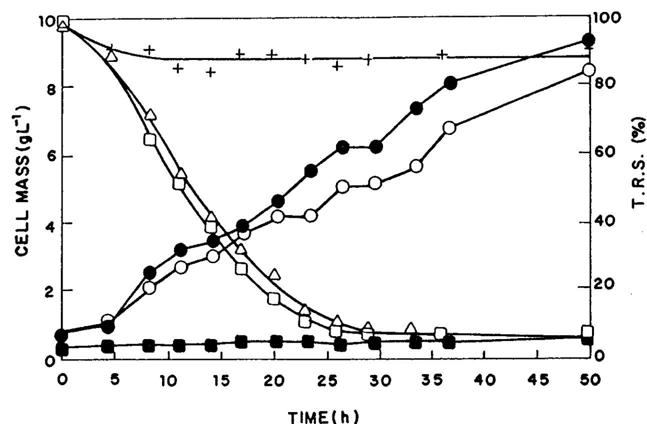

Figure 1 Cell mass (g L^{-1}) at pH 4.0 (-■-), pH 5.0 (-○-), pH 6.0 (-●-) and TRS concentration (%) at pH 4.0 (-+-), pH 5.0 (-△-), pH 6.0 (-□-) *vs* time in cultivation of *C. tropicalis* IZ 1824 carried out under the following conditions: sugar cane bagasse hemicellulose hydrolyzate containing; rice bran (20.0 g L^{-1}); $MgSO_4 \cdot 7H_2O$ (1.0 g L^{-1}); $(NH_4)_2HPO_4$ (2.0 g L^{-1}); 30°C and 200 revolutions min^{-1} of agitation.

After defining pH 6.0 as the most appropriate for *C. tropicalis* cultivation, the influences of the concentrations of the medium components on cell performance were evaluated. As shown in Table 4, media 9, 10 and 14 provided the highest efficiencies. This shows that the hydrolyzate does not need supplementation with Mg^{2+}, K^{+}, microelements and vitamins. Likewise the nitrogen source did not influence cell growth and TRS consumption rate.

Using hydrolyzate without nutritional supplementation (medium number 1) cell mass reached 5.5 g L^{-1} and 45.4% of the TRS was consumed during cultivation. The data suggest that nutrient(s) became limiting in the hydrolyzate, eg nitrogen and phosphate. The necessity of phosphorus supplementation can be explained by the acid hemicellulosic hydrolyzate neutralization. This reaction occurred using calcium oxide and promoted removal of phosphate ions from the medium [7]. Medium number 14 (Table 4) is considered the most advantageous, since it is less expensive.

Conclusions

The potential of sugar cane bagasse hemicellulosic hydrolyzate as a substrate for microbial growth was demonstrated. Sugar cane hemicellulosic bagasse can be efficiently hydrolyzed and the sugar solution can be used by several microorganisms as a cultivation medium to obtain commercial biotechnological products. Compared to other microorganisms tested, *C. tropicalis* IZ 1824 showed a better performance in medium supplemented with phosphate and nitrogen.

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