CONTINUOUS ETHANOL PRODUCTION USING YEAST IMMOBILIZED ON SUGAR-CANE STALKS

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Abstract - Sugar-cane stalks, 2.0 cm long, were used as a support for yeast immobilization envisaging ethanol production. The assays were conducted in 38.5 L fermenters containing a bed of stalks with 50% porosity. The operational stability of the immobilized yeast, the efficiency and stability of the process, as well as the best dilution rate were evaluated. Molasses from demerara sugar production was used in the medium formulation. It was diluted to obtain 111.75 ± 1.51 g/L without any further treatment. Sulfuric acid was used to adjust the pH value to around 4.2. Every two days Kamoran HJ (10 ppm) or with a mixture containing penicillin (10 ppm) and tetracycline (10 ppm), was added to the medium. Ethanol yield and efficiency were 29.64 g/L.h and 86.40%, respectively, and the total reducing sugars (TRS) conversion was 74.61% at a dilution rate of 0.83 h^{-1}. The yeast-stalk system was shown to be stable for over a 60 day period at extremely variable dilution rates ranging from 0.05 h^{-1} to 3.00 h^{-1}. The concentration of immobilized cell reached around 10^9 cells/gram of dry sugar-cane stalk when the fermenter was operating at the highest dilution rate (3.00 h^{-1}).
Keywords: alcoholic fermentation, continuous fermentation, yeast, sugar-cane stalks, yeast immobilization, ethanol.

INTRODUCTION

Most of the ethanol produced in Brazil is manufactured using the old, discontinuous, Melle-Boinot process. Some disadvantages of this method of ethanol production are the high fermenter capacity required; the variations in medium composition during the process; the occurrence of unproductive steps, i.e., charge and discharge; and the need for continuous centrifuging for yeast recycling (Carvalho Neto et al., 1990).

Time of fermentation, ethanol yield, and fermentation efficiency improved with optimization of the fermentation process and new technologies implemented in the National Alcohol Program (PROÁLCOOL) (Amorim, 1982; Stupiello, 1984). Fermentation time was reduced from 18-24 hours to 5-8 hours and process efficiency increased from 70-80% to 88-92%. In spite of these improvements, the existence of unproductive time resulted in low yields. Only 30% of all the alcohol produced in Brazil during that decade was produced by the

continuous process system (Andrietta and Stupiello, 1990).

As a general solution to reduce production costs and improve process efficiency and ethanol yield, the continuous fermentation method is the most appropriate, no matter which process we choose: free cells, flocculent cells, or immobilized cells.

When compared with processes using immobilized cells, alcoholic fermentation using free cells offers some advantages: the larger area of contact between cells and nutrient medium and the management of current technology. However, disadvantages include the higher costs of microbial recycling and installation, high contamination risks, susceptibility to environmental variations, and the limitations of the dilution rate in continuous fermentation due to wash out.

The supports for immobilization discussed in those studies seem to be infeasible for the process of industrial ethanol production. An important consideration is the absence of an abundant supply in the producing area; in most cases it must be imported.

In previous work (Vasconcelos et al., 1998), results were presented on the use of sugar-cane stalks, as a support for yeast cells in alcoholic fermentation as well as the best immobilization technique. The stalks were demonstrated to be satisfactory for 220-consecutive-day cycles, a period much longer than the sugar-cane harvest.

In Brazil the efficiency of industrial fermentation has already reached levels that will be difficult to surpass without further technological innovation. Instrumentation and process control, utilization of flocculent yeast, and processes with immobilized cells will have important roles in this technological evolution.

Adoption of the continuous process of alcoholic fermentation using flocculent yeast has been shown to be an alternative, and it is already being employed in industrial units in several ethanol-producing regions (Carvalho Neto et al., 1990). This form of conduction eliminates the need for centrifuges, whose installation and maintenance are onerous. The current advantages, derived from elimination of the separation stage, remain when this process is compared to the free-cell process. However, when compared to the continuous fermentation process using immobilized yeast, it entails additional expenses for the equipment necessary for sedimentation and recycling of the microorganisms responsible for the alcoholic fermentation.

Based on the available literature, it is evident that to achieve greater savings on ethanol production by fermentation, it is necessary to have a continuous process without the need for cell recycling. An economical system, which incorporates lower fixed and operational costs, would probably reduce production costs. The elimination of continuous centrifuges would bring additional savings in the construction and operation of industrial unit. The development of immobilized-cell processes, using low-cost support and low operational immobilization, and the use of identical fermentation media that are similar to those used in Brazilian industrial facilities would be fully justified.

MATERIALS AND METHODS

Organism Employed

Pressed Fleischmann yeast, always acquired one day prior to the experiment and kept in the refrigerator until the time of use. The cell suspensions used in all the experiments had about 2×10^8 yeast cells/mL.

Fermentation Conditions

Molasses, extracted in the production of demerara sugar, diluted to 111.67 ± 1.51 g/L TRS. The pH factor was adjusted by addition of sulfuric acid to 4.21 ± 0.14. Both urea and triple superphosphate were added to the molasses medium at concentrations of 0.5 g/L. Kamoran HJ (10 ppm) and V-acid penicillin (10 ppm) + tetracycline (10 ppm) were used as antimicrobial agents by alternating them at two-day intervals.

Support for Cell Immobilization and Fermentation Process

Sugar-cane stalks, 2 cm long, 2.57 ± 0.34 cm in diameter, and with a density of 1.02 ± 0.01 g/cm^3, picked at the moment of immobilization. The bed density was similar to the peeled stalk density (1.02 ± 0.01 g/mL). Average porosity of the bed, formed of peeled, 2 cm long stalks was $49.26 \pm 2.34\%$.

The fermenter configuration used is shown in Figure 1. It basically consisted of two reservoirs for the fermentation medium [1]. While one was in operation, the other was being cleaned. The two reservoirs were alternated every 12 hours, independently of the dilution rate. These reservoirs fed, continually and by gravity, a third reservoir [1a], Which contained a buoy [2] to maintain the level. In this third reservoir [11], it was possible to control the fermenter's feed flow with a globe valve.
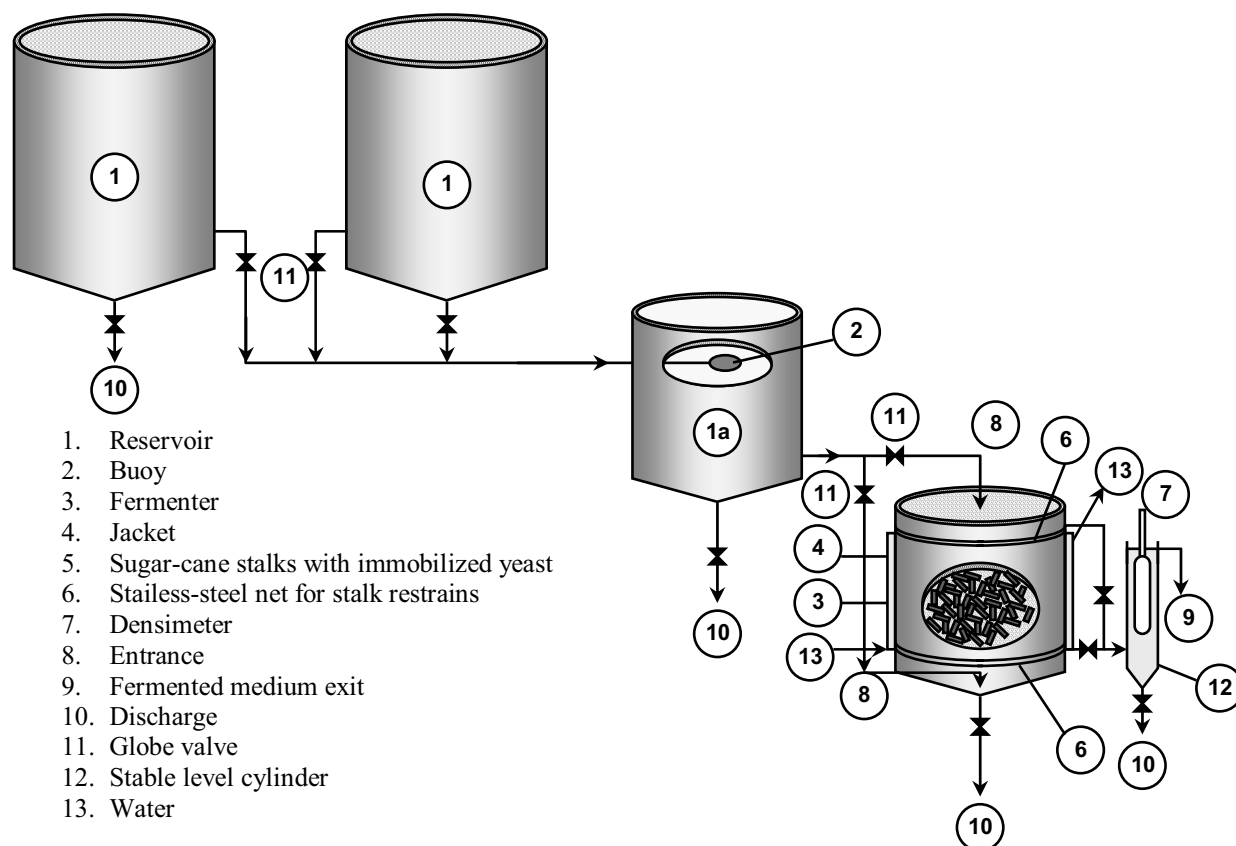
Figure 1: Flow of the continuous alcoholic fermentation process with yeast immobilized on sugar-cane stalks.

1. Reservoir
2. Buoy
3. Fermenter
4. Jacket
5. Sugar-cane stalks with immobilized yeast
6. Stailess-steel net for stalk restrains
7. Densimeter
8. Entrance
9. Fermented medium exit
10. Discharge
11. Globe valve
12. Stable level cylinder
13. Water

The temperature, kept around $33 \pm 1 \,^{\circ}C$, was reached by a refrigerated jacket [4]. A reservoir connected to the fermenter [12] was used for in-line quantification of total diluted solids, measured with a Brix densimeter [7]. The strong linear correlation between total reducing sugars (TRS) content and Brix enabled an ideal TRS content to be fixed in the fermentation medium. The top and bottom fermenter screens [6] were used to keep the bed packed [5]. The dilution rates varied from very low value of $D = 0.05 \, h^{-1}$ to a very high value of $D = 3.0 \, h^{-1}$. The working volume was of 38.5 L.

Analytical Determinations

TRS was estimated according to the methodology described by ICUMSA (1994), and the content of total diluted solids was estimated using a Brix densimeter. Ethanol concentrations were determined by gas chromatography, using a CG-3537D gas chromatograph manufactured by Instrumentos Científicos CG LTDA with a flame ionization detector and a CG-300 integrator. The pH was determined with a B272 pH digital meter, manufactured by MICRONAL.

Yeast Cell Counts

The cells were quantified according to the methodology suggested by Lee et al. (1981) using a methylene blue solution, counted with a Neubauer camera. To count the yeast cell in the stalks, 50g of stalks containing the immobilized microorganisms were ground at 3470 rpm for 5 minutes in 1 L of purified water under aseptic conditions, using appropriate equipment (Figure 2). The cells were thereby freed and identified in the residual medium.
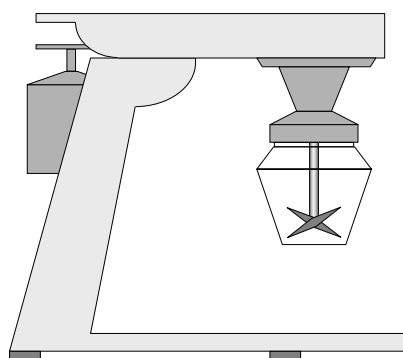
Figure 2: Equipment used for grinding stalks.

RESULTS AND DISCUSSION

The microbial data refers to the stalks containing the immobilized yeast and to the fermentation medium (Tables 1 and 2). The number of viable cells and total cells immobilized/g of dry sugar-cane stalk, about 3×10^9, remained approximately constant at all the dilution rates studied. The concentration of viable cells and total cells/mL in suspension in the fermentation medium reached values on the order of 4×10^7.

The cells from the cane stalks had values lower than 10^7, taking into consideration the free yeast from molasses. Therefore, the effluent losses can be considered small and easily compensated for by cell multiplication.

The result for concentration of cells was similar to that obtained in many industrial alcoholic fermentation processes (2 to 6 $\times 10^8$ live cells/mL of fermentation medium) working with free cells and continuous or batch processes.

The number of cells retained in the stalks was similar to those obtained by Tyiagi et al. (1992) when using sugar-cane bagasse, 0.445 g of treated bagasse cells/g and 0.346 g of untreated bagasse cells/g.

For $D = 0.33$ h^{-1} and converting the total number of yeast cells to dry mass, a value of 0.4776 g of dry cells/g of dry stalk was obtained. This value is higher than that obtained by Tyiagi et al. (1992) using sugar-cane bagasse.

Table 1: Microbial parameters in the steady-state process of continuous alcoholic fermentation with yeast immobilized on sugar-cane stalks (dry stalks).

D (h^{-1})	Dry stalks			
	CV/g ($\times 10^9$)	CT/g ($\times 10^9$)	Brot (%)	V.C. (%)
0.05	1.04	1.27	3.94	81.89
0.06	1.32	1.62	7.36	81.48
0.07	1.35	1.66	4.77	81.32
0.11	2.11	2.98	6.43	70.80
0.13	2.27	3.27	4.06	69.41
0.14	2.49	3.51	3.40	70.94
0.20	2.69	3.84	3.96	70.05
0.23	2.80	4.07	4.81	68.80
0.33	3.37	4.77	4.75	70.65
0.43	3.37	4.31	5.36	78.19
0.54	2.61	3.52	4.87	74.15
0.70	2.66	3.49	7.66	76.22
0.83	2.43	3.39	5.17	71.68
1.25	2.20	3.03	4.75	72.60
1.50	1.54	2.62	7.87	58.78
1.98	1.46	2.51	8.38	58.17
3.00	1.41	2.63	7.34	53.61

D-dilution rate; CV-live cells; CT-total cells;
Brot-budding; V.C.-Cell viability. All results are an average of three repetitions.

Table 2: Microbial parameters in the steady-state process of continuous alcoholic fermentation with yeast immobilized on sugar-cane stalks (fermented medium).

D (h^{-1})	Fermented medium			
	CV/mL (x10^7)	CT/mL (x10^7)	Brot (%)	V.C. (%)
0.05	0.17	0.18	7.80	94.44
0.06	0.64	0.68	7.96	94.12
0.07	0.83	0.86	7.51	96.51
0.11	2.69	2.81	7.09	95.73
0.13	4.43	4.59	9.35	96.51
0.14	2.64	2.75	7.20	96.00
0.20	3.05	3.21	5.63	95.01
0.23	2.40	2.57	7.71	93.38
0.33	6.52	6.84	5.81	95.32
0.43	2.76	2.87	8.33	96.17
0.54	2.98	3.14	5.24	94.90
0.70	4.04	4.25	6.75	95.06
0.83	5.44	5.88	8.16	92.52
1.25	5.16	5.32	6.41	96.66
1.50	4.98	5.28	6.73	94.32
1.98	2.45	2.65	8.42	92.45
3.00	2.22	2.38	8.82	93.28

D-dilution rate; CV-live cells; CT-total cells;
Brot-budding; V.C.-Cell viability. All results are an average of three repetitions.

The viability of free cells at all dilution rates (about 95%) remained basically constant; a similar behavior was seen in the cell budding (about 7%).

The budding of immobilized cells was similar to that which occurred in free cells. However, the cell viability maintained was constant (about 81.5%) for very low dilution rates (D = 0.05 h^{-1} to 0.07 h^{-1}). It became stable at a lower rate of around 70% for a wide range of dilution rates (D = 0.11 h^{-1} to D = 1.25 h^{-1}) and, from that point on, showed a decreasing trend according to dilution rate. These trends were probably due to the effect of substratum and product diffusion through the support.

Walsh et al. (1993) assumed that a microbial population grows in a heterogeneous way, multiplying more quickly at the surface, given its easy access to the substratum and other nutrients to the detriment of cells located inside the calcium alginate sphere. Eventually, a dense external biomass layer is formed around a dispersely populated nucleus. The authors attributed this heterogeneous cell distribution inside the support to the diffusion effects of the substratum and the product through the gel particles, which contributes decisively to the alteration of intra-support cell viability. Given the internal and complex structures of this natural support, the distribution of cells on the sugar-cane stalk would also be heterogeneous.

One of the big problems related to the process of alcoholic fermentation with immobilized cells is the large CO$_2$ detachment. In this research, this phenomenon was not considerable due to the 1:1 height to diameter ratio of the fermenter dimensions and to the decreasing flow rate of the fermentation medium. The high porosity of the bed containing immobilized yeast (49.26±0.70%) might be another factor minimizing problems arising from the considerable CO$_2$ detachment during alcoholic fermentation.

In this research the population of contaminated bacteria was maintained at very low levels, by addition of Kamoran HJ (10 ppm) and Tetracycline (10 ppm) + V–acid penicillin (10 ppm), alternated at two-day intervals. Prince and Barford (1982) eliminated the contamination on the tubular base of the fermenter by reducing the pH factor from 4.0 to 3.5 and increasing the dilution rate.

The low pH value slows bacterial growth without damaging immobilized cells because these systems are not sensitive to variations in pH (Buzás et al., 1989). Fast flow can be used to remove contaminating bacteria without dragging the immobilized cells, as illustrated in Tables 1 and 2.

The data obtained from five of a total of 17 dilution rates (Figures 3 to 7) shows the stages corresponding to transient and permanent regimes. The first was characterized by oscillations in cell concentration, in substratum and ethanol, because in this stage the system reacts to the new dilution rate and oscillates until reaching a new level of equilibrium. The second stage was characterized by constant parameters.
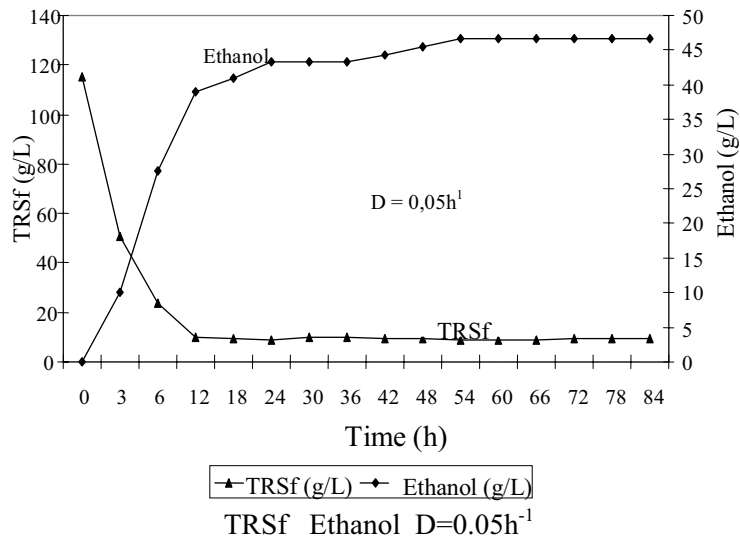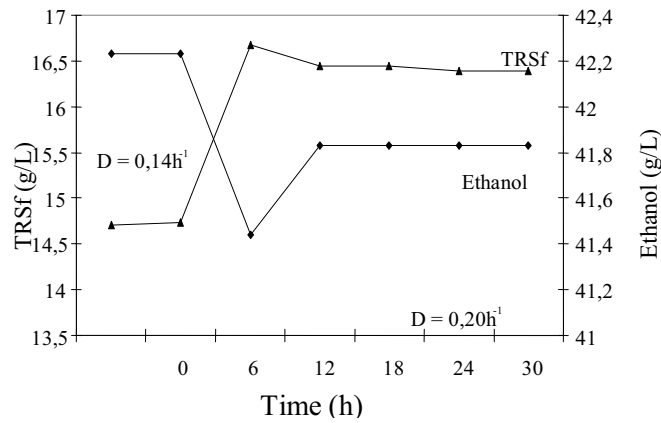
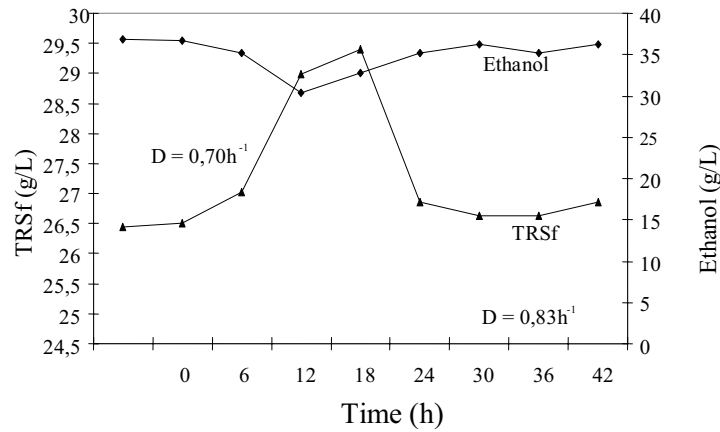**Figure 3:** Continuous alcoholic fermentation with immobilized yeast on sugar-cane stalks at D = 0.05 h^{-1}.
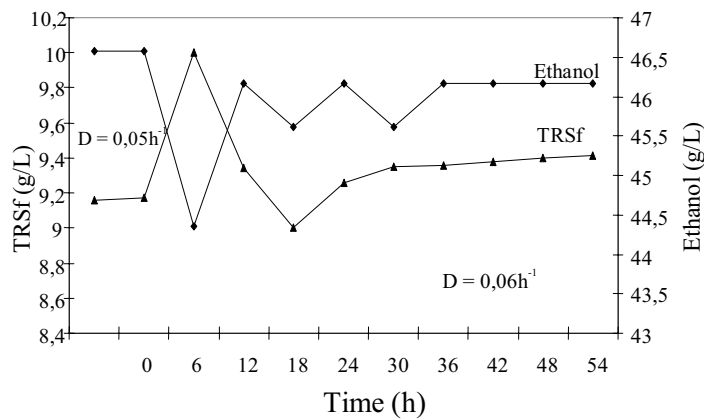
Figure 4: Continuous alcoholic fermentation with immobilized yeast on sugar-cane stalks at D = 0.06 h^{-1}.
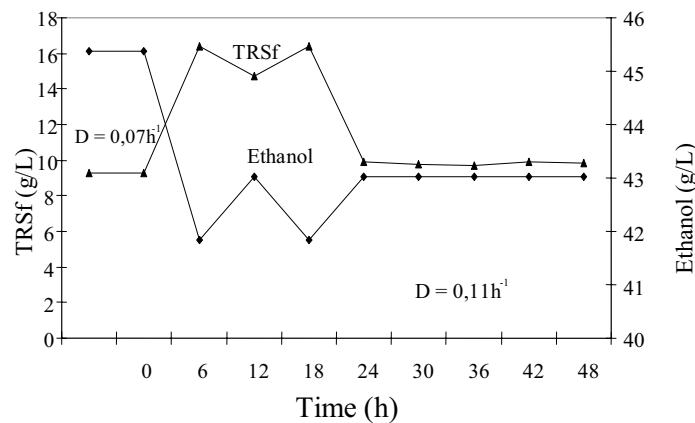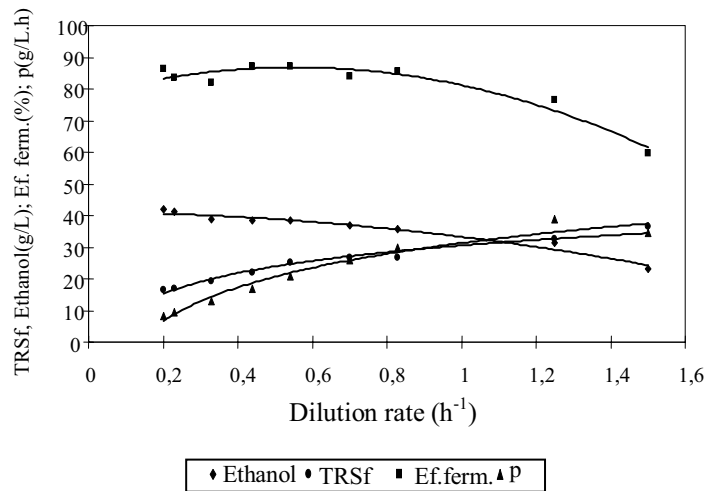

Figure 5: Continuous alcoholic fermentation with immobilized yeast on sugar-cane stalks at D = 0.11 h^{-1}.

Figure 6: Continuous alcoholic fermentation with immobilized yeast on sugar-cane stalks at D = 0.20 h^{-1}.

Figure 7: Continuous alcoholic fermentation with immobilized yeast on sugar-cane stalks at D = 0.83 h^{-1}.

Figure 8: Evaluation parameters of continuous alcoholic fermentation with yeasts immobilized on sugar-cane stalks for dilution rate function.

The fermentation efficiency values (Figure 8) were around 85% and were stable up to a dilution rate of 0.83 h^{-1}. There were small variations in the ethanol concentration of 36 to 40 g/L. A sharp drop was observed when the dilution rate exceeded 0.83 h^{-1}. At 0.83 h^{-1}, productivity was 29.64 g/L.h in relation to the volume of the medium, the residual reducing substances reached 26.74 g/L, and fermentation efficiency was 85.56%.

It's pertinent to point out that some of the reduced substances quantified by the adopted method are not fermentable. Considering this fact and the microorganisms' metabolic efficiency, it is assumed that the most appropriate dilution rate was 0.83 h^{-1}, despite the high concentration of TRS in the fluid. This dilution rate can be the starting point for improvement of the process on a pilot or industrial scale.

Maximum yield was obtained with a dilution rate of 1.25 h^{-1}; however, a large drop in fermentation efficiency was also observed as well as a large reduction in ethanol concentration and a residue with significant amounts of reducing substances.

These consequences can be explained by the increase in the dilution rate, causing the system to react to reach a new equilibrium, or steady-state. However, when the dilution rate increases excessively and the sugars are added at a speed faster than the speed of the substratum feeding, the loss of sugar increases and the ethanol concentration decreases, as does efficiency.

Working with sugar-cane bagasse and a clarified molasses medium (Tyiagi et al., 1992) obtained a maximum yield of 31.8g ethanol/L.h with 200 g/L of initial TRS and a D of 0.53 h^{-1}, maintaining the operational system stable for 76 days.

The fermentation efficiencies and yield obtained in several studies are difficult to compare due to the wide diversity of conditions during the fermentation process.

On an industrial scale, yields from continuous fermentation and free cells, varying from 4 to 8 g/L.h, are mentioned, when fermentation medium with 140 to 160 g/L of total sugars, a fermentation efficiency of 80 to 85%, a cell concentration from 10 to 12 g/L and a residence time of 5 to 8 hours are used (Manual..., 1990). Adopting flocculent yeast, the industrial processes using 12 to 20% (p/v) initial TRS, a pH of 3.6 to 4.0 and a concentration of cells in the fermenter of 10-12% (v/v) obtained productivities of around 12.34 g/L.h and fermentation efficiencies of around 89.00% (Carvalho Neto et al., 1990).

Continuous production is more advantageous than the batch fermentation process due to the unproductive time (charging, discharge, cleaning, etc.) in each fermentation cycle, which is about 50% longer than the time necessary for alcoholic fermentation. In the case of continuous alcoholic fermentation, unproductive time was not noticed. In spite of the high fermentation efficiencies (around 88-90%) and low fermentation times (5 to 8 hours), yield is very low.

In Brazilian alcohol industries, residence time varies according to the process and to the area used, depending on the technologies appropriate for the process. It can be estimated that residence time generally varies between 8 and 10 hours wich is considerable better than values obtained in this study. The fermentation efficiencies are quite compatible with batch process a well-conducted. The difference lies in the higher yield and in the smaller volumetric capacity for the same production.

An advantage of using a process of alcoholic fermentation with yeast cells immobilized on sugar-cane stalks is the possibility of working with high dilution rates, which is not possible in Brazilian industrial units. The disadvantage of the immobilized-cell process in comparison to the correspondent use of free cells lies in the volume of the support used, which can be compensated for the short residence time when using immobilized cells.

Despite our restricted range of dilution rates (0.2 to 1.5 h^{-1}) the experiments developed used both very low (D = 0.05 h^{-1}) and very high dilution rates (D = 3.00 h^{-1}), proving that the stalk-yeast system functions well for these variations.

CONCLUSIONS

The sugar-cane stalk is suitable for yeast cell immobilization in the continuous alcoholic fermentation process. The stalk-yeast system showed operational stability in up to 60-day cycles, even with extreme variations in dilution rate of 0.05 h^{-1} to 3.0 h^{-1}. High concentrations of immobilized cells of around 10^{9}cells/g of dry stalk were obtained. They were stable during alcoholic fermentation, even at the extremely high dilution rate of 3.0 h^{-1}.

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NOMENCLATURE

D	dilution rate, ratio of effluent volumetric flow (F) to the volume of liquid in the fermenter (V). Calculated using the equation: $D = F/V$, h^{-1}
P	ethanol productivity, calculated by considering the volume of liquid medium in the fermenter, using the following equation: $p = Ef\ V/V$, where Ef is the ethanolconcentration, g/L.h
ε	bed porosity, defined as the ratio of the liquid volume (V) to the work volume of the fermenter (Vf), represented by the equation: $\epsilon = V/Vf$, %
TRSf	total reducing sugar in fermented medium, g/L
Ef. ferm.	fermentation efficiency, calculated based on the contents of consumed sugar, %
Ef. proc.	fermentation efficiency process, calculated based on the theoretical ethanol from the initial substratum, %

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