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Alternative Approach for Utilization of Pentose Stream from Sugarcane Bagasse by an Induced Flocculent *Pichia stipitis*

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Abstract

A new approach for the utilization of hemicellulosic hydrolysate from sugarcane bagasse is described. This approach consists of using the hydrolysate to dilute the conventional feedstock (sugarcane juice) to the usual sugar concentration (150 g/L) employed for the industrial production of ethanol. The resulting sugar mixture was used as the substrate to evaluate the performance of a continuous reactor incorporating a cell recycle module, operated at several dilution rates. An induced flocculent pentose-fermenting yeast strain was used for this bioconversion. Under the conditions used, the reactor performance was satisfactory at substrate feed rates of 30 g/(L·h) or less, corresponding to an ethanol productivity of about 11.0 g/(L·h) and an overall sugar conversion >95%. These results show real advantages over the existing alternatives for a better exploitation of surplus bagasse to increase industrial alcohol production.

Index Entries: Sugarcane bagasse; sugar mixture; *Pichia stipitis*; flocculation; ethanol.

Introduction

The main objective of the studies related to pentose fermentation is to establish favorable process conditions, which can also be applied to hemicellulosic hydrolysate obtained from agricultural residues. The preparation of this kind of hydrolysate often generates inhibitory components (acetic acid, hydroxymethylfurfural), which have negative effects on the fermentation process *(1,2)*. Because the formation of these components

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cannot be avoided, several methodologies have been employed to minimize their undesirable effects. These include, detoxification strategies for removing these components *(3–4)*, improvement of the medium composition by adding special nutrients *(1)* or microorganism acclimatization to the toxic inhibitors *(1,5)*.

Under batch cultivation, these methodologies have proven to be efficient using several types of microorganisms, although they increase the cost of the process. In the evolution of the studies carried out in our laboratory, these strategies showed limited efficiency to achieve satisfactory reactor performance under continuous operation when the most promising pentose-fermenting yeast (*Pichia stipitis*) was used. This was related not only to the chemical environmental composition available to the biocatalyst but also to the poor ability of the selected yeast strain to maintain high cell concentrations inside the reactor. A different behavior, however, was observed when the pentose stream was combined in different proportions with sugarcane juice and used as feed medium in the fermentation system *(6)*. Under these conditions, the selected yeast strain was shown to have very strong flocculating properties, which allowed maintenance of high cell concentrations inside the reactor. This suggested that sugarcane juice could be used as an inducer of flocculation in *P. stipitis*.

Taking advantage of this property, a new approach for the utilization of the pentose component obtained from acid hydrolysis of sugarcane bagasse is proposed (Fig. 1). By diluting the sugarcane juice with sufficient hemicellulose hydrolysate to attain the usual level of sugar concentration of ethanol plants (150 g/L), a substrate containing a sugar mixture (sucrose, glucose, and xylose) was used to evaluate the performance of a continuous reactor incorporating a cell recycle module, operated at several dilution rates. Since high cell densities were achieved, high conversion of the mixed substrate was possible. In this article, we give the flocculating properties of *P. stipitis* growing under continuous operation. We also present the steady-state concentrations of relevant process variables.

Materials and Methods

Preparation of Microorganism and Inoculum

P. stipitis strain CBS 5773 was employed. Stock culture was maintained at 4°C on malt agar slants. Yeast was grown semiaerobically at 30°C for 24 h in Erlenmeyer flasks containing 20 mL of presterilized 10% sucrose broth (sugarcane juice) at pH 5.5. Amounts of additional nutrients/L of media prepared were 5.0 g of $(NH_4)_2 SO_4$, 1.0 g of KH_2PO_4, 1.5 g of $MgSO_4 \cdot 7H_2O$, 0.1 g of $CaCl_2$, and 0.5 g of yeast extract.

Preparation of Sugarcane Bagasse Hydrolysate and Fermentation Medium

Acid hydrolysis of sugarcane bagasse was carried out at 190°C for 15 min in a 25-L working volume steel reactor (AISI 316), with agitation. The

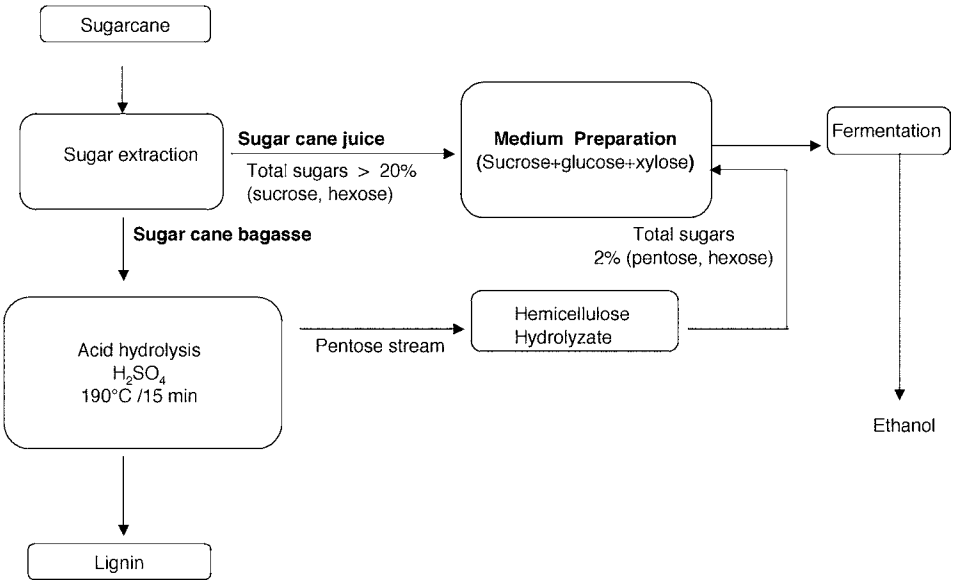

Fig. 1. Flow chart for ethanol process by *P. stipitis* based on utilization of sugarcane juice diluted with pentose stream from sugarcane bagasse.

Table 1
Substrate Characteristics[a]

Substrate type	Reducing sugars (g/L)	Total sugars(g/L)	Furfural (g/L)	Acetic acid (g/L)
Sugarcane juice	15.00	237.00	—	0.49
Bagasse hydrolysate	23.00	23.00	1.10	3.37
Sugarcane juice diluted with bagasse hydrolysate	19.80	149.90	0.66	0.92

[a]Average values are given.

reactant mixture consisted of 70 mg of concentrated (98% [w/v]) H_2SO_4/g (dry wt) of sugarcane bagasse and sufficient water to provide a liquid-to-solid ratio of 10:1. After hydrolysis, the liquid fraction was used to dilute fresh crushed sugarcane juice to a desirable sugar concentration (150 g/L of total reducing sugars). Then, the medium was supplemented with nutrients at the same concentrations used for the inoculum preparation. The chemical composition of the feedstock sources and the resulting mixed substrate is shown in Table 1. Under the conditions used, a sugar mixture containing 20% monosaccharide and 80% disaccharide was obtained. The concentrations of acetic acid and furfural originally present in the bagasse hydrolysate were significantly reduced to levels <1 g/L.

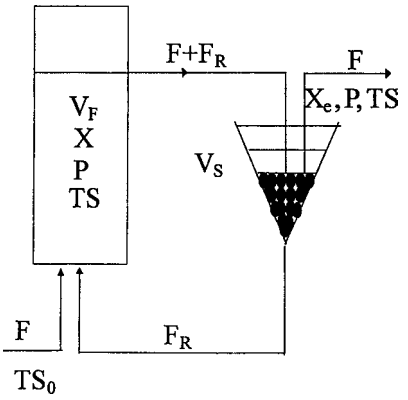

Fig. 2. Schematic diagram of experimental system used in continuous fermentation runs (V_F = 0.22l, V_S = 0.025 L; F_R = recycle rate).

Fermentation Conditions

Fermentation runs were carried out in a simple column reactor fitted with a separate settling device and working volume of 0.245 L. The fermentation system utilized appropriate sensors to control the temperature and pH, as shown schematically in Fig. 2. Continuous fermentation was started by loading the reactor with substrate medium at a low sugar concentration and adjusting the control parameters (pH 5.0, temperature of 30°C, and airflow rate of 0.1 vvm). Then the reactor was inoculated with cell suspension using five precultured conical flasks. A start-up period of 4–6 d was required to accumulate a working cell density (X > 80 g/L, dry wt), and for this purpose, the fermentation system was continuously fed with substrate medium at increased sugar levels ranging from 50 to 150 g/L. Several dilution rates (D = 0.1 to 0.4 h^{-1}) were imposed in order to determine the limit performance of the reactor for this specific substrate. During the continuous runs, periodic samples were taken from the reactor vessel for analysis of the relevant variables, such as sugar output, biomass density, and product concentration. For each tested dilution rate, the fermentation parameters were determined when the concentrations within the reactor remained relatively constant over a period corresponding to at least three residence times. For comparison, the reactor was also run with pure sugarcane medium containing 150 g/L of total sugars.

Analytical Procedures

The cell concentrations in the overflow were determined from a calibration curve relating the optical density (600 nm) to cellular biomass. Cell density in the reactor was directly determined by centrifuging 10 mL of culture, resuspending cells in distilled water, recentrifuging, and then drying at 105°C to constant weight. The degree of flocculation, expressed as

sedimentation rate, was determined by measuring the height of sediment cells in a millimetrically graduated cylinder as it settled at 1 min intervals *(7)*. Total reducing sugars were assayed following the method described by Nelson *(8)*. Xylose and acetic acid concentrations were analyzed by high-performance liquid chromatography (HP 1082B) using a refractive index detector and a Bio-Rad HPX 87H (300×7.8 mm) column, and employing the following conditions: H_2SO_4 ($5 \cdot 10^{-3}$ *M*) eluent, 0.6 mL/min flux, 45°C column temperature, 16X detector attenuation, 20-mL sample volume. Ethanol concentration was measured by gas chromatography (GC model 35) equipped with an FFAP 20% Chromosorb W column and flame ionization detector with an injection temperature of 110°C *(9)*.

Results and Discussion

Flocculation and Cell Retention Within Reactor

Flocculation is a widespread phenomenon in the microbial world, that is frequently used as an alternative way to obtain a high-cell-density fermentation system *(10,11)*. The most common application of flocculating microorganisms can be found in the production of ethanol for beverage and nonbeverage uses, employing several reactor configurations *(12,13)*. Contrary to hexose-fermenting yeasts (i.e., typically *Saccharomyces cerevisiae*), which are reasonably well-understood systems *(10,11)*, flocculation in pentose-fermenting yeasts is a very recent method *(14–17)*. In these yeasts, flocculation has been observed in cells of *Pachysolen tannophilus (15)* and *Pichia stipitis (16,17)*. However, the feasibility of using such yeasts in continuous fermentations is still limited by their poor stability and weak flocculation tendencies *(17,18)*. If floc retention inside the reactor could be increased, then the phenomenon would have a great technological importance for ethanol production from hemicellulosic wastes. This has been achieved by using the system described here. High cell concentrations within the reactor were attained in a period no longer than 4 d. The ratio of the cell concentration inside the reactor (X) to that in the overflow (X_e) defined by Denverell and Clark *(18)* was used to measure the flocculation intensity of *P. stipitis* cells during the start-up period. As shown in Table 2, there was a noticeable increase in the yeast settling accompanied by a buildup of dense particle flocs. Therefore, a gradual increase in the flocculation intensity (X/X_e) was achieved, resulting in a cell concentration ratio of about 36.5 after 48 h. This allowed the reactor to run with virtually total biomass retention and clear effluent. A sample of the flocculation culture, taken from the reactor, was used to conduct a set of standard flocculation tests *(7)*, as shown in Table 3. No attempt was made to explain the mechanism by which cell aggregation occurred; however, the characteristics of this cellular aggregate are similar to those attained by the flocculent yeast strain *S. cerevisiae (19,20)*. Despite the characteristics of the substrate used, no negative effects on yeast flocculation properties or activity were observed during the continuous runs.

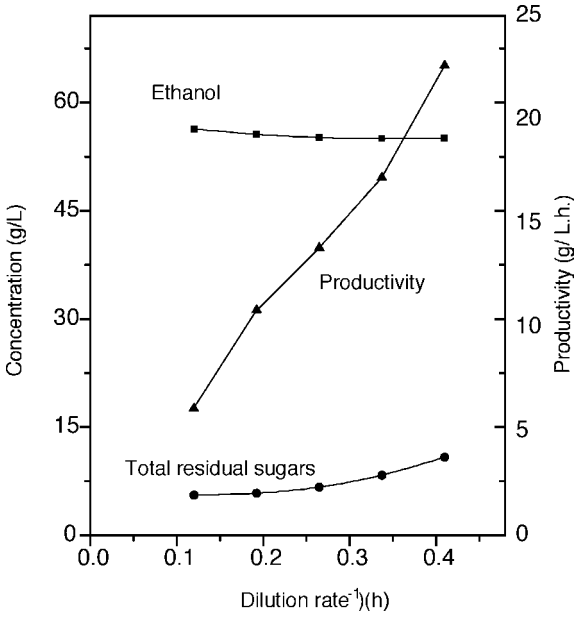

Fig. 3. Steady-state variables of continuous ethanol production by *P. stipitis* using sugar mixture substrate.

Continuous Runs

The performance of the fermentation system was evaluated by plotting ethanol productivity (Q_p), residual sugar concentration (S), and product concentration (P) as a function of the dilution rate (D) (Fig. 3). An average ethanol concentration of 56 g/L was achieved at dilution rates ranging from 0.12 to 0.4 h^{-1}, which corresponded to ethanol productivities from 6.6 to 22.6 g/(L·h).

Table 4 shows that sugars metabolized as hexoses (sucrose and glucose) were almost fully converted, while pentose (xylose) conversion markedly decreased with the increase in dilution rate. Although both sugars were consumed simultaneously, the hexose consumption rate by *P. stipitis* was much higher than the pentose one. As a consequence, a progressive loss of sugars in the output stream (as pentose) was observed when the dilution rate increased. This is in agreement with findings that xylose metabolism in *P. stipitis* is inhibited but not repressed by hexoses. Thus, in our case, the rate of xylose fermentation was found to be the limiting step of the process, and maximum dilution rate for effective conversion was 0.20 h^{-1}. Increasing dilution rate above this level resulted in a progressive loss of sugars, namely, pentose in the output stream.

No significant differences were observed in the apparent product yield ($Y_{P/S}$) throughout the continuous runs (Table 5), with an average value of 0.39 g/g. Since most of the ethanol was produced from hexoses,

Table 2
Retention and Accumulation of *Pichia stipitis* Cells Within Reactor Using Substrate Based on Sugarcane Juice Diluted with Pentose Stream

Duration (h)	Cell concentration inside reactor, X (g/L)	Output cell concentration X_e (g/L)	X/X_e ratio
7	5.2	ND	5.2
24	6.9	1.1	6.3
48	14.6	0.4	36.5
72	37.7	ND	37.7
96	55.2	2.5	22.1
120	76.6	1.7	45.0
144	97.0	2.8	34.0
148	107.4	4.1	26.1

[a]ND, not detected.

Table 3
Flocculent Properties of *P. stipitis* Using Sugarcane Juice Diluted with Hemicellulose Hydrolysate

Characteristics	Values
Sedimented cells in 5 min (%)	75
Flocculation velocity (cm/s)	0.14
Specific rate of sedimentation (mL/g)	27.5
Washout resistant	D = 0.4 1h^{-1} , X = 93.0 g/L

Table 4
Influence of Substrate Feed Rate on Individual and Overall Sugar Conversion

	Substrate input (g/L)			Substrate output (g/L)			Substrate conversion (%)		
D (h^{-1})	Xyl	Suc + Glu	TS	Xyl	Suc + Glu	TS	Xyl	Suc + Glu	Overall
0.12	18.5	129.2	147.7	4.9	0.3	5.2	73.5	99.7	96.5
0.14	13.8	139.1	152.9	5.9	0.6	6.5	57.2	96.6	95.7
0.20	11.4	141.9	152.4	4.9	0.5	5.4	57.0	99.6	96.4
0.37	20.2	131.0	151.2	7.4	0.5	7.9	62.0	99.7	94.7
0.41	15.1	141.3	156.4	10.1	0.7	10.8	33.1	99.5	93.1

[a]Xyl, xylose; Suc, sucrose; Glu, glucose; TS, total sugar.

the average ($Y_{P/S}$) value was unexpectedly lower than the one observed for hexose-fermenting yeasts, such as *S. cerevisiae* ($Y_{P/S}$ = 0.45 g/g) *(20)*. This suggests that there are distinct physiologic differences between pentose and hexose yeasts.

Table 5
Performance of Bioreactor Characteristics
on Different Substrates Using *Pichia stipitis*

Reactor parameters	Substrate type	
	Sugarcane juice[a]	Sugarcane juice diluted with pentose stream
Dilution rate (h^{-1})	0.36	0.37
Substrate input (g/L)	147.9	151.2
Residual sugars (g/L)	3.3	7.9
Product (g/L)	59.9	55.9
Biomass (g/L)	90.0	94.7
Sugar conversion rate (%)	97.8	94.7
$Y_{P/S}$ (g/g)	0.41	0.39
Q_p (g/L·h)	21.6	20.7
μ_p (g/g·h)	0.24	0.22
DS_0 (g/[L·h])	53.2	55.9

[a]Data obtained using the same reactor system running on sugarcane medium.

Conclusions

Research and development studies of alcohol fermentation technology have been conducted over the last two decades to make alcohol production more efficient. In this context, there are clear advantages in using surplus bagasse from ethanol plants as a raw material for the same end product. Alcohol fermentation of pentose has become an attractive research topic and publications are numerous. In principle, biochemical and microbiologic problems, such as finding strains and establishing the somewhat unusual (e.g., semiaerobic) optimal conditions, have been solved at a laboratory-research level. However, so far, a suitable process technology has not been established.

Given our background in developing very effective ethanol technology based on disaccharide feedstock *(19,20)*, it would be logical to continue this research program using pentose feedstocks. The present study investigated the feasibility of using hemicellulosic hydrolysate to dilute the conventional raw material (sugarcane juice) to a standard sugar concentration employed for the industrial production of ethanol. Under the conditions used here, the reactor performance was satisfactory at substrate feed rates of 30 g/(L·h) or less, corresponding to an ethanol productivity of about 11.0 g/(L·h) and an overall sugar conversion >95%. On a laboratory scale, this process has shown to be an attractive alternative for utilization of the bagasse generated by the ethanol plants. Moreover, this process has the potential to attain high ethanol levels; avoid treatment for removing inhibitory compounds normally found in the hemicellulosic acid hydrolysate; decrease the addition of microbial nutrients; and require

minimal changes on ethanol plants for its implementation, i.e., substitution of the yeast genus *S. cerevisiae* by *P. stipitis*. However, a successful application of this novel approach is limited by the implementation of an adequate dilution rate on the reactor in order to achieve an efficient pentose conversion. Optimization of the dilution rate is currently the main potential of further developments of this technology.

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References

1. Olsson, L. and Hahn-Hagerdal, B. (1996), *Enzyme Microb. Technol.* **18,** 312–331.
2. Clark, T. and Mackie, K. L. (1984), *J. Chem. Biotechnol.* **34B,** 101–110.
3. Palmqvist, E., Hahn-Hagerdal, B., Szengyel, Z., Zacchi, G., and Rczey, K. (1997), *Enzyme Microb. Technol.* **20,** 286–293.
4. Silva, S. S., Ribeiro, J. D., Felipe, M. G., and Vitolo, M. (1997), *Appl. Biochem. Biotechnol.* **63/65,** 557–564.
5. Parekh, S., Parekh, R., and Wayman, M. (1987), *Process Biochem.* **22,** 86-–91.
6. de Castro, H. F., Castro, L. A. B., and Nunes, A. L. L. (1989), in *Biomass for Energy and Industry*, vol. 2, Grassi, G., Gosse, G., and dos Santos, G., eds., Elsevier Applied Science, London, UK, pp. 2318–2322.
7. de Castro, H. F. and Oliveira, P. C. (1998), *Revista Microbiologia* **29,** 27–30.
8. Nelson, N. A (1944), *J. Biol. Chem.* **153,** 357–380.
9. Roberto, I. C., Mancilha, I. M., Felipe, M. G. A., Silva, S. S., and Sato, S. (1994), *Arq. Biol. Tecnol.* **37,** 55–63.
10. Calleja, G. B. (1987), in *The Yeasts*, vol 2, Rose, A. H. and Harrison, J. S. , eds., Academic Press, New York, NY, pp.165–201.
11. Stratford, M. (1996), *Cerevisiae* **1,** 38–45.
12. Grenshields, R. N. and Smith, E. L. (1974), *Process Biochem.* **9,** 11–28.
13. Jones, S. T., Korus, R. A, Admassu, W., and Heimsch, R. C. (1984) *Biotechnol. Bioeng.* **26,** 742–747.
14. Weeb, S. R. and Lee, H. (1990), *Biotechnol. Adv.* **8,** 685–697.
15. Chung, I. S. and Lee, Y.Y. (1986), *Biotechnol. Bioeng. Symp.* **17,** 391–400.
16. Pereira Jr., N. and Bu'Lock, J. D. (1993), *Revista Microbiologia* **24,** 132–139.
17. Grootjen, D. R. J., Vleesenbeek, R., Windmeijer, M. G. A, van der Lans, R. G. J. M., and Luyben, K.C.A. M. (1991), *Enzyme Microb. Technol.* **13,** 734–739.
18. Denverell, K. F. and Clark, T. A. (1985), *Biotechnol. Bioeng.* **27,** 1608–1611.
19. Paiva, T. C. B., Sato, S., Visconti, A. E. S., and Castro, L. A. B. (1996), *Appl. Biochem. Biotechnol.* **57/58,** 535–541.
20. Oliveira, S. C., Paiva, T. C. B., Visconti, A. E. S., and Giudici, R. (1999), *Appl. Biochem. Biotechnol.* **74(3),** 161–172.