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Continuous Fermentation of D-Xylose by Immobilized *Pichia stipitis*

Comparison Between CSTR and CPFR

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ABSTRACT

The main purpose of this work was to compare the performance of two different kinds of reactors (CSTR and CPFR) in order to enhance the ethanol productivity in the fermentation of D-xylose by *Pichia stipitis* immobilized in κ-carrageenan. Immobilization was carried out in a 4% aqueous suspension of κ-carrageenan, which was mixed with the inoculum. The bioparticles were treated with $Al(NO_3)_3$ as hardening agent. The fermenters operated during a long period of time (about 30 d). Best results were obtained in the packed-bed reactor (CPFR), which allowed operation at high final ethanol concentrations, this fact having been explained because of the observed strong product inhibition. The overall productivity reached values higher than 3.8 g/(L·h). This supposed an interesting improvement with relation to the productivities found in the literature, which as an average did not exceed 1 g/(L·h). However, the specific productivities of yeast in the continuous stirred tank reactor (CSTR) were always greater because the bioparticles were kept in close contact with the broth, whereas in the CPFR, there were at least two problems: (a) the possibility that the produced gas could prevent the intimate contact between the substrate and the particles and (b) the possible existence of preferential paths.

Index Entries: D-xylose; yeast; immobilization; hardening; bioreactors.

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Abbreviations: P, ethanol concentration (g/L); S, D-xylose concentration (g/L); D, dilution rate (h^{-1}); Q_p, overall ethanol productivity (g/L·h); Q_s, overall D-xylose consumption rate (g/L·h); q_p, specific ethanol productivity (g/g·h); q_s, specific D-xylose consumption rate (g/g·h); q_{po}, maximum specific ethanol productivity (g/g·h); $Y_{p/s}$, yield in ethanol; P_m, toxic ethanol concentration (g/L); β, parameter of Luong's model indicating the inhibition degree; K_s, affinity constant (g/L); μ_m, maximum specific growth rate (h^{-1}).

INTRODUCTION

Many fermentation processes of great interest cannot be realized because of their low productivities, resulting in high investment and running costs. Among these processes, pentoses fermentation can be noted; the feasibility of this transformation could favor the economics of the lignocellulosics to ethanol process. One of the microorganisms capable of directly fermenting xylose (major constituent pentose of lignocellulosics) is *Pichia stipitis*, although its overall productivity (Q_p) is too low to achieve the economic viability of the process.

Once the specific productivity (q_p) has been optimized, the sole way to step up Q_p is to increase the cell concentration in the fermenter through high density cultures, these being obtained by such techniques as immobilization, flocculation, or cell recycle. In this work, fermentation of xylose to ethanol by *Pichia stipitis* immobilized in κ-carrageenan was studied in packed-bed and stirred tank reactors, the behavior of both types of equipment being analyzed and compared.

MATERIALS AND METHODS

Microorganisms

Pichia stipitis NRRL-Y7124 was kindly supplied by the Northern Regional Research Center, US Department of Agriculture, Peoria, IL.

Culture Media

Fermentation media had the following composition: xylose 50 g/L; yeast extract 3 g/L; $(NH_4)_2SO_4$ 3 g/L; $MgSO_4 \cdot 7H_2O$ 1.1 g/L; KH_2PO_4 2 g/L, and micronutrient solution (*1*) 10 mL/L. pH was maintained at 4.5 by citrate buffer in the experiments realized in fixed-bed reactors.

Analytical Methods

Xylose was followed by the dinitrosalicylic acid method with a Hitachi U-2000 Spectrophotometer at 540 nm. Ethanol was determined by gas chromatography in a Perkin-Elmer Sigma 3B apparatus equipped with a

TCD and an 80/100 mesh Porapak Q column. A 45 mL/min flow of helium as carrier gas was applied, with oven, injector, and detector temperatures of 145, 175, and 175°C, respectively. Isopropanol was the external standard. Cell mass in the bioparticles was determined as dry weight after being dissolved in citrate.

Immobilization

Cell suspensions of about 40 g/L were added to a previously sterilized 4% w/v solution of κ-carrageenan (Genugel X0828 from Kobenkavns Rektinfabrik, Denmark) at 35°C. Cell-gel beads about 2.5 mm in diameter were produced by using a 19G/11/2 in syringe to drip this mixture into a stirred solution of KCl (20 g/L) and $CaCl_2$ (0.11 g/L) at 4°C. After 30 min the beads were recovered, washed with 9 g/L NaCl, immersed in a 0.075*M* $Al(NO_3)_3$ solution (2) for 5 min, and washed once again before use.

Equipment

Experiments in continuous stirred tank reactors were carried out in a 1-L Braun fermenter with 800 mL of working volume, always maintaining an agitation of 150 rpm. Temperature (30°C), pH (4.5), and aeration were kept under automatic control. In microaerobic conditions, the oxygen concentration was 10% of the saturation level, approximately with a flow rate of 0.1 vvm.

Plug-flow reactors for continuous experiments had a volume of 160 mL (4.5 cm in diameter and 10 cm high), with a bed set up by beads of yeast immobilized in κ-carrageenan gel. Temperature was kept at 30°C by means of a thermal jacket. In both reactors, culture medium was fed using a variable-flow peristaltic pump.

RESULTS AND DISCUSSION

Fermentation in CSTR

Two series of successive experiments in CSTR were realized, the first in absence of oxygen and the second with a microaeration corresponding to 10% of the saturation level. A greater aeration could have provoked a change of the yeast metabolism towards other fermentation products different from ethanol (3). The stirred tank model allowed study of the behavior of immobilized yeasts at the operational conditions fixed for each steady state, such as ethanol concentration and its possible inhibitory effect on the process. When three consecutive measures of substrate concentration, at intervals not less than twice the residence time, were essentially equal, it was considered that a steady state was reached.

The fermentation process was followed by measuring the substrate concentration in the effluent. Product concentration was also analyzed for

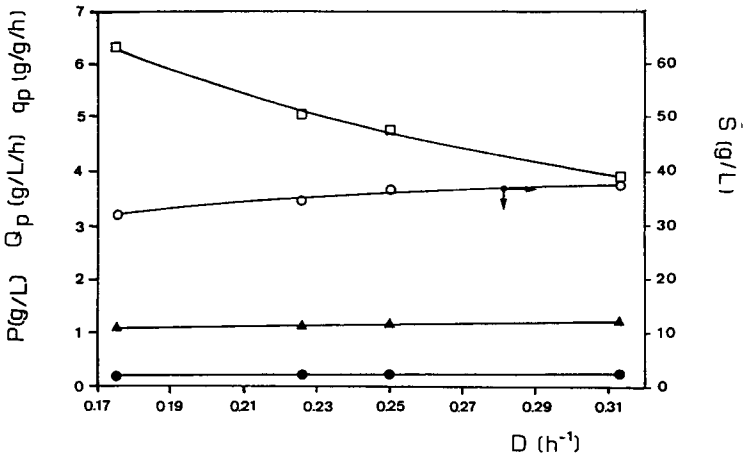

Fig. 1. Evolution of operation parameters with dilution rate for anaerobic experiments: P (□), S (○), Q_p (▲), and q_p (●).

samples taken at each steady state. 250 g of bioparticles, previously proliferated until matrix saturation, were used as inoculum; a higher quantity could have caused alterations in fluidity and, therefore, in the homogeneity of the medium. The initial concentration of yeast in bioparticles was 1.8% w/w, equivalent to a concentration of 5.6 g/L in the reactor.

At the end of all the experiments (26 d), 252 g of bioparticles, 1.75% w/w, were obtained, so that concentration of microorganisms in the medium, as well as the integrity of the particles, remained unaltered. These facts can be explained by the good retention (enhanced by hardening) of the yeasts in the matrix, which reached its maximum capacity; in addition, operating at high dilution rates, cells that had been eluted from the matrix are washed out.

Comparison of Microaerobic and Anaerobic Processes

Figures 1 and 2 reveal the evolution of some of the operation parameters with different dilution rates (D) for anaerobic and microaerobic fermentations. In the former process, D varied between 0.175–0.313 h^{-1}, whereas in the latter, the range of variation was 0.16–0.26 h^{-1}. Significant concentration of yeast was not detected in the effluent, because except for the minor D in microaerobic conditions, dilution rates were greater than the maximum specific growth rate, μ_m, for the soluble yeast (0.19 h^{-1} and 0.07 h^{-1} with and without oxygen, respectively) (4).

From the figures, low conversions can be inferred, although they could increase at lesser D. However, the high values of affinity constant, K_s, for *Pichia stipitis* (in solution 9 g/L and 19 g/L for anaerobic and microaerobic conditions) will suppose a limitation in the final conversion. For the immobilized yeast, perhaps K_s is different, but in any case, it would be even

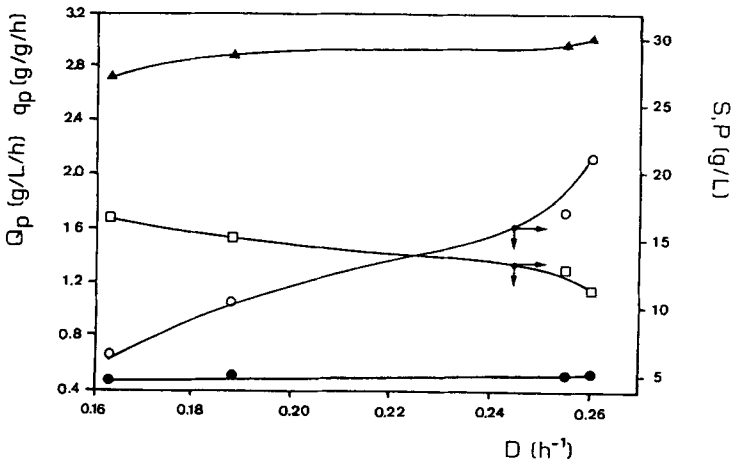

Fig. 2. Evolution of operation parameters with dilution rate for microaerobic experiments: P (□), S (○), Q_p (▲), and q_p (●).

higher, especially because of diffusional problems. A decrease in yields $Y_{p/s}$ can be deduced when ethanol concentration increases, possibly because then the yeast needs more substrate for its maintenance. As an average, $Y_{p/s}$ was 0.34 and 0.40 for anaerobic and microaerobic processes; the lesser value in the first case was likely the result of the production of metabolic substances other than ethanol, such as xylitol.

Specific and overall productivities are much higher in the presence of oxygen. These are the values superior to the ones appearing in literature (*5,6*), which, for the most cases, do not exceed 1 g/L·h for Q_p. When overall productivity is highest (3.02 g/L·h), the ethanol concentration and, therefore, the conversion efficiency are low. Ethanol concentration increases faster than productivity decreases (11.6–16.7 in front of 3.02–2.27), as D lessens. Thus, the optimal operation point (compromising situation between ethanol concentration and productivity) is not necessarily the point of maximum productivity.

Inhibition by Product

By doing an analysis of the experimental data, a decrease in relative productivity can be noted when ethanol concentration increases, this being a typical fact of a process inhibited by product. For the case of the anaerobic fermentation, it is not possible to model this behavior in an accurate manner, because P values are always very low (3.9–6.3 g/L), so relative productivities decrease by a small percentage. By this fact, the performance of *P. stipitis* immobilized in κ-carrageenan is only studied for the different ethanol concentration reached in steady state under microaeration.

q_{po}, which represents the maximum specific productivity obtained when ethanol concentration tends toward zero, can be calculated from extrapolation of experimental data (Fig. 3). This value (q_{po}=0.552 g/g·h) is

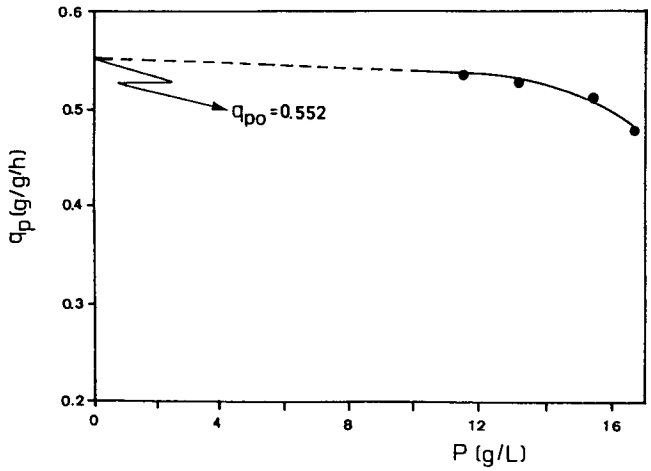

Fig. 3. Fitting of experimental data (●) to Luong's model (——).

necessary to fit experimental data to several models referred to in literature (*7*), being the best result obtained employing the Luong's model (*8*):

$$q_p/q_{po} = q_p^* = 1 - (P/P_m)^\beta$$

Experimental data adjust well enough with this model (*see* Fig. 3), with a correlation coefficient of 0.98. Value of P_m was 29.8 g/L, indicating that, for higher concentrations, alcohol production was very slow; value for β, 3.334, superior to 1, signified that, at the beginning of the process, the inhibition was small, but increased in a continuous and quick manner until it became almost total when P exceeded 30 g/L.

Fermentation in a Plug-Flow Reactor

The reactor operated at the same environmental conditions as the CSTR (T=30°C, pH=4.5, S_o=50 g/L), and the operational strategy to achieve the steady state was also the same. Medium was saturated in O_2 prior to feeding to the reactor, assuring microaeration conditions. Reactors were inoculated with particles previously proliferated in successive batches. The three flow rates of operation were 10, 15, and 30 mL/h, equivalent to dilution rates of 0.09, 0.136, and 0.272 h^{-1}, respectively. The latter value corresponds to the highest used in the CSTR. Dilution rates were attributed to the void volume of the reactor, so results could be compared with others appearing in the same manner in literature. During experiments, only a slight compression of the bed was observed, with bioparticles maintaining their spheric shape. This proved that the optimized hardening treatment previously done minimized the pressure drop along the column, thereby preserving the beads' integrity. Results showed an almost constant value for the initial and final fractions. Yeast concentration in the support (1.8%) was in the order of the value obtained when the fermentation finished, this again indicating the existence of a maximum retention capacity for the matrix.

Table 1
Kinetic Parameters for the Continuous Fermentation
With Immobilized *Pichia stipitis* (packed-bed reactor)

Flow [mL/h]	D h^{-1}	Q_p g/(L·h)	Q_s g/(L·h)	q_p g/(g·h)	q_s g/(g·h)	$Y_{p/s}$
10	0.090	1.75	4.07	0.10	0.23	0.416
15	0.136	2.44	5.81	0.14	0.33	0.420
30	0.272	3.84	9.25	0.22	0.53	0.415

(*) Feed xylose concentration: 50 g/L

Table 2
Operational Parameters for Fermentation Process
With Immobilized *Pichia stipitis* (packed-bed reactor)

Operation parameters	Values
Initial bed volume	110 mL
Final bed volume	107 mL
Volume occupied by gas	5 mL
Initial void fraction	0.46
Final void fraction	0.45
Initial cell concentration	17.30 g/L
Final cell concentration	17.50 g/L

(*) measures at the end of 24 d.

In Tables 1 and 2, several kinetic and operational parameters are shown, the latter taken at the end of 24 d. Specific ethanol productivity, q_p, and specific substrate consumption rate, q_s, can only be calculated in a direct way for the last flow rate (30 mL/h), because the concentration of immobilized yeast in bioparticles is determined when the fermentation process ends; however, since yeast concentration is nearly unvariable, these parameters can be adequately estimated for all employed flows.

Productivities rise with dilution rates, although at the same time, ethanol concentration lessens. q_p and q_s were in the same order as that found in batch experiments (9).

From Table 2, a relatively easy evolution of gas can be seen, as reflected in the volume that CO_2 fills up in the reactor (4.5%, equivalent to a 10% of useful volume). Doubtless, this fact is related to the good mechanical strength of bioparticles.

Table 3
Evolution of S and P Along the Reactor

Reactor Zone	S	P	S	P	S	P
	30 mL/h		15 mL/h		10 mL/h	
In [g/L]	50	0	50	0	50	0
Middle	24.6	10.1	36.7	13.4	19.9	12.8
Out	15.8	14.2	7.6	17.8	4.8	19.4

(*) Flow: 30 mL/h

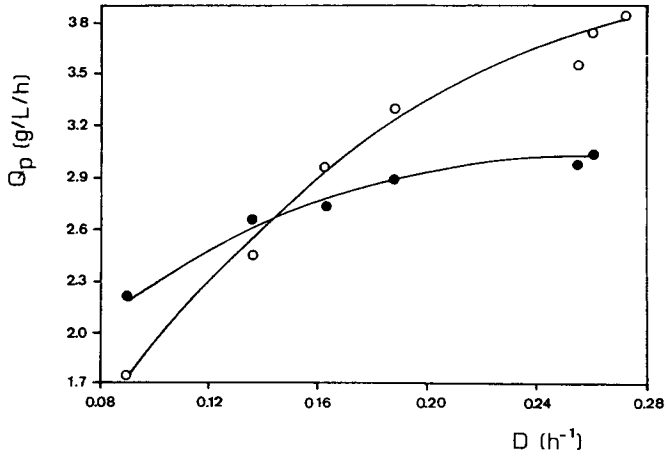

Fig. 4. Comparison between CSTR (●) and CPFR (○).

To analyze variation of substrate and product concentration along the bioreactor, samples at the bottom, middle, and top zones were taken. Bioparticles of lower, middle, and upper zones of the reactor were also analyzed, the yeast concentration being similar in all of them. This result confirms the advantage of proliferating the yeast prior to fermentation. Table 3 shows the results for *S* and *P* at the different flows, emphasizing the fact that, as mean, product concentration in the middle of the reactor is already 70% of that encountered at the top. The system does not have a homogeneous behavior, presenting higher overall and specific ethanol productivities in the feeding zone (at the bottom). Low effectiveness at the top can be explained by ethanol inhibition as well as by a deficient contact bioparticles-substrate in this zone, where big "bags" of gas exist.

Comparison Between CSTR and CPFR

The overall productivities vs dilution rates for both systems appear in Fig. 4. Q_p was higher in general for packed-bed fermenter (except at very low *D*), although q_p was lesser. This latter fact is the result of better con-

tact culture medium-bioparticles in the stirred tank reactors; as mentioned before, the plug-flow reactor has a top zone where the CO_2 gas avoids the "capture" of substrate by microorganisms. In addition, the existence of preferential paths causes additional problems. It is also significant to emphasize that the aeration in the packed-bed reactor proceeds from an O_2 saturation of culture medium, previous to its feed; for this reason, not all the yeasts are equally aerated, thereby causing different productivities. However, the overall ethanol productivity, Q_p, the really important parameter, is always higher in the plug-flow model for the same dilution rate, with the additional advantage of a higher ethanol concentration in the effluent. If comparison of both reactors is done at equal P, differences in Q_p are even higher. Lesser Q_p for CPFR at a low dilution rate can be caused by a worse wetting of bioparticles, because of preferential paths, thereby provoking a minor effectiveness for the system.

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