

ETHANOL PRODUCTION FROM XYLOSE BY *Pichia stipitis* NRRL Y-7124 IN A STIRRED TANK BIOREACTOR

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Abstract - The ethanol production by *Pichia stipitis* was evaluated in a stirred tank bioreactor using semi-defined medium containing xylose (90.0 g/l) as the main carbon source. Experimental assays were performed according to a 2² full factorial design to evaluate the influence of aeration (0.25 to 0.75 vvm) and agitation (150 to 250 rpm) conditions on ethanol production. In the studied range of values, the agitation increase and aeration decrease favored ethanol production, which was maximum (26.7 g/l) using 250 rpm and 0.25 vvm, conditions that gave a volumetric oxygen transfer coefficient ($k_L a$ value) of 4.9 h⁻¹. Under these conditions, the ethanol yield factor, ethanol productivity, and the process efficiency were 0.32 g/g, 0.32 g/l.h, and 63%, respectively. These results are promising and contribute to the development of a suitable process for ethanol production from xylose by *Pichia stipitis*.

Keywords: Ethanol; *Pichia stipitis*; Xylose; Stirred tank bioreactor; Volumetric oxygen transfer coefficient.

INTRODUCTION

Ethanol production by fermentation has gained great importance in the last few years due to its increased use as fuel and as a complement to gasoline (Ratnam et al., 2005; Sun and Cheng, 2005). Besides being a renewable energy source, ethanol has other important advantages when compared to gasoline. Since it is an oxygenated fuel (contains 35% oxygen), the emission of NO_x and particulate materials from its combustion is lower. Lignocellulosic materials have been pointed out as being promising and very attractive raw materials for this bioconversion process because of their low cost and abundance and non-competition with foodstuffs (Duff and Murray, 1996; Lin and Tanaka, 2006). Xylose is the main sugar obtained by hydrolysis of the hemicellulosic fraction and its bioconversion is an important step in the use of lignocellulosic materials (Guo et al., 2008; Nakamura et al., 2001).

The ability of microorganisms to produce ethanol from xylose has been investigated by some researchers (Hahn-Hägerdal et al., 1994; Millati et al., 2005; Sanchez et al., 2004) since *Saccharomyces cerevisiae*, the most commonly used ethanol producing microorganism, cannot ferment pentoses (Kumar et al., 2009). In several works, *Pichia stipitis* has presented interesting results for xylose conversion to ethanol from lignocellulosic hydrolysates (Agbogbo et al., 2008; Nigam, 2001). However, the environmental parameters like pH, temperature, oxygen availability, among others, and the culture medium composition are important factors affecting the bioconversion processes (Sunitha et al., 1999). Among these factors, aeration is considered to be the most important factor affecting xylose fermentation by yeasts, since it determines the partitioning of the carbon flow substrate between growth and product formation (du-Preez, 1994).

Oxygen has low solubility in water when compared to carbon, nitrogen and phosphorus

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sources. Therefore, in submerged fermentations where it is necessary, oxygen transfer to the fermentation medium must be performed during the entire process time. Such transfer can be performed in different ways; among these, bioreactors with mechanic agitation and air bubble dispersion to the medium are the most commonly used, corresponding to 93% of the applications (Schmidell et al., 2001). Studies in bioreactors are also advantageous since they permit the evaluation of fermentation parameters that cannot be well controlled in rotatory incubators and promote conditions more similar to those employed on an industrial scale. Nevertheless, for the development of an efficient large scale process for xylose bioconversion to ethanol, it is of fundamental importance to understand the influence of the environmental parameters in bioreactors operated under controlled conditions.

In this sense, the present study aimed to evaluate the influence of agitation and aeration conditions on ethanol production from xylose by *Pichia stipitis* NRRL Y-7124 in a stirred tank bioreactor. Experimental assays were performed according to a 2² full factorial design, varying the aeration and agitation conditions in the range of 0.25 to 0.75 vvm and 150 to 250 rpm, respectively. The best experimental condition to perform this bioconversion process was established by using statistical tools.

MATERIALS AND METHODS

Microorganism and Inoculum

Pichia stipitis NRRL Y-7124 was the microorganism used in the experiments. Cultures of this yeast were maintained on malt extract agar slants at 4°C, the cells being transferred to a new medium each week. For inoculum preparation, cells of the yeast in the maintenance medium were transferred to 500-ml Erlenmeyer flasks containing 100 ml of the medium composed of (g/L): xylose (30.0), glucose (5.0); arabinose (5.0); urea (2.3), MgSO₄.7H₂O (1.0),

and yeast extract (3.0). The inoculated flasks were incubated at 30°C, 200 rpm, during 24 h. After this time, when the end of the exponential growth phase had been achieved, the cells were recovered by centrifugation (2000 × g, 20 min) and resuspended in the fermentation medium to obtain an initial concentration of 1 g/L.

Fermentation Medium and Conditions

The fermentation medium was composed of (g/L): xylose (90.0), glucose (15.0), arabinose (15.0); urea (2.3), MgSO₄.7H₂O (1.0), and yeast extract (3.0). Fermentation assays were performed in a 1.6-L stirred tank bioreactor (Autoclavable Benchtop Fermenter Type R'ALF, Bioengineering AG, Wald, Switzerland), containing 1.2 L fermentation medium inoculated with 1 g/l of initial cell concentration. The runs were performed at 30°C for 96 h. The agitation and aeration conditions used in each experiment are shown in Table 1. During the experiments, samples were taken each 12 h for sugars, ethanol, and cell growth determinations.

Evaluation of k_La

The determination of the volumetric oxygen transfer coefficient was carried out by the static method proposed by Wise (1951). The polarographic oxygen probe was calibrated at atmospheric pressure by setting zero and 100% saturation under nitrogen and air sparging, respectively. In order to evaluate k_La, the fermentor was configured as used in the fermentation experiment, but was not inoculated. Oxygen was removed from the fermentor medium by sparging with nitrogen. The oxygen saturation time course was then monitored under the air flow and stirring conditions chosen for use during fermentation. For the k_La value calculation, the integrated form of the equation proposed by Stanbury et al. (1995) was used, where the value of -k_La is equal to the slope of the resulting straight line representation of ln(C* - C_L) versus time.

Table 1: Experimental design for evaluation of the agitation and aeration effect on ethanol production by *Pichia stipitis* in a stirred tank bioreactor.

Assay	Variables coded levels		Variables original values		k _L a (h ⁻¹)	Responses *			
	Aeration	Agitation	Aeration	Agitation		Y _{P/S} (g/g)	Q _P (g/L.h)	P (g/L)	S (%)
1	-1	-1	0.25	150	0.7	0.33	0.24	23.0	70
2	1	-1	0.75	150	3.2	0.29	0.30	17.7	63
3	-1	1	0.25	250	4.9	0.32	0.32	26.7	83
4	1	1	0.75	250	12.1	0.31	0.34	16.2	52
5	1	1	0.75	250	11.7	0.30	0.33	16.1	56
6	0	0	0.50	200	2.1	0.33	0.32	19.4	58

* P: maximum ethanol production; S: total substrate consumed; Y_{P/S}: ethanol yield factor; Q_P: ethanol volumetric productivity

Analytical Methods

Cell growth was determined by measuring the fermentation broth UV-spectrophotometric absorbance at 600 nm, which was correlated with a calibration curve (dry weight \times optical density [OD]). Glucose, xylose, arabinose, and ethanol concentrations were determined by high-performance liquid chromatography (HPLC) in a Jasco chromatograph equipped with a refractive index detector and a Bio-Rad Aminex HPX-87H column (300 \times 7.8 mm). Operating conditions included: temperature of 60°C, 0.005 M sulfuric acid as eluent at a flow rate of 0.7 mL/min and sample volume of 20 μ L.

Ethanol yield factor ($Y_{P/S}$, g/g) was defined as the ratio between ethanol concentration (g/L) and substrate (glucose + xylose) consumed (g/L). Ethanol volumetric productivity (Q_P , g/L.h) was calculated as the ratio between the maximum ethanol concentration (g/L) and the respective fermentation time (h). The efficiency of sugar conversion to ethanol (η , %) was determined as the ratio between $Y_{P/S}$ (g/g) and the theoretical value (0.51 g/g) of this parameter (Hahn-Hägerdal et al., 1994).

Statistical Analysis

Fermentation assays were performed according to a 2^2 full factorial design (Table 1) to evaluate the influence of agitation and aeration variables on xylose bioconversion to ethanol by *P. stipitis*. The

ethanol concentration (P), yield factor ($Y_{P/S}$), and productivity (Q_P), were taken as responses of the experimental design. Statistical analysis of the data was carried out using Statgraphics 6.0, Design-Expert 5.0, and Statistica 6.0 softwares.

RESULTS AND DISCUSSION

During the experiments, the level of oxygen dissolved in the medium was zero for all the levels of oxygenation, possibly indicating that all the oxygen supplied was quickly consumed by the yeast. The fermentation profiles for sugars consumption, cell growth, and ethanol production are shown in Figure 1. *P. stipitis* was able to consume glucose and xylose sugars in all the fermentation runs; nevertheless, no arabinose consumption was observed during the considered fermentation time. In all the assays, glucose was consumed in the initial 12 h of fermentation, except in the run of lowest volumetric oxygen transfer coefficient (assay 1), in which the substrate consumption was slower than in the other runs, requiring around 24 h for total consumption of this hexose. Xylose was also slowly consumed in this assay (65% consumption at the fermentation end). On the other hand, the fermentation condition that proportioned the highest substrate consumption (90%, being 100% glucose and 87% xylose) was not that of highest volumetric oxygen transfer coefficient, but an intermediate value ($k_L a = 4.9 \text{ h}^{-1}$, assay 3).

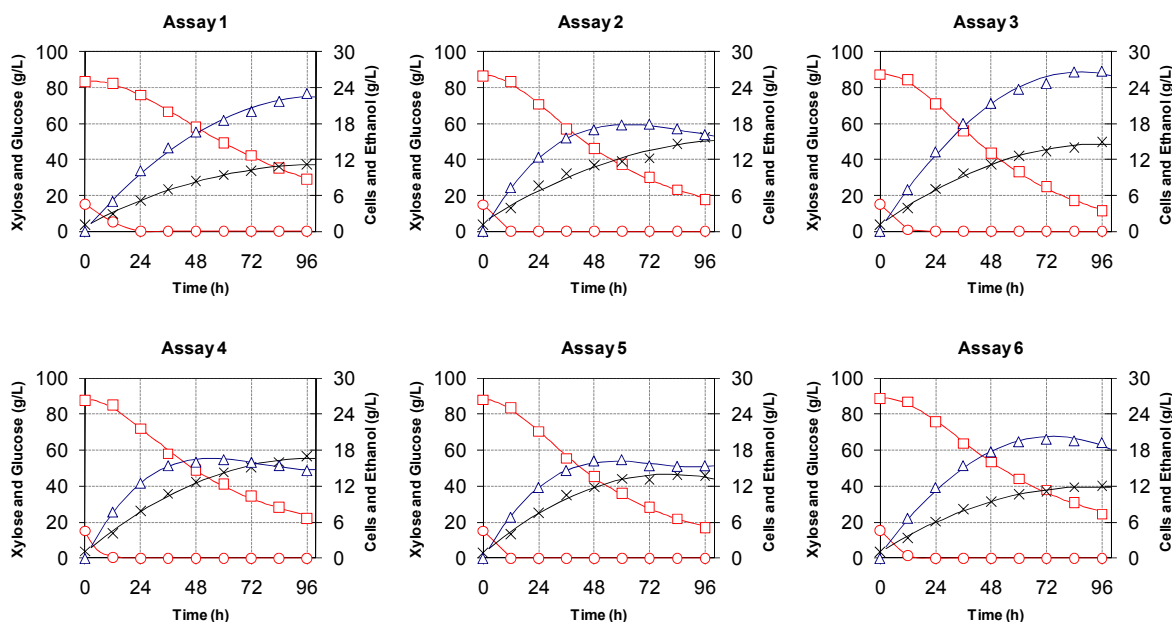


Figure 1: Kinetic behavior of cell growth (—x—), xylose (—□—) and glucose (—○—) consumption, and ethanol production (—Δ—) by *Pichia stipitis* in a stirred tank bioreactor under different agitation and aeration conditions, according to a 2^2 full factorial design.

P. stipitis was also able to grow and produce ethanol under all the evaluated fermentation conditions. The final cell concentration varied between 11 and 17 g/L, these values being found for the conditions of lowest (0.7 h^{-1}) and highest (12.1 h^{-1}) k_La values, respectively (assays 1 and 4). Similar results were observed during ethanol production by *P. stipitis* CBS 6054 (Agbogbo and Wenger, 2007) and *P. stipitis* NRRL Y-7124 (Telli-Okur and Eken-Saraçoglu, 2008), where the oxygen transfer increase (due to the agitation increase) also favored cell growth.

In the present work, the condition of highest k_La value favored cell growth and affected ethanol production, which presented the lowest values (assays 4 and 5 - Figure 1). However, the highest ethanol production (26.7 g/L) was not obtained in the assay of lowest values of volumetric oxygen transfer coefficient, but using an intermediate k_La value (4.9 h^{-1} , assay 3). According to Klinner et al. (2005), *P. stipitis* is a respiration-fermentative yeast and thus, excess oxygenation can lead to low ethanol yields. Some studies have shown that *P. stipitis* produces ethanol under anaerobic conditions, but microaerobic conditions appear to be optimal for ethanol production (Agbogbo and Coward-Kelly, 2008).

Table 1 summarizes the maximum ethanol concentration, substrate consumption and fermentation parameters values ($Y_{P/S}$ and Q_P) obtained for each fermentation assay. Despite the differences in the studied oxygen transfer levels, significant variations were not observed in the $Y_{P/S}$ results, which varied between 0.29 and 0.33 g/g. Similarly, the Q_P results also presented few variations (between 0.30 and 0.34 g/L.h) for all the evaluated conditions (except for assay 1). In this case, such behavior is due to the fact that the assays that promoted less ethanol accumulation attained the maximum production in a short time, yielding Q_P values similar to the assays that produced more

ethanol, but that also required a longer time to achieve the maximum concentration. The low ethanol yield variation observed for the studied k_La values (0.7 to 12.1 h^{-1}) is in agreement with the findings of Liu et al. (2002), who reported that the ethanol yield was practically constant for k_La values less than 14 h^{-1} . According to these authors, the k_La value must be controlled between 0 and 14 h^{-1} to obtain high ethanol yield.

A Pareto chart was used to perform a statistical analysis of these experimental data. Figure 2 represents the estimated effects of the agitation and aeration on ethanol yield factor (a) and ethanol volumetric productivity (b). The length of each bar was proportional to the standardized effect. Bars extending beyond the vertical line correspond to effects statistically significant at 90% confidence level. It can be observed that, in the range of values studied, the aeration (factor A) and agitation (factor B) did not influence $Y_{P/S}$. However, the agitation presented an effect significant at 90% confidence level for Q_P , which had a positive signal, promoting an increase in the values of this response when the agitation was increased from 150 to 250 rpm.

Figure 3 shows the maximum ethanol concentration obtained in each fermentative assay as a function of the agitation and aeration used in the bioconversion process. It can be easily noted that, in the range of values studied, ethanol accumulation was favored by conditions of low aeration (0.25 vvm), for any of the agitation conditions used. However, an agitation increase only influenced ethanol accumulation when the aeration was employed at the lowest level (0.25 vvm), little influence of this variable on ethanol production being observed when the aeration was used at the highest level (0.75 vvm). The best ethanol production (26.7 g/L) was achieved using 250 rpm of agitation and 0.25 vvm of aeration, conditions that gave a k_La value of 4.9 h^{-1} .

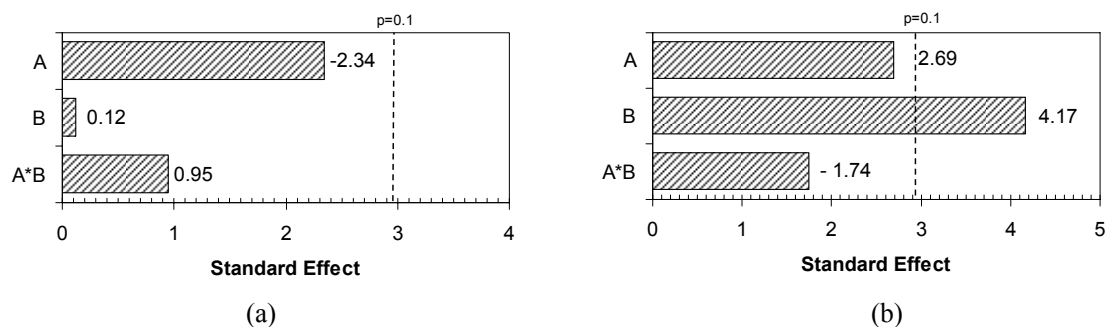


Figure 2: Pareto charts for estimation of the aeration (factor A) and agitation (factor B) effects on the responses: ethanol yield factor (a) and ethanol productivity (b).

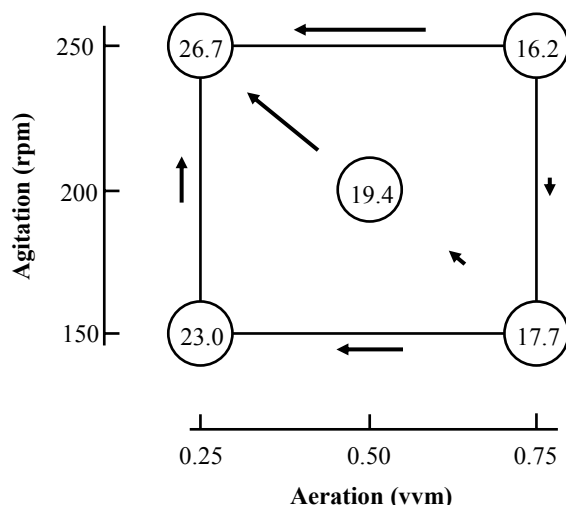


Figure 3: Maximum ethanol production (g/L) from xylose by *Pichia stipitis* in a stirred tank bioreactor under different agitation and aeration conditions, according to a 2^2 full factorial design.

The volumetric oxygen transfer coefficient is a parameter of great influence during the ethanol production from xylose by *Pichia stipitis*. The $k_{L}a$ value of 4.9 h^{-1} , which gave the best ethanol production (26.7 g/L) in the present study, is similar to those reported by Taniguchi et al. (1997) and Furlan et al. (1994). Taniguchi et al. (1997) investigated the effect of oxygenation on ethanol production from xylose or glucose by *Pichia stipitis* NRRL Y-7124 in batch culture. For xylose based cultures, the highest ethanol concentration (20 g/L) and $Y_{P/S}$ (0.40 g/g) were attained when using a $k_{L}a$ of 2.3 h^{-1} . The use of smaller (1.2 h^{-1}) or larger (6.1 h^{-1}) $k_{L}a$ values caused a reduction in the maximum ethanol concentration produced. Furlan et al. (1994) investigated different yeasts, including *Pichia stipitis* NRRL Y-7124, to select the most suitable strain to convert xylose either to ethanol or to xylitol, with little or no formation of by-products, and the aeration rate was used as a variable parameter during this process. Fermentation runs were carried out initially under microaerobic (0.08 vvm and 250 rpm: $k_{L}a = 4.8 \text{ h}^{-1}$) and aerobic (0.9 vvm and 250 rpm: $k_{L}a = 35.4 \text{ h}^{-1}$) conditions. For a $k_{L}a$ of 4.8 h^{-1} , the highest ethanol concentration (15 g/L) was attained with *P. stipitis* at 104 h. Increasing the $k_{L}a$ value to 35.4 h^{-1} led to an increase in the maximum specific growth rate and growth yield at the expense of ethanol formation (6 g/L of ethanol in 38 h). The results obtained in this set of experiments suggest that microaerobic conditions ($k_{L}a = 4.8 \text{ h}^{-1}$) are the most suitable operating conditions for xylose fermentation with *P. stipitis*.

CONCLUSIONS

Pichia stipitis NRRL Y-7124 was able to grow and produce ethanol from xylose in a stirred tank bioreactor under different aeration and agitation conditions, which gave $k_{L}a$ values varying between 0.7 and 12.1 h^{-1} . Although few differences in the fermentative parameters values were observed for the different fermentation conditions, the maximum ethanol production varied according to the volumetric oxygen transfer coefficient level employed, the maximum value (26.7 g/L) being obtained after 84 h fermentation using a $k_{L}a$ of 4.9 h^{-1} . Under this condition, the ethanol yield factor, ethanol productivity, and the process efficiency were 0.32 g/g, 0.32 g/L.h, and 63%, respectively. These results are of great relevance and contribute to the development of a suitable process for ethanol production from xylose by *Pichia stipitis*.

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NOMENCLATURE

$k_{L}a$	volumetric oxygen transfer coefficient	h^{-1}
η	efficiency of xylose conversion to ethanol	%

P	ethanol concentration	g/L
Q _P	ethanol volumetric productivity	g/L.h
S	substrate consumption	%
vvm	volume of air per volume of medium per minute	mL/mL/min
Y _{P/S}	ethanol yield factor	g/g

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