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EFFECT OF INITIAL SUBSTRATE CONCENTRATION AND AGITATION ON XYLITOL PRODUCTION BY FERMENTATION OF HYDROLYZED TAMARIND SEED MEDIA WITH *Kluyveromyces marxianus*

EFECTO DE LA CONCENTRACIÓN INICIAL DE SUSTRATO Y AGITACIÓN SOBRE LA PRODUCCIÓN DE XILITOL POR FERMENTACIÓN DE MEDIO HIDROLIZADO DE SEMILLA DE TAMARINDO CON *Kluyveromyces marxianus*

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Abstract

Tamarind seed consists of 50 to 72 % of a branched heteropolysaccharide, called xyloglucan. By fragmenting xyloglucan with a coupled process of acid hydrolysis and thermal treatment, it is possible to obtain considerable amounts of fermentable sugars, glucose and xylose being the most abundant. Xylose is the precursor of xylitol, a sugar with similar characteristics to sucrose. Chemical synthesis of xylitol is very expensive and of low-yield. On the other hand, xylitol can be obtained by fermentation, using yeasts that incorporate xylose in their metabolism, such as *Kluyveromyces marxianus*. Being a biological process, xylitol production by fermentation depends on different environmental factors. In this paper, the effect of two factors on xylitol production was evaluated based on a 3^2 factorial experimental design: initial substrate concentration (20-80 g/L) and agitation (120-240 rpm). Both factors considerably influenced xylitol production of *K. marxianus*, where the optimization of the experimental design predicted a yield of 0.57 g of xylitol/g of xylose, with an initial substrate concentration of 50 g/L and an agitation of 177 rpm, from a source substrate of which there are no reports of its use in this field, such as tamarind seed.

Keywords: xylose, fermentation, xylitol, initial substrate concentration, agitation.

Resumen

La semilla de tamarindo está compuesta en un 50 a 72 % por un heteropolisacárido ramificado, llamado xiloglucano. Mediante su fragmentación con un proceso acoplado de hidrólisis ácida y tratamiento térmico, es posible obtener cantidades considerables de azúcares fermentables, siendo la glucosa y la xilosa los más abundantes. La xilosa es el precursor del xilitol, un azúcar con características similares a la sacarosa. La síntesis química del xilitol es costosa y de bajo rendimiento. Por otro lado, se puede obtener xilitol por fermentación usando levaduras que incorporan la xilosa a su metabolismo, como *Kluyveromyces marxianus*. Al ser un proceso biológico, la producción de xilitol depende de diferentes factores ambientales; en este estudio, se evaluó el efecto de dos factores usando un diseño experimental factorial 3^2: concentración inicial de sustrato (20-80 g/L) y agitación (120-240 rpm). Ambos factores tuvieron un impacto considerable sobre la producción de xilitol de *K. marxianus*, donde la optimización del diseño experimental predijo un rendimiento de 0.57 g de xilitol/g de xilosa, a una concentración inicial de sustrato de 50 g/L y agitación de 177 rpm, usando una fuente de sustrato que no ha sido reportada: la semilla de tamarindo.

Palabras clave: xilosa, fermentación, xilitol, concentración inicial de sustrato, agitación.

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1 Introduction

Xylitol is a natural carbohydrate, chemically classified as an alcohol-sugar of high added value owning to its dietetic and technological properties: it does not produce Maillard browning (Silva and Roberto, 2001), it increases both taste and color in food without affecting its properties (Mushtaq *et al*., 2010), it has the same sweetness as sucrose, while being suitable for diabetic use, since its metabolism is not regulated by insulin (Ahmed, 2001), it possesses anti-caries properties and facilitates the mineralization of teeth, thereby preventing cavity formation (Bahador *et al*., 2012; Diz *et al*., 2002). Nowadays, xylitol is obtained by catalytic hydrogenation at high pressure and temperature, using commercial-type xylose or corncob. Its chemical synthesis is not only expensive but also produces undesirable byproducts, as shown by its low-level yield (Granström, 2002; Vanegas *et al*., 2004).

The conversion of xylose to xylitol can be obtained by a fermentation process, in which yeasts are recognized as the best producers of said compound, employing a fermentation medium with high xylose content as substrate. The most studied yeasts in the production of xylitol are of the genus *Candida*, where *Candida guilliermondii* is notable for its high fermentation productivity in hydrolyzed media of organic residues (Ghindea *et al*., 2010). Another less studied yeast of this genus is *Candida magnoliae*, although it could be a good candidate for the production of xylitol together with acceptable fermentation results in corncob hydrolyzed media (Kiyoshi *et al*., 2004).

Some other organic sources composed of rich-xylose polysaccharides which have been used for obtaining xylitol by fermentation are: hydrolyzed eucalyptus and wheat straw (Martínez *et al*., 2002), hydrolyzed rice straw (Roberto *et al*., 1999), hydrolyzed sugar cane (Santos *et al*., 2003), barley-malt residue (Solange and Inês, 2005), corncob with added xylose and rice husk (Villalba *et al*., 2009).

The use of hydrolyzed tamarind seed media has not been reported to obtain xylitol. Tamarind seed is composed of an elevated amount of carbohydrate 50 to 72 % (El-Siddig *et al*., 2006) which are found in the form of a heteropolysaccharide, known as xyloglucan. Xyloglucan, or tamarind gum, consists principally of D-glucose, D-xylose, D-galactose and L-arabinose in proportions of 8: 4: 2: 1, respectively (Kaur *et al*., 2006); it is formed by a base chain of D-glucans joined by β-links (1-4) partly substituted in position 6 of D-glucopyranosyl residue by α-D-xylopyranose or 2-0-β-D-galactopyranosyl α-D-xylopyranose (Kumar and Bhattacharya, 2008). Due to its high content of xylose, the tamarind seed constitutes a potential substrate source for yeast producers of xylitol (González-Hernández *et al*., 2012).

Xylitol production by fermentation depends on various factors, such as the amount of oxygen, amount of inoculum, concentration of substrate, components of the fermentation media used, temperature and pH (Srivani and Pydi, 2011). In this study, the effect of initial substrate concentration and agitation with reference to xylitol production by fermentation of *K. marxianus* in hydrolyzed tamarind seed media was evaluated.

2 Materials and methods

2.1 Experimental design

The influence of two factors, namely the initial substrate concentration (reducing sugars) and agitation - which affect yeast growth, the yield and production of xylitol - was evaluated by a factorial 3^2 experimental design. Table 1 shows the experimental design with 9 factorial treatments, which were duplicated.

2.2 Hydrolyzed tamarind seed media

The tamarind seed without its coat was ground and sieved so as to obtain a particle of 0.125 mm maximum diameter. The resultant powder was hydrolyzed with 3% nitric acid, heating in an autoclave at 121 °C for 20 min, as per González-Hernández *et al*. (2012). In order to eliminate the remaining solids, the hydrolyzed medium was centrifuged at 4000 rpm for 15 min and subsequently filtered.

2.3 Microorganism and preparation of inoculum

The yeast *K. marxianus* ITMLB03, was grown in Petri plates at 30 °C for 48 h with a medium of YPD-hydrolyzed tamarind seed (10 g/L glucose, 10 g/L sugars in the hydrolyzed tamarind seed media, 10 g/L bactopeptone, 10 g/L yeast extract, 10 g/L agar), as the pre-adaptation of the yeast to the culture medium, and kept in refrigeration. The stored yeast was transferred

by a bacteriological loop to 25 mL of fermentation medium (initial substrate concentration depending on the treatment in the experimental design) in a 50 mL Erlenmeyer flask for the preparation of the inoculum, then incubated at 30 °C for 24 h and shaken according to the corresponding treatment.

2.4 Fermentation conditions

The pH of the hydrolyzed tamarind seed media was adjusted to 6.0 with tris $NH_2C(CH_2OH)_3$ 2M; but was not controlled during fermentation. The concentration of reducing sugars of the hydrolyzed medium was adjusted by diluting with distilled water according to the different treatments of the experimental design for the initial substrate concentration (Table 1). The medium was enriched with ammonium phosphate salts $(NH_4)_2H_2PO_4$ 1 g/L as the nitrogen source.

For each test, 3×10^6 cells/mL was inoculated into 100 mL of the fermentation medium in a pre-sterilized 250 mL Erlenmeyer flask and then incubated at 30 °C for 92 h agitation in each treatment being in accordance with the experimental design. Samples were taken every 4 h, following cellular growth by which yeast doubling time was calculated (t_d), substrate (glucose and xylose) consumption and the production of xylitol, ethanol and glycerol were also followed with reference to the conversion factor of xylose to xylitol ($Y_{p/s}$).

Table 1. Treatments and coded values for the factorial 3^2 experimental design

Treatment	Agitation (rpm)	Initial substrate concentration (g/L)	Coded values	
			A	B
1	120	20	-1	-1
2	180	20	0	-1
3	240	20	1	-1
4	120	50	-1	0
5	180	50	0	0
6	240	50	1	0
7	120	80	-1	1
8	180	80	0	1
9	240	80	1	1

2.5 Analytical methods

The concentration of reducing sugars in the hydrolyzed medium plus the diluting process was measured using the DNS oxide-reduction method taken and modified by Pérez *et al.* 2013: in the presence of reducing sugars, 3, 5-dinitrosalicylic acid is reduced to 3-amino-5-nitrosalicylic acid, resulting in a change of colour which is measured by absorbance at a wavelength of 540 nm.

The concentration of sugars constituting the hydrolyzed medium and being the substrates in the fermentation samples were estimated every 12 h using enzyme assays (Megazyme® kits) for glucose (with glucosidase and peroxidase enzymes) and xylose (by the catalyzed reaction for xylose dehydrogenase), with a resultant change in absorbance of the sample measured in an UV-Visible JENWAY 6305® spectrophotometer. Quantification of furfurals in the hydrolyzed medium of tamarind seed was carried out according to the method proposed by Martínez *et al.* (2000). The furfural and hydroximethylfurfural show an united absorbance peak in the UV spectrum at 284 nm. Some other components present in the residual organic hydrolyzed media absorb in the same region but maintain an absorbance code at 320 nm. Using the subtraction of both absorbances and comparison with a standard curve, the presence of the components in the hydrolyzed tamarind seed media was measured.

The main fermentation products were calculated by enzymatic tests with Megazyme® kits. For xylitol (xylitol dehydrogenase and diaphorase reactions), ethanol (in the catalyzed reactions by alcohol dehydrogenase and aldehyde dehydrogenase) and, finally, glycerol (with glycerokinase and lactate dehydrogenase), from the absorbance changes of the sample measured in a UV-Visible JENWAY 6305® spectrophotometer.

2.6 Statistical analysis

Result analyses were carried out on Statgraphics Centurion XV® software so as to evaluate 3 response variables of the experimental design: doubling time (t_d), product-substrate yield ($Y_{p/s}$) and xylitol production. Furthermore, a Tukey-Kramer test using JMP 6.0® software was performed on the respective response variables together with the production of ethanol and glycerol.

Table 2. Composition of the hydrolyzed tamarind seed media with different levels of initial substrate concentration

Level	Initial substrate concentration (g/L)	Glucose (g/L)	Xylose (g/L)	Furfurals (g/L)
-1	20	15.580 ± 0.344	5.912 ± 0.473	0.052 ± 0.010
0	50	35.289 ± 0.750	11.621 ± 1.222	0.128 ± 0.008
1	80	49.271 ± 1.167	14.558 ± 1.585	0.225 ± 0.010

Data show the means ± standard deviation for $n = 6$

3 Results and discussion

The composition of hydrolyzed tamarind seed and its dilutions to establish levels of the initial substrate concentration factor are shown in Table 2. The hydrolyzed tamarind seed prepared according to conditions established by González-Hernández *et al*. (2012) contained high concentrations of both glucose and xylose, compared to those of same monosaccharides in other organic hydrolysates which have been used as a source of substrate for the production of xylitol. Martínez *et al*. (2002) report on four hydrolyzed hemicellulosic composites: sugar cane bagasse with concentrations of glucose and xylose of 1.70 and 22.71 g/L, respectively; rice straw at 3.29 and 18.33 g/L; hydrolyzed eucalyptus at 1.53 and 24.32 g/L, and for wheat straw at 2.79 and 10.65 g/L.

The production of xylitol by fermentation of hydrolyzed hemicellulosics is hindered by toxic compounds resulting from the conditions in which hydrolysis is carried out. Such toxins can be: acetic acid, phenolic derivatives, furfural and hydroximehylfurfural (Fernández *et al*., 2007). Those furans result from the Maillard reactions of pentoses and hexoses during the acid hydrolysis and form the principal inhibitor group of hydrolyzed media; furfural results from dehydration of pentoses, while hydroximethylfurfural results from hexoses (Palmqvist and Hahn-Hägerdal, 2000). Few authors have reported on the concentration of furfurals in hydrolyzed media obtained from organic residues. Concentrations produced of furfurals, due to the hydrolytic conditions present in this study (Table 2) are slightly higher than those reported by Martínez *et al*. (2002) for sugar cane bagasse (0.19 g/L), but lower than those obtained by the same authors for rice straw (0.27 g/L), eucalyptus wood (0.64 g/L) and wheat straw (0.43 g/L).

In general, *K. marxianus* adapted well to the hydrolyzed tamarind seed media in the majority of experiments. The doubling time is a response variable calculated from yeast growth curves (data not shown) and allows *K. marxianus* adaptation to be studied under different treatments. A lower doubling time would indicate an accelerated growth of the yeast, suggesting a greater adaptation to the fermentation medium.

Table 3. Average results of factorial 3^2 experimental design for different parameters compared by Tukey-Kramer statistical test

Treatment	Factor A	Factor B	$Y_{p/s}$ (g xylitol/g xylosa)	t_d (h)	Xylitol (g/L)	Ethanol (g/L)	Glycerol (g/L)
1	-1	-1	0.4121^{CD}	2.44^{D}	1.57^{CD}	12.09^{E}	1.47^{A}
2	0	-1	0.6354^{A}	2.74^{D}	4.36^{AB}	19.11^{BC}	1.79^{A}
3	1	-1	0.0552^{F}	-	0.01^{E}	0.01^{G}	0^{B}
4	-1	0	0.2183^{E}	2.89^{D}	1.15^{CD}	14.47^{D}	1.58^{A}
5	0	0	0.6098^{A}	2.80^{D}	3.62^{B}	18.30^{C}	1.67^{A}
6	1	0	0.4445^{C}	7.04^{A}	4.35^{AB}	22.04^{A}	1.69^{A}
7	-1	1	0.2036^{E}	2.76^{D}	0.81^{D}	11.92^{E}	1.85^{A}
8	0	1	0.3043^{DE}	3.46^{C}	1.74^{C}	9.18^{F}	2.86^{A}
9	1	1	0.5043^{BC}	4.91^{B}	4.44^{A}	20.91^{AB}	2.38^{A}

Means followed by the same letter do not differ at a significance level α=0.05. Table representative for n = 2

Table 4. ANOVA decomposed to effects with one degree of freedom for the doubling time

Source of variation	Sum of Squares (SS)	Degrees of Freedom (df)	Mean Square (MS)	F-value	P-value
A:Initial substrate concentration	4.98482	1	4.98482	2.56	0.1379
B:Agitation	11.8125	1	11.8125	6.07	0.0315
AA	0.448587	1	0.448587	0.23	0.6406
AB	10.5407	1	10.5407	5.41	0.0401
BB	9.29996	1	9.29996	4.78	0.0514
Blocks	0.00963272	1	0.00963272	0	0.9452
Error	21.4133	11	1.94666		
Total	58.5096	17			

Null hypotheses are rejected when the P-value is less than the established significance level (α=0.05)

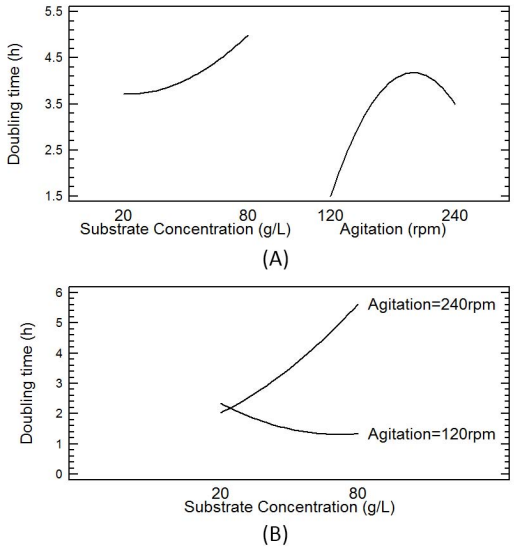

Fig. 1. Effects of initial substrate concentration and agitation on the doubling time (td): (A) main effects of the factors, and (B) interaction effect.

Doubling times obtained from various treatments are shown and statistically compared in Table 3. The only treatment where neither growth nor consumption of substrates present in the fermentation medium were observed, was that at an initial concentration of 80 g/L and agitation of 120 rpm.

Table 4 shows the analysis of variance (ANOVA) at a significance level α of 0.05, decomposed to view effects with one degree of freedom. Agitation showed the largest effect on the response variable, its lineal component being as significant as the quadratic one. This fact can be confirmed in Figure 1A, where an increase in agitation also considerably increased doubling time to maximum level after which it began to decrease. The curvature in this area would correspond to the quadratic component of the effect. On the other hand, the effect of the A factor (initial substrate concentration) is not significant, in spite of a slight linear effect observed in Figure 1A. However, a low level of initial substrate concentration would have less doubling time as a consequence.

The interaction of the different factors was significant on a confidence level of 95 % (Figure 1B). This interactive effect could be verified by using the Tukey-Kramer comparative test on the average treatment results (Table 3). Treatments at 120 rpm did not show statistically significant differences and represent the lowest doubling times (together with treatments 4, 5 and 7). Treatments with largest effect were those performed at a high level of initial substrate concentration (80 g/L), where the values were elevated and statistically different from the already mentioned (treatments 6 and 9) - among which we could find those conditions unfavorable for cell growth, corresponding to treatment 3.

At high levels of initial substrate concentration (i. e. 80 g/L) we found the highest concentration of inhibitory substances in the fermentation medium (Table 2), which are the furfurals produced during the hydrolysis process (Fernández *et al*., 2007). These concentrations could prove lethal in conditions where there is little substrate diffusion, as in treatment 3 (at 120 rpm), where there was no cell growth. Various methods to purify hemicellulosic hydrolyzed media have been employed in order to lower the concentration of these components, which might help to improve the adaptation of yeast to high sugar concentrations. Exposure of the medium to activated carbon or ionic-exchange resins, have given the best results (Nápoles *et al*., 2006; Viñals *et al*., 2006).

Another factor to be considered is the osmotic stress generated by high concentrations of substrate in the fermentation medium. Catabolic repression (or that of glucose) has been identified in many

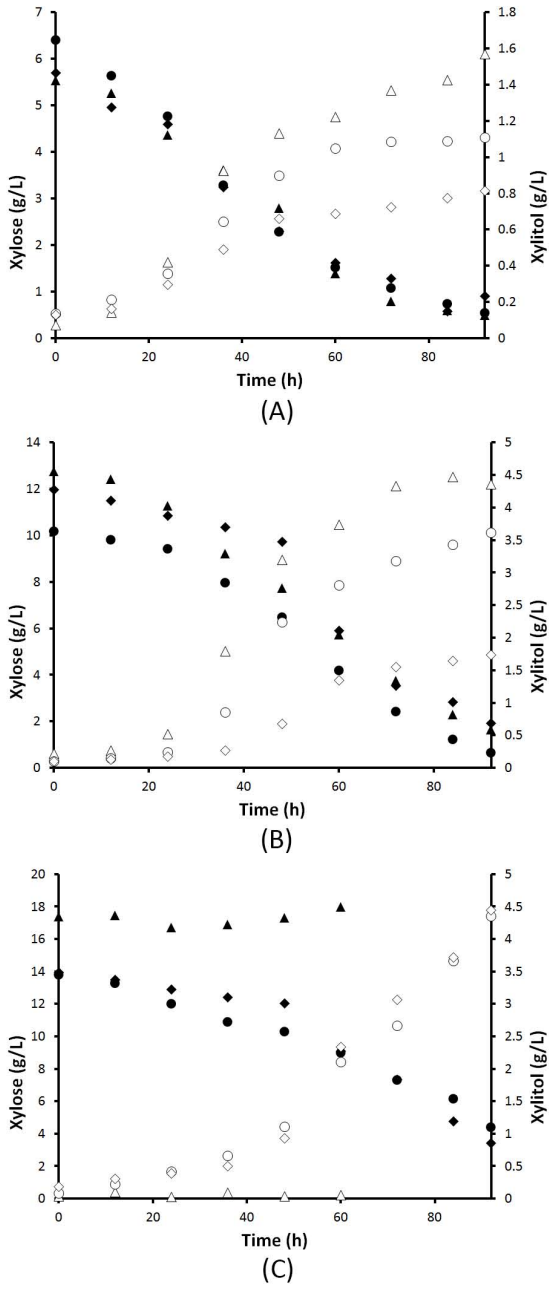

Fig. 2. Consumption of xylose and xylitol production of the treatments of the factorial 32 experimental design. Agitation of (▲, △) 120 rpm, (●, o) 180 rpm and (♦, ◊) 240 rpm, for different initial concentrations of substrate: (A) 20 g/L, (B) 50 g/L and (C) 80 g/L. Representative data for n=2.

microorganisms, including yeasts. Accordingly, transcription of genes whose products are essential for the catabolism of carbon sources slowly fermentable is inhibited in the presence of a rapidly-consumed carbon source, such as glucose. This phenomenon occurs in those genes involved in respiration, gluconeogenesis and the use of alternate carbon sources, as in xylose. The consequence of this fact is the inhibition of the production of components useful in the regulation of osmotic pressure by yeasts, thereby reducing its activity (Gancedo, 2008; Santagelo, 2006). Glucose consumption by yeast was similar with the various treatments, practically using it all up (almost to 100 %) after 48 h of fermentation, with the exception of treatment 3.

At low level initial substrate concentration (20 g/L) the consumption of xylose among treatments with different levels of agitation resulted similar over time demonstrating a linear tendency (Figure 2A), with an approximate consumption of 90 % regarding available xylose in all cases. The production of xylitol was the most affected by varying in agitation at this initial substrate concentration. Xylitol production was favored at lower agitation, 120 rpm, reaching maximum production among these three treatments (1.57 g/L) at the end of fermentation. Notably, the product generated at 240 rpm was the lowest, achieving only 0.8 g/L of xylitol.

Total consumption of xylose by *K. marxianus*, showed only a slight decrease when the initial substrate concentration was changed to an intermediate value (50 g/L) with an approximate consumption of 88 % for all treatments at this level (Figure 2B). Again, the production of xylitol was favored at an agitation of 120 rpm reaching a final concentration of 4.36 g/L quite the opposite to a high level agitation, where product concentration is seriously affected, resulting in 1.74 g/L xylitol.

High levels of initial substrate concentration (80 g/L) resulted in the lowest consumption of xylose. Intermediate and high levels of agitation showed consumption of approximately 72 % of available xylose in the fermentation medium (Figure 2C). In this latter case, no effect was observed concerning intermediate to high levels of agitation. At the end of the fermentation process, xylitol concentrations of 4.35 g/L and 4.44 g/L respectively were obtained.

The product-substrate yield was measured through the consumption of xylose and production of xylitol, which is shown and statistically compared together with the final production of xylitol from each treatment in Table 3.

Table 5. ANOVA decomposed to effects with one degree of freedom for the product-substrate yield

Source of variation	Sum of Squares (SS)	Degrees of Freedom (df)	Mean Square (MS)	F-value	P-value
A:Initial substrate concentration	0.00962767	1	0.00962767	0.54	0.4779
B:Agitation	0.00272707	1	0.00272707	0.15	0.7033
AA	0.176666	1	0.176666	9.9	0.0093
AB	0.216186	1	0.216186	12.12	0.0051
BB	0.0205683	1	0.0205683	1.15	0.3059
Blocks	6.96889E-06	1	6.96889E-06	0	0.9846
Error	0.196201	11	0.0178364		
Total	0.621983	17			

Null hypothesis are rejected when the P-value is less than the established significance level (α=0.05).

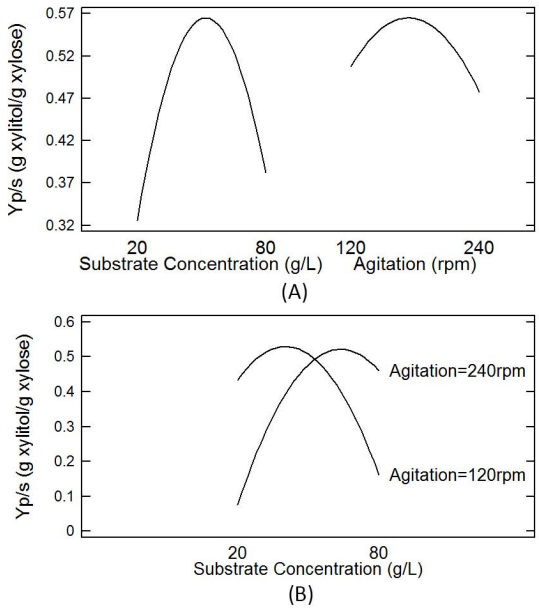

Fig. 3. Effects of initial substrate concentration and agitation on the product-substrate yield ($Y_{p/s}$): (A) Principal effects of the factors and (B) interaction effect.

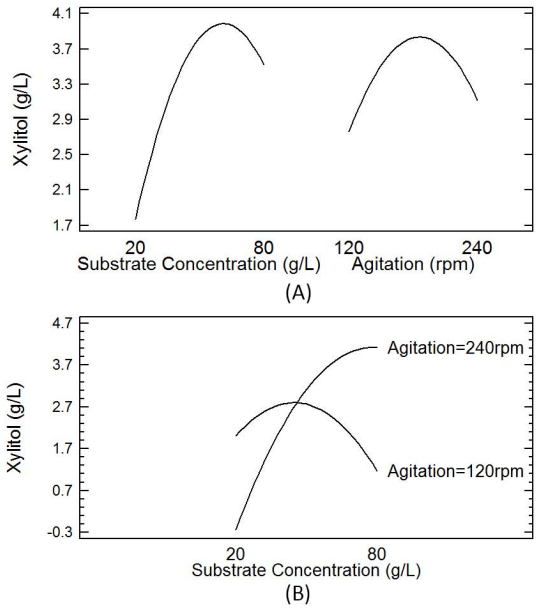

Fig. 4. Effects of initial substrate concentration and agitation on the production of xylitol: (A) Principal effects of the factors and (B) interaction effect.

Analysis of variance applied to product-substrate yield decomposed to effects with one degree of freedom, is shown in Table 5. This allows to conclude that the main effects on the factors are due to quadratic component of the initial substrate concentration and its interaction with agitation.

The quadratic effect of the initial substrate concentration factor is clearly shown in Figure 3A, where the maximum is seen to be very close to the intermediate level used in the experimental design (50 g/L). In spite of the fact that ANOVA gave no significance to agitation, a quadratic graphic effect of this factor can be observed, also reaching a maximum point at a mid-level (150 rpm).

The main effect found was the factor interaction (Figure 3B), where at a low agitation the response variable is benefited by an initial substrate concentration between 20 and 50 g/L, while at a high agitation, concentrations from 50 to 80 g/L should be used. However, the tendency towards a mid level agitation is not seen in the graph, where as has been already mentioned more favorable yield conditions may be found.

Table 6. ANOVA decomposed to effects with one degree of freedom for the xylitol production

Source of variation	Sum of Squares (SS)	Degrees of Freedom (df)	Mean Square (MS)	F-value	P-value
A:Initial substrate concentration	9.26115	1	9.26115	6.04	0.0318
B:Agitation	0.370095	1	0.370095	0.24	0.6329
AA	5.57952	1	5.57952	3.64	0.0829
AB	13.4753	1	13.4753	8.79	0.0129
BB	3.13007	1	3.13007	2.04	0.1809
Blocks	0.0356623	1	0.0356623	0.02	0.8816
Error	16.8702	11	1.53365		
Total	48.722	17			

The null hypothesis are rejected when the P-value is less than the established significance level (α=0.05).

Employing the Tukey-Kramer statistic test, in Table 3, it can be proved that the conditions with the best average yield ($Y_{p/s}$) value were those with a mid-level of initial substrate concentration (50 g/L) and low/medium levels of agitation (120 rpm to 180 rpm) corresponding to treatments 2 and 5, which are statistically distinct to the rest with a significance level α of 0.05.

The analysis of variance with respect to the initial substrate concentration and agitation on the production of xylitol is shown in Table 6.

The initial substrate concentration both in its lineal component and in its quadratic one corresponds to the principal effects on the production of xylitol, due only to the separate factors as indicated in the analysis of variance. These effects can be clearly seen in Figure 4A, where there is a significant lineal increase owing to a rise in the initial concentration of substrate, reaching a maximum level and thereafter decreasing, consequently provoking a curve (i. e. quadratic effect) in the response variable.

The graph showing principle effects (Figure 4A) would suggest an important curvature, caused by agitation. Nevertheless, this effect was not found to be significant when looking at the analysis of variance in Table 6.

The principal effect on the response variable, the production of xylitol, is due once more to the interaction of those factors employed in the experimental design, and can be seen in Figure 4B.

Treatments resulting in higher xylitol production were those carried out at a high levels of initial substrate concentration, with the exception of that with a low agitation level, where there was no production (Table 3). Treatment 9 was the one resulting in the highest production of xylitol, but was not statistically different to treatments 2 and 6. In fact, these two treatments were no statistically different to number 5, where high production values of xylitol at initial substrate concentrations of 50 g/L were found.

When relating yield ($Y_{p/s}$) to xylitol production, it can be concluded that the most favorable treatments for both variables were those of a mid level of initial substrate concentration (50 g/L) and low medium levels of agitation (120 rpm to 180 rpm).

The factorial 3^2 experimental design has the advantage of allowing optimization of the factors studied, within the limits of each one. Therefore, optimization of the principal response variable, product substrate yield has been carried out here employing the StatGraphics Centurion® software. The graph of surface contours estimated response is shown in Figure 5. In this way, a maximum value can be predicted for the response variable of 0.5653 g of xylitol/g of xylose at an initial substrate concentration of 51.43 g/L and agitation of 177 rpm.

The highest yields obtained experimentally, correspond to treatments 2 and 5 of 0.64 and 0.61 g of xylitol/g of xylose respectively, using *K. marxianus* fermenting hydrolyzed tamarind seed media, and are similar than those reported by

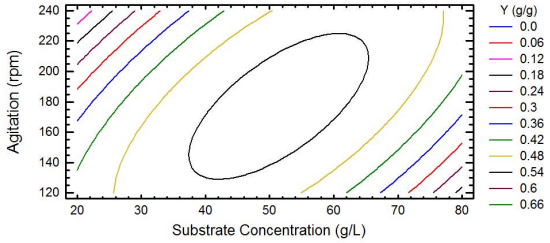

Fig. 5. Surface contours estimated response to product-substrate yield ($Y_{p/s}$) for the factorial 3^2 experimental design.

Martínez *et al*. (2002) employing hydrolyzed sugar cane bagasse as substrate, which was 0.63 g of xylitol/g of xylose. Furthermore, yields quoted in this study are higher than others reported using different sources of organic substrates for fermentation at the flask level: 0.55 g of xylitol/f of xylose from rice straw with *C. guilliermondii* (Silva and Roberto, 2001), 0.57 f of xylitol/g of xylose from eucalyptus wood with *D. hansenii* (Diz *et al*., 2002), plus a synthetic medium of xylose of 0.57 g of xylitol/g of xylose with *C. magnoliae* (Wannawilai *et al*., 2007).

Final concentrations of other products resulting from fermentation namely ethanol and glycerol are shown and statistically compared in Table 3.

The treatments showing a larger production of ethanol were those performed with an initial substrate concentration of 80 g/L (owing to a high glucose concentration), together with med/high agitation levels (Table 3). Treatment 9 produced a greater quantity of ethanol, but was not statistically different to treatment 6, which in turn showed no significant difference to treatments 2 and 5 i. e. those with a medium initial substrate concentration. It would appear that the production of ethanol does not affect that of xylitol, as seen precisely in treatments 6 and 9 (according to the statistical analysis) where the highest concentrations of both are reached.

According to Table 3, no significant differences were noted in any of the treatments with respect to glycerol production by *K. marxianus*, with exception of 3, in which there was no cell growth. Despite the fact that greater values were found with treatments with a high level of agitation and med/high levels of initial substrate concentration, the Tukey-Kramer statistic test did not show a significant difference with reference to other treatments.

Conclusions

Significant concentrations of fermentable sugars (49.27 g/L of glucose and 14.56 g/L of xylose) were obtained by hydrolysis of tamarind seed, these results being higher than those by hydrolysis of other organic sources. Tamarind seed, an organic residue resultant from agro-industrial activity, could well be an important source of substrate for the production of ethanol and xylitol.

Factors studied here such as agitation and initial substrate concentration had a significant influence with reference to the production of xylitol by fermentation of a hydrolyzed medium of tamarind seed with *K. marxianus*, as well as its growth. *K. marxianus* showed reduced doubling times in the majority of treatments carried out, principally in those at a low level of agitation (120 rpm). A high concentration of substrate considerably affected both growth and productivity of yeast in fermentation of hydrolyzed tamarind seed media, which may have been due to the high content of inhibitory components in said concentration or that osmotic pressure of this sugar known to affect the yeast.

The highest yield reached (0.63 g of xilitol/g of xylose) resulted even greater than those reported by other authors, employing hydrolyzed hemicelluloses from various organic sources. With reference to the aforementioned yield at flask level, this is high and in some cases similar to those reported by a few authors.

In accordance with the factorial 3^2 experimental design, taking into account the factors studied optimum conditions for the production of xylitol, give a product substrate yield result of 0.5653 g of xylitol/g of xylose at an initial substrate concentration of 51.43 g/L and agitation of 177 rpm.

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