

Evaluation of Nutrient Supplementation to Charcoal-Treated and Untreated Rice Straw Hydrolysate for Xylitol Production by *Candida guilliermondii*

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ABSTRACT

Xylitol was produced by *Candida guilliermondii* from charcoal-treated and untreated rice straw hemicellulosic hydrolysate with or without nutrients (ammonium sulphate, calcium chloride, rice bran extract). Both, xylitol yield and volumetric productivity decreased significantly when the nutrients were added to treated and untreated hydrolysates. In the treated hydrolysate, the efficiency of xylose conversion to xylitol was 79% when the nutrients were omitted. The results demonstrated that rice straw hemicellulosic hydrolysate treated with activated charcoal was a cheap source of xylose and other nutrients for xylitol production by *C. guilliermondii*. The non-necessity of adding nutrients to the hydrolysate media would be very advantageous since the process becomes less costly.

Key words: Rice straw hydrolysate, nutrient, activated charcoal, *Candida guilliermondii*, xylitol

INTRODUCTION

Rice straw is a fibrous lignocellulosic material that differs from most crop residues in its high content of silicon dioxide (SiO₂). Ash content on a dry weight basis ranges from 13 to 20%, varying according to the state of conservation of the straw after harvest. Ash generally contains 75% SiO₂, 10% K₂O, 3% P₂O₅, 3% Fe₂O₃, 1.3% CaO and smaller amounts of Mg, S, and Na (Kadam et al., 2000). Rice straw also contains a large amount of hemicellulose fraction that can be easily hydrolysed to its constituent carbohydrates, a mixture of sugars, containing mainly xylose (Roberto et al., 2003). Xylose solution obtained can be used as a fermentation medium to produce xylitol (Silva and Roberto, 1999, 2001; Mussatto and Roberto, 2003), a five-carbon sugar alcohol and a food edulcorant with clinical properties and

considerable pharmaceutical applications (Mussatto and Roberto, 2002).

All published data on xylitol production by yeasts have demonstrated that xylitol accumulation is influenced by a number of experimental conditions. Studying the effect of these conditions is of particular interest as a prerequisite for higher xylitol yields and productivities (Winkelhausen and Kuzmanova, 1998). Major improvements in the productivity of bioconversion processes are generally ascribed to the development of superior microbial strains by mutation. However, other parameters such as the nutritional and physical environment to which an organism is exposed are also known to alter product yield significantly (Greashan and Inamine, 1996). Hydrolysate treatment with activated charcoal has also been considered as a good method to overcome microbial inhibition caused by toxic substances,

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improving the fermentability of the hydrolysate (Winkelhausen and Kuzmanova, 1998; Mussatto and Roberto, 2001; Mussatto, 2002). However, besides removing toxic substances, the charcoal can also adsorb compounds that are essential to the microbial growth.

The present study evaluated the effect of nutrient supplementation to rice straw hydrolysates on xylitol production by *Candida guilliermondii*.

MATERIALS AND METHODS

Hemicellulosic Hydrolysate

Rice straw containing (% w/w) 43.5 cellulose, 22.0 hemicellulose, 17.2 lignin and 11.4 ash (Mussatto, 2002) was dried in the sun and milled to attain particles of about 1 cm in length and 1 mm in thickness. The hemicellulosic hydrolysate was obtained by acid hydrolysis in a 350-l stainless-steel pressure reactor as described by Roberto et al. (2003) and subsequently was concentrated (under vacuum) in a 4-l evaporator at $70 \pm 5^\circ\text{C}$ to obtain a xylose content of approximately 150 g/l. The concentrated hydrolysate was over-titrated with NaOH (pellets) to pH 8.0 and adjusted to pH 6.5 with concentrated sulphuric acid. This was termed as untreated hydrolysate. The hydrolysate was mixed with activated charcoal (40 g of hydrolysate per g of charcoal) and after agitation at 200 rpm, 30°C for 1 h, the charcoal was removed by centrifugation at 2000 $\times g$ for 20 min. This was termed as treated hydrolysate.

Inoculum Preparation

Strain of *Candida guilliermondii* FTI 20037 was maintained at 4°C on malt extract agar slants. The inoculum was prepared by growing cells in 125 ml flasks containing 50 ml medium composed of (g/l): xylose 20, $(\text{NH}_4)_2\text{SO}_4$ 3, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.1 and rice bran extract (20% v/v). A 10% w/v suspension of rice bran was sterilised by autoclaving at 121°C for 20 min, and cooled to ambient temperature. This suspension was then aseptically centrifuged at 2000 $\times g$ for 20 min. The liquid fraction (rice bran extract) was stored at 4°C for one day. The solutions were sterilised by autoclaving at 121°C for 20 min, except xylose solution, which was autoclaved at 112°C for 15 min. The microorganism was cultivated in a rotatory shaker (200 rpm) at 30°C for 24 h and the cells were

separated by a 20 min centrifugation at 2000 $\times g$ and resuspended in sterilised water to obtain a dense cell suspension (about 30 g/l).

Media and Culture Conditions

Assays were performed using 125-ml Erlenmeyer flasks closed with cotton-wool plugs, containing 50 ml of medium consisting of charcoal-treated and untreated hydrolysates, diluted to 85 g/l xylose, supplemented with or without nutrients (ammonium sulphate (3 g/l), calcium chloride (0.1 g/l) and rice bran extract (20% v/v)) and inoculated to obtain an initial cell concentration of 3.0 g/l. Fermentation was carried out on an orbital shaker at 250 rpm, 30°C for 96 h. A 2^2 factorial design in order to determine the effect of the charcoal treatment (factor A) and nutrient supplementation (factor B) on the xylitol production was employed. The levels of the factors were conveniently coded into minus and plus signs.

Analytical Methods

Sugars and xylitol concentrations were determined by HPLC using a refractive index (RI) detector and a Bio-Rad HPX - 87H (300 \times 7.8 mm) column, a temperature of 45°C ; 0.005 M sulphuric acid as the eluent, flow of 0.6 ml/min and sample volume of 20 μl . Cell growth was evaluated spectrophotometrically at 600 nm, and estimated by means of a calibration curve (dry weight \times optical density at 600 nm) obtained from growing cells on hydrolysate medium on a rotatory shaker at 250 rpm, 30°C for 72 h.

RESULTS AND DISCUSSION

Expensive nitrogen sources (e.g. yeast extract and peptone) and various minerals have been used to supplement xylose culture media for xylitol production by yeasts (Azuma et al., 2000; Walter et al., 2001; Canettieri et al., 2001). However, when hemicellulose hydrolysates are used as culture media, the need for supplementation should be minimised, since they contain not only fermentable sugars, but also compounds that are essential for cell growth (Preziosi-Belloy et al., 2000; Canettieri et al., 2001). Considering that the cost of the culture medium is a decisive factor in determining the economic viability of the bioconversion process, it is very important to

define the minimal nutrient requirements for the hydrolysate bioconversion.

Recently, some studies have demonstrated that the hemicellulosic hydrolysates fermentability can be significantly improved by removing inhibitors compounds (Mussatto and Roberto, 2001; Mussatto, 2002) and by supplementing with nutrients (Preziosi-Belloy et al., 2000; Almeida e Silva et al., 2003).

The results of treated and untreated rice straw hydrolysates supplemented with or without nutrients are shown in Table 1. It could be observed that the nutrient supplementation did not affect xylose consumption, but in media without nutrient addition, both xylitol production and cell growth were favoured. After 96 h of bioconversion without nutrients, xylitol concentration was 40 g/l in the untreated hydrolysate and 59 g/l in the treated hydrolysate, showing an increase of almost 50%. However, in both media supplemented with nutrients, xylitol concentration was about 30 g/l. These results suggested that the charcoal adsorption removed the potential bioconversion inhibitors, but not the compounds that were essential to the microbial metabolism. Indeed, the nutrient supplementation had a dramatic inhibiting effect on xylitol production, probably due to imbalance between the ionic nutrition, which was reflected in low tolerance to the environment. Silva and Roberto (1999) suggested that the utilisation of ammonium sulphate by the microorganisms could promote a liberation of sulphate ions, which generated H_2SO_4 , decreasing the pH of the fermentation medium and thus, affecting the yeast activity.

A Pareto chart was used to perform a statistical analysis of the experimental data. Fig. 1 (a, b and c), represents the estimated effects of the charcoal treatment and nutrient supplementation on xylitol and biomass production, and on xylose utilisation, in descending order. The length of each bar was proportional to the standardised effect. Bars extending beyond the vertical line corresponded to effects statistically significant at 95% confidence level. It could be observed that the charcoal treatment (factor A) influenced positively all responses studied. However, nutrients supplementation (factor B) did not affect xylose utilisation and biomass concentration, showing a strong negative influence only with xylitol production. Fig. 1a also revealed that factor A (hydrolysate treatment) interacted negatively with

factor B (nutrient supplementation) during xylitol production. This meant that the best results were provided by the hydrolysate treated with activated charcoal without the addition of ammonium sulphate, rice bran extract and calcium chloride.

The effects of nutrient supplementation on the bioconversion parameters of *C. guilliermondii* grown on hydrolysates treated with activated charcoal or on untreated hydrolysates are shown in Table 2. The volumetric productivity (Q_P) and yield factor ($Y_{P/S}$) varied strongly from 0.32 to 0.62 g/l.h and from 0.40 to 0.72 g/g, respectively.

The highest yield (0.72 g/g) and productivity (0.62 g/l.h) were obtained when *C. guilliermondii* was cultivated in treated hydrolysate (initial xylose = 85 g/l) without nutrient supplementation. Similar values ($Y_{P/S} = 0.80$ g/g and $Q_P = 0.58$ g/l.h) were reported by Preziosi-Belloy et al. (2000) for bioconversion process by *C. guilliermondii* from aspenwood hemicellulose hydrolysate (initial xylose = 50 g/l). Nevertheless, according to these authors, the addition of yeast extract (2 g/l) to the hydrolysate was fundamental to improving bioconversion rate and xylitol yield.

Several agricultural residues can be used to produce xylitol; however, the necessity of adding nutrients in the fermentation media varies for each lignocellulosic material employed. According to Almeida e Silva et al. (2003), xylitol production from eucalyptus hemicellulosic hydrolysate was dependent on the ammonium sulphate addition in the culture medium. Pessoa Jr et al., (1996) concluded that oligoelements and vitamins were present in sugarcane bagasse hydrolysate, but no phosphate.

Thus, when compared with the other lignocellulosic materials, rice straw appeared as a more economical alternative to produce xylitol, due the non-necessity of adding nutrients to the media formulated from its hydrolysate.

The necessity of adding nutrients in hydrolysate medium formulated from other lignocellulosic materials could be due to their low quantity of ash (around 4% w/w in sugarcane bagasse, wood and others, Kuhad and Singh, 1993). According to Kaddam et al. (2000), rice straw contained a high quantity of minerals, oligoelements and vitamins, in the ash (corresponding to 11.4% w/w). For this reason, the hydrolysate obtained from rice straw could be a rich source of nutrients essential for the growth of microorganisms in fermentative process

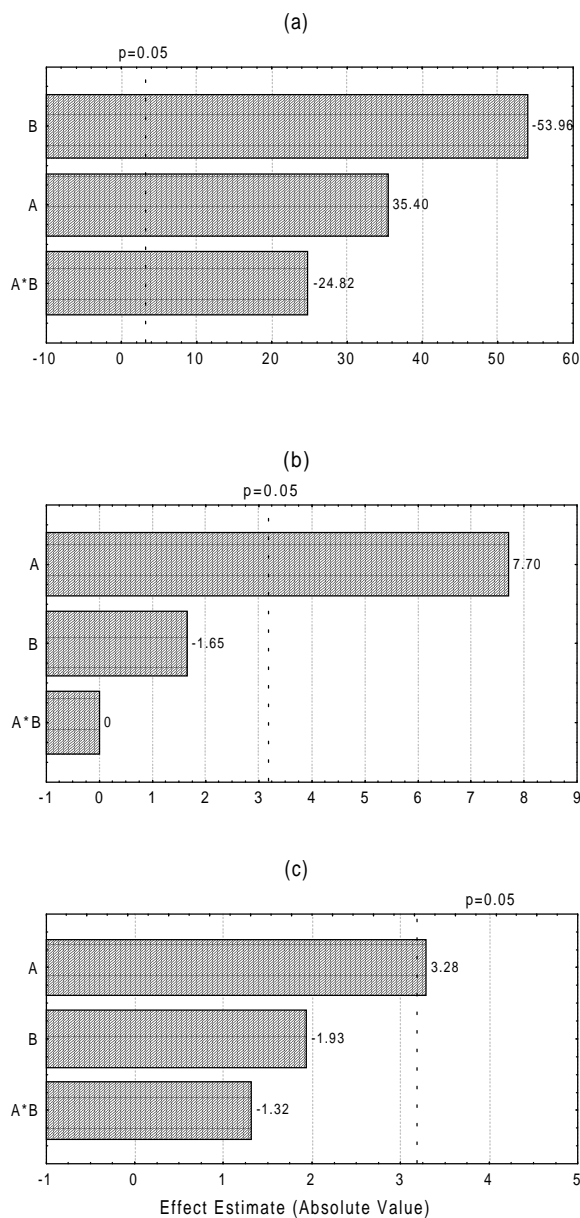


Figure 1 - Pareto chart for the effects of charcoal treatment (factor A) and nutrient supplementation (factor B) on the production of xylitol (a), and biomass (b) and on the utilisation of xylose (c) by *C. guilliermondii*.

Table 1 - Results of bioconversion of *C. guilliermondii*, after 96 h cultivation, from charcoal treated and untreated hydrolysate in response to addition of nutrients

Factors	Runs N°			
	1	2	3	4
Charcoal treatment (A)	- (without)	+ (with)	- (without)	+ (with)
Nutrients (B)	- (without)	- (without)	+ (with)	+ (with)
Responses				
Xylitol (g/l)	40.0	59.1	30.8	34.1
Xylose utilised (%)	82	94	79	93
Biomass (g/l)	8.0	12.6	7.4	9.4

Table 2 - Effect of nutrient supplementation on the bioconversion parameters of *C. guilliermondii* in the experiments with treated and untreated hydrolysates, after 96 h

Parameters	Untreated Hydrolysates		Treated Hydrolysates	
	with Nutrients	without Nutrients	with Nutrients	without Nutrients
$Y_{P/S}$ (g/g)	0.45	0.56	0.40	0.72
$Y_{X/S}$ (g/g)	0.05	0.06	0.06	0.10
Q_P (g/l.h)	0.32	0.42	0.36	0.62
η (%)	49	61	44	79

$Y_{P/S}$, xylitol yield coefficient, g xylitol per g xylose consumed; $Y_{X/S}$, cell yield coefficient, g drycell mass per g xylose and glucose consumed; Q_P , volumetric xylitol production rate, g/l.h; η , efficiency of bioconversion (considering 0.91 g/g the theoretical value).

CONCLUSIONS

The results of this study demonstrated that rice straw hemicellulose hydrolysate treated with activated charcoal was a cheap source of xylose and other nutrients for xylitol production by *Candida guilliermondii*. The non-necessity of adding nutrients to the hydrolysate media could be very advantageous since the process becomes less costly. This opens up new possibilities for using this substrate in other microbial process for ethanol and enzymes production, lowering the production costs.

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RESUMO

Este trabalho avaliou a produção de xilitol pela levedura *Candida guilliermondii*, a partir de hidrolisado hemicelulósico de palha de arroz não tratado e tratado com carvão ativo, ambos suplementados ou não com nutrientes (sulfato de amônio, cloreto de cálcio e extrato de farelo de arroz). Os resultados mostraram que tanto o rendimento como a produtividade volumétrica em xilitol diminuíram quando os nutrientes foram adicionados em ambos hidrolisados, tratado e não tratado. Em hidrolisado tratado, a eficiência de conversão de xilose em xilitol foi de 79% quando em ausência de nutrientes. Estes resultados

mostram que o hidrolisado hemicelulósico de palha de arroz tratado com carvão ativo é uma fonte barata de xilose e outros nutrientes, para a produção de xilitol por *Candida guilliermondii*. A não necessidade de adicionar nutrientes ao meio a base de hidrolisado é muito vantajosa, uma vez que o processo se torna mais econômico.

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