Vol.55, n. 5: pp.771-777, September-October 2012
ISSN 1516-8913 Printed in Brazil

BRAZILIAN ARCHIVES OF
BIOLOGY AND TECHNOLOGY
A N I N T E R N A T I O N A L J O U R N A L

Evaluation of Oat Hull Hemicellulosic Hydrolysate Fermentability Employing *Pichia stipitis*

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ABSTRACT

Oat hull hemicellulosic hydrolysate obtained by diluted acid hydrolysis was employed as fermentation medium for Pichia stipitis *cultivation. A comparison between the use of treated hydrolysate with 1% activated charcoal to reduce the toxic compounds generated during the hydrolysis process and untreated hydrolysate as a control was conducted. In the cultures using treated hydrolysate the total consumption of glucose, low xylose consumption and ethanol and glycerol formation were observed. The medium formulated with untreated hydrolysate showed morphological cell modifications with consequently cell death, no ethanol formation and formation of glycerol as byproduct of fermentative process, probably as a response to stressful conditions to yeast due to presence of high concentration of toxic compounds. Thus, further studies are suggested in order to determine the best conditions for hydrolysis and detoxification of the hydrolysate to improve the fermentative performance of* P. stipitis.

Key words: *Pichia stipitis*, hemicellulosic hydrolysate, oat hull, ethanol, activated charcoal

INTRODUCTION

The growing interest in the biotechnological use of byproducts generated by the agribusiness have been based on the premises of environmental conservation, low-cost feedstock and obtaining products of high added value through controlled processes.

In this context, new investigations in biotechnology have a fundamental importance to determine the behavior of microorganism grown on plant biomass, in solid form (Pinto 2007) or in cellulosic and hemicellulosic hydrolysates (Felipe et al. 1997; Mussato and Roberto 2002; Canilha et al. 2003; Tamanini et al. 2004; Marton et al. 2006; Jeon et al. 2010).

Oats, cereal member of the genus *Avena*, is a grass (Ceres 2011) with multiple possibilities for use as food, feed, forage, cover soil and green manure, besides the inhibition of weed infestations (Sá 1995). In Brazil, the estimated production for the 2010/2011 crop is about 379,000 tons of oat grains (Conab 2011).

The oat hull, a byproduct of grain milling, is equivalent to about 25-30% of grain weight (Wang and Klopfenstein 1993), whose main function is maintaining the grains clean and protected from mechanical destruction and pathogen attacks. This byproduct has traditionally been discarded and becoming a pollutant to the environment, although it can be utilized for the production of industrial solvents, sweeteners and animal feed, as well in food industry, in the production of breads, crackers and pasta (Galdeano 2006; González-Alvarado et al. 2010).

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According to Tamanini et al. (2004), oat hulls are composed of cellulose (29.26%), hemicellulose (28.35%) and lignin (22.22%), among others. Due to the high sugar content in the hemicellulose fraction of oat hulls, it may also be used in the bioprocesses to produce the products of high added value, such as ethanol (Lawford et al. 2001) and xylitol (Tamanini et al. 2004).

However, during diluted acid hydrolysis process, usually employed for the deconstruction of the hemicellulosic polysaccharides into fermentable sugars (D-xylose, D-glucose, L-arabinose), toxic compounds to microorganisms (acetic acid, phenolic compounds, furfural and hydroxymethylfurfural) are also generated that inhibit the microbial activity (Felipe et al. 1995; Palmqvist and Hahn-Hägerdal 2000; Felipe 2004; Silva et al. 2004; Diaz et al. 2009). According to Tamanini et al. (2004), oat hull hemicellulosic hydrolysate presents high concentrations of toxic compounds. Thus, it is essential to treat the hydrolysate before using it in fermentation medium.

Different detoxification procedures have been evaluated to remove or reduce the toxic compounds concentrations such as pH adjustment combined with activated charcoal adsorption (Marton et al. 2006), adsorption onto ion-exchange resins (Canilha et al. 2010) and utilization of plant polymer (Chaud 2010). The use of 1% activated charcoal has been shown an effective and inexpensive alternative in the detoxification of hemicellulosic hydrolysates (Silva et al. 2007).

Due to heterogeneous composition of the hemicellulosic hydrolysates, it is necessary to screen for an efficient microorganism able to ferment a variety of sugars (pentoses, and hexoses) as well as to tolerate stress conditions, in order to optimize the production of the product of interest (Zaldivar et al. 2001).

Among the xylose-fermenting yeasts, *Pichia stipitis* has been considered promising for industrial applications due to its ability to ferment xylose with a high ethanol yield (Agbogbo et al. 2006). In this context, the aim of this work was to evaluate the fermentative performance of *Pichia stipitis* cultivated in treated or untreated oat hull hemicellulosic hydrolysate.

MATERIAL AND METHODS

Microorganisms and inoculum preparation

The experiments were conducted with *P. stipitis* NRRL Y-7124 maintained at 4°C on malt-extract agar slants. A loopful of cells grown on a malt-extract agar slant was transferred to the medium used for inoculum preparation containing xylose (30.0g/L), rice bran extract (20.0g/L), $(NH_4)_2SO_4$ (2.0g/L) and $CaCl_2.2H_2O$ (0.1g/L). Erlenmeyer flasks (125ml), each containing 50mL medium, were incubated on a rotary shaker (200rpm) at 30°C for 24h. Afterwards, the cells were separated by centrifugation (2000xg; 20min), rinsed twice with distilled water, and then the cell pellet was once again suspended in an adequate volume of distilled water. The initial cell concentration for all the experiments was around 1.0 g/L.

Diluted acid hydrolysis and treatment of oat hull hemicellulosic hydrolysate

Oat hull was hydrolysed in a 1L steel reactor at 156°C for 27 min with H_2SO_4 (0.35%, w/v) at 1:4.5 solid/liquid ratio (Canettieri et al. 2001). Afterwards, its pH was initially adjusted to 7.0 with CaO (commercial grade) and then to 2.5 with H_3PO_4, followed by the addition of 1.0% (w/v) activated charcoal (refined powder), for 30 min, under agitation (200 rpm, 60 °C). The precipitate formed as a result of this treatment was removed by vacuum filtration (Silva et al. 2007). The untreated hydrolysate also was used in the experiments and its pH was adjusted to 5.5 with NaOH. The hydrolysates were autoclaved at 111°C, under 0.5 atm.

Medium and fermentation conditions

For fermentation media preparation, the treated and untreated hydrolysates were supplemented with (g/L): rice bran extract 20.0, $(NH_4)_2SO_4$ 2.0 and $CaCl_2.2H_2O$ 0.1. The media (50mL) were placed in Erlenmeyer flasks (125ml) and incubated at 30°C and 200rpm for 72h (Silva et al. 2010), with initial pH adjusted to 5.5 (Sun et al. 2011). The experiments were conducted in duplicate.

Analytical methods

Xylose, glucose, arabinose, xylitol, ethanol, glycerol, acetic acid, furfural and hydroxymethylfurfural concentrations were determined by HPLC (Waters, Milford, MA) with

a refraction index detector on a Bio-Rad Aminex HPX-87H at 45ºC, with 0.01N H_2SO_4 as the eluent at 0.6 mL/min flow rate. A Hewlett-Packard RP 18 column at 25°C with acetonitrile:water (1:8) and 1% acetic acid as the eluent, and a 0.8 mL/min flow rate was employed for determination of furfural concentration in a visible ultraviolet-light detector (SPD-10^A UV-VIS). The total phenolic compounds concentration was estimated by the ultraviolet spectroscopy at 280 nm (Gouveia et al. 2009). The concentrations of metallic ions were determined by the flame atomic-absorption spectroscopy as described by Soares et al. (2010).

Cell growth was monitored by measuring the absorbance at 600 nm (Beckman-DU 640B spectrophotometer). The cell concentration was calculated based on the on the relation of optical density and cell dry weight through a calibration curve. Cell number was determined directly by counting in a NEUBAUER chamber (area=1/400mm^2; height=0.100mm).

RESULTS AND DISCUSSIONS

Oat hull hemicellulosic hydrolysate composition

Table 1 presents the contents of sugars, toxic compounds and metallic ions in the oat hull hemicellulosic hydrolysate before and after the toxic compounds with the treatment using 1% charcoal, except in the case of the acetic acid, whose concentration remained almost unchanged. However, the treatment also resulted in the loss of about 20% of sugars, which was totally undesirable.

Table 1 - Characteristics of oat hull hemicellulosic hydrolysate before and after treatment process.

Components	Original Hydrolysate	Treated Hydrolysate	Not Treated Hydrolysate*
Sugars (g/L)			
Xylose	58.56	46.80	53.02
Glucose	11.48	8.32	10.36
Arabinose	8.00	6.44	7.68
Toxic Compounds (g/L)			
Acetic Acid	5.40	5.10	5.40
Total Phenols	2.50	1.19	2.50
Furfural	1.19	0.48	0.92
Hydroxymethylfurfural	0.32	0.20	0.26
Inorganics (mg/L)			
Na	52.40	6948.70	6896.20
K	1677.00	1.25	1575.00
Mg	318.20	299.80	328.60
Ca	187.30	79.62	199.30
Cr	nd	nd	nd
Mn	nd	nd	nd
Fe	43.10	2.82	32.50
Ni	4.20	10.05	13.20
Zn	nd	nd	nd

nd: not detected

*hydrolysate with pH adjustment

According to Palmqvist and Hahn-Hägerdal (2000), the presence of toxic compounds can impair cellular metabolism, either by acidifying the cytosol, such as acetic acid or the loss of membrane biological integrity, like the phenolic compounds and furans, furfural and hydroxymethylfurfural, which can inhibit the microbial activity at low concentrations.

A reduction was also observed in the metallic ions concentrations: Mg (5.78%), Ca (20%), Fe (93.4%) and K (99.9%), while the concentrations of Na and Ni increased in the treated and untreated hydrolysates (Table 1). This probably occurred due to NaOH used to adjust the pH of the hydrolysates before its use as fermentation media. The increase in Ni concentration could be related to the equipment used for the homogenization of the hydrolysate during the treatment process and pH adjustment. The removal of metallic ions and phenolic compounds employing the activated

charcoal was also observed by Mussato et al. (2010) in brewer's spent grain hemicellulosic hydrolysate.

It has been reported that many metals influence positively the yeast fermentation performance as they are required for the growth and metabolism. Besides, yeasts are able to very effectively accumulate essential minerals and exclude or detoxify the non-essential minerals (Walker et al. 2006). Some metal ions (K, Na, Zn) can change the rate of glycolysis and subsequently the conversion of pyruvate to ethanol. Metal ions are vital for all the organisms, as they play an important role in the cellular metabolism primarily due to their requirements as cofactors for a large number of enzymes (Soares et al. 2003). However, the presence of these compounds in the toxic concentrations can be a significant problem during the fermentation of various substrates into useful chemical products (Tosun and Ergun 2007).

For example, sodium ion concentration has significant effect on ethanol production by *S. cerevisiae*, and there is interactive effect only between calcium and magnesium in complex media (Soyuduru et al. 2009). Thus, further studies should be made on the effect of ions concentration on the *P. stipitis* metabolism.

Fermentation of oat hull hemicellulosic hydrolysate

Figure 1A shows the sugars consumption by *P. sitipis* grown in oat hull hemicellulosic hydrolysates.

The use of untreated hydrolysate resulted in the consumption of only 4% of glucose and 2.15% of xylose after 72h of fermentation. The use of the treated hydrolysate, resulted a total consumption of glucose and 16.79% of xylose after 48 and 72h of fermentation, respectively (Fig. 1A). Arabinose was not assimilated by the yeast, regardless of conditions employed. Thus, the use of treated hydrolysate resulted in the consumption of xylose and glucose, 8-fold and 25-fold higher respectively than that observed with untreated hydrolysate.

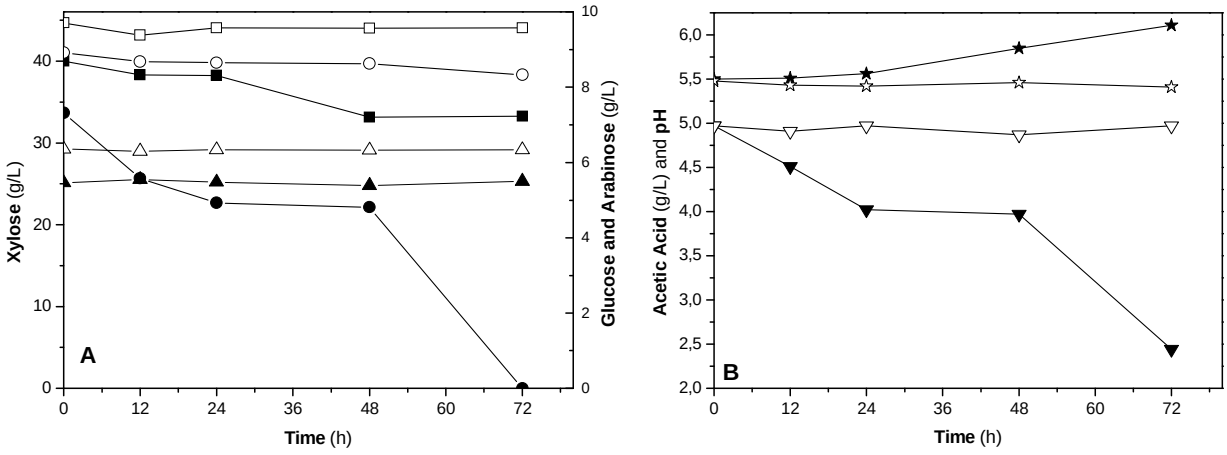

Figure 1 - (**A**) Consumption of xylose (square), glucose (circle) and arabinose (up triangle); (**B**) acetic acid (down triangle) and pH variation (star) by *P. stipitis* grown in oat hull hemicellulosic hydrolysate treated (black symbol) or not (white symbol) with 1% activated charcoal.

It is important to note that even in the treated hydrolysate, sugar consumption was very low, probably due to the toxic compounds present in concentrations considered inhibitory to yeast metabolism. In both the hydrolysates, acetic acid concentration was higher than 5 g/L. It is known that acetic acid in concentrations higher than 3g/L can inhibit xylose metabolism (Felipe et al. 1995). Besides this, the concentrations of furfural, 5-HMF and phenols were also high. According to Diaz et al. (2009), inhibitory compounds can affect the fermentation performance by *P. stipitis* in a synergistic way. They found that in the experiments performed with *P. stipitis* grown in the synthetic medium containing 20g/L glucose and 15g/L xylose in the presence of 2g/L furfural and 3g/L acetic acid, no sugar consumption was

detected and cellular growth was completely inhibited.

Decrease in acetic acid concentration was also observed in this work when treated hydrolysate was employed as fermentation medium and was accomplished by raising the medium pH (Fig. 2B). In the cultures with untreated hydrolysate neither acetic acid consumption nor change in pH was observed.

The low assimilation of sugars by *P. stipitis* appeared be directly related to low cell growth as shown in Figure 2.

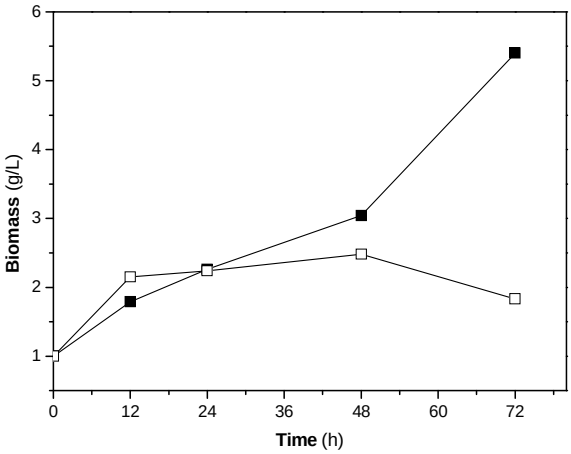

Figure 2 - Cell growth during *P. stipitis* fermentation on oat hull hemicellulosic hydrolysate treated (black symbol) or not (white symbol) with 1% activated charcoal.

The fermentation medium formulated with untreated oat hull hemicellulosic hydrolysate resulted in lower cell growth (54%) compared with the treated hydrolysate. Besides, the use of untreated hydrolysate resulted in the morphological changes in yeast cells (data not showed), with consequently cell death after 48h of fermentation, which was not the case with the use of the treated hydrolysate, even with the partial removal of toxic compounds.

Figure 3 shows the formation of ethanol and glycerol during the fermentation of oat hull hemicellulosic hydrolysate by *P. stipitis.*

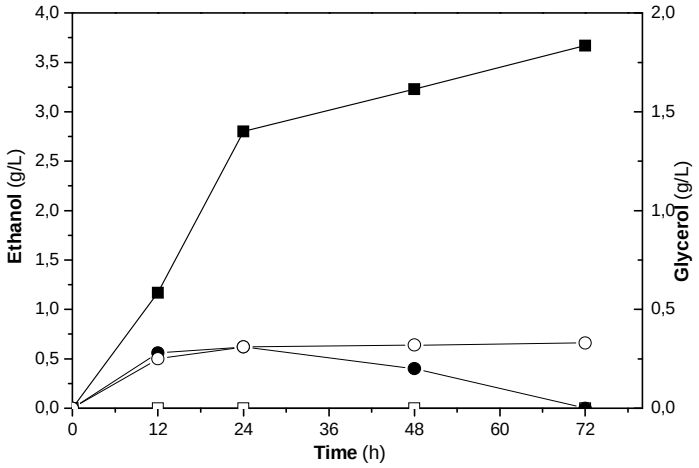

Figure 3 - Ethanol (square) and glycerol (circle) formation by *P. stipitis* grown on oat hull hemicellulosic hydrolysate treated (black symbol) or not (white symbol) with 1% activated charcoal.

The high concentration of toxic compounds resulted in low sugar consumption by the yeast (Fig. 1A) and consequently also resulted in low ethanol production (Fig. 3). Ethanol formation was observed only by employing the medium formulated with the treated hydrolysate. In this condition, maximum ethanol formation (3.67g/L) was close to that reported by Klinner et al. (2005) in the study with *P. stipitis* in the synthetic medium containing only glucose (30g/L) as carbon source (ethanol around 5.0g/L) and considerably lower than that reported by Agbogbo et al. (2006), with the same yeast grown in a synthetic medium containing glucose (30g/L) and xylose (30g/L) (ethanol 23g/L) and that observed by Nigam (2001) in wheat straw hemicellulosic hydrolysate with xylose (30g/l) and glucose (3g/L) (ethanol 15g/L).

The low ethanol production employing the treated hydrolysate and no formation in untreated hydrolysate together with the formation of by-product glycerol (Fig. 3) were directly related to high concentrations of toxic compounds in the hydrolysate. The formation of glycerol, a compatible solute has been observed as a response to stressful conditions in yeast, imposed by the toxic compounds present in the hemicellulosic hydrolysates (Arruda and Felipe 2009).

CONCLUSIONS

The current work showed the fermentative performance of *P. stipitis* cultivated on oat hull hemicellulosic hydrolysate. The yeast was able to grow using the sugars of the hydrolysate with consequent production of ethanol and also glycerol as the by-product. Due to the high concentration of toxic compounds to microbial metabolism present in the hydrolysate, the production of ethanol was very low. To improve the fermentative performance of *P. stipitis* on oat hull hemicellulosic hydrolysed, further studies should be done to improve the hydrolysis and detoxification processes. Besides, it is necessary to evaluate an adequate supplementation of nutrients and also methods for cell adaptation to toxic compounds in order to increase ethanol production.

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Received: May 10, 2011;
Revised: October 28, 2011;
Accepted: May 14, 2012.

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Branco