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Application of Wheat Straw Autohydrolysis Liquor to Xylanase and β-Xylosidase Large-Scale Production in a Stirred Tank Bioreactor

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ABSTRAT

Research into microbial xylanases production has increased due to its several applications. In this context, studies that make this practice feasible are important. Wheat bran is an inexpensive byproduct, which contains around 28% hemicellulose; however, the wheat bran particles suspended in the cultivation medium have to be decomposed to soluble compounds to be used by the fungi and the treatment of lignocellulosic materials in autohydrolysis processes makes this easier. The inclusion of these treated materials in the nutrient media can be a strategy to increase and undervalue xylanase production. The best conditions for xylanase and β-xylosidase production were observed when A. ochraceus *was cultivated with 1% wheat bran added 10% wheat straw autohydrolysis liquor as carbon source, this substrate was more favorable when compared with xylan, wheat bran and wheat straw autohydrolysis liquor used separately. The application of this substrate in a stirred tank bioreactor shows the need for improvements of the fermentation process.*

Keywords: Xylanase; β-xylosidase; Wheat Straw Autohydrolysis Liquor; Fermentation.

INTRODUCTION

Biomass from plant material is the most abundant and widely spread renewable raw material for the sustainable production of clean and affordable biofuels, biopower, and high-value bioproducts. Lignocellulosic materials (LCM) from forest, agriculture, set-aside lands, industry or urban solid wastes, mainly made up lignin, cellulose and hemicelluloses, are potential feedstocks for chemical utilization.

LCM are heterogeneous and present a complex chemical nature. Their integral benefit can be achieved by chemical fractionation, following the "biomass refining" philosophy (Myerly et al., 1981; Moure et al., 2006), based on the selective separation of the main components to yield a variety of high added-value bioproducts. This operational method allows a complete utilization of the raw materials by means of sequential treatments. In this field, autohydrolysis (in which the feedstock and water are the only reagents) causes a selective solubilisation of hemicelluloses, leading to liquors containing sugar oligomers, sugars and sugar-decomposition products and to a solid phase enriched in cellulose and lignin, which can be subjected to further processing (Garrote et al., 2002; 2007).

The most abundant hemicellulose is xylan, made up of xylose units. Xylans represent an immense resource of biopolymers for practical applications, accounting for 25-35% of the dry biomass of woody tissues of dicots and lignified tissues of monocots, and occur up to 50% in some tissues of cereal grains. The structure of xylans depends on the source considered. The most common xylans are made up of a main backbone of xylose linked by β-1,4 bonds, where the structural units are often substituted at positions C2 or C3 with arabinofuranosyl, 4-*O*-methylglucuronic acid, acetyl or phenolic substituents (Moure et al., 2006).

Xylanases play a key role in xylan hydrolyzation to xylooligosaccharides. Microbial xylanases mainly include xylanase or endoxylanase (1,4-β-D-xylan xylanohydrolase, E.C. 3.2.1.8) that cleaves β-1,4-linked xylan backbone and β-xylosidase (1,4-β-xylan xylohydrolase, E.C. 3.2.1.37) that hydrolyses xylooligomers (Ghosh et al., 1993; Liu et al., 2008). From a commercial viewpoint, xylanases are an important group of carbohydrolases and have a worldwide market of around $200 million each year (Liu et al., 2008). Xylanases have been widely applied in food animal feed, bioconversion, textile, and in paper and pulp industries (Subramaniyan and Prema, 2002; Liu et al., 2008).

The successful use of a microbial enzyme at an industrial scale will depend on the viability of its large-scale production. Xylanase is an industrially useful enzyme and investigations of the optimal conditions and the effects of scale-up procedures for its optimal production are thus important (Reddy et al., 2002). Thus, the aim of the present work was to investigate the xylanase production using wheat straw autohydrolysis liquor and/or wheat bran as substrate by *Aspergillus ochraceus* fungus and the application of this substrate on large-scale production of xylanolytic enzymes in stirred tank bioreactor (STB).

MATERIALS AND METHODS

Wheat residues were kindly supplied by a local farmer (Portugal). The wheat bran stored at room temperature and the wheat straw (material for autohydrolysis in this work), after being dried at 40 ºC in an oven for 12 h, was cut into small pieces (1-3 cm), milled in a knives mill to pass through a 0.4 mm screen (for chemical composition) and 1.0 mm (for hydrothermal pre-treatments). Wheat straw samples and water were mixed in a closed and pressurized vessel in order to obtain a solid/liquid ratio of 1:10 w/v. The system was heated to 200 ºC during 15 min. The liquid phase or liquor (hemicelluloses rich fraction) was separated from the solids by filtration. The hemicelluloses were then precipitated with three volumes of 95% ethanol (20 °C, 24 h) and dried for yield determination (4.9%), or used directly as liquid substrate.

The microorganism used in this work was the fungal strain *Aspergillus ochraceus*. It was collected from decomposing fruits and leafs, in the Ribeirão Preto region – SP, Brazil, and classified by the Fungi Collection of Pernambuco Federal University (Brazil). This fungus was maintained at 30 °C, on slants of solid PDA media (Difco).

Conidia from 7 day-old cultures, with cell concentration of 2×10^8 cells.mL^{-1}, were inoculated into 250 mL Erlenmeyer flasks containing 50 mL of the liquid medium described by Adams (1990), pH 6.0, containing the carbon source: 1% (w/v) birchwood xylan; 1% (w/v) wheat bran; 100% (v/v) wheat straw autohydrolysis liquor; and combination of 1% (w/v) wheat bran and 10% (v/v) wheat straw autohydrolysis liquor. The cultures were incubated at 30 °C, for 100 rpm, for until seven days. During fermentations, samples were taken in each 24 h and the mycelial and residues were removed by centrifugation at 10000 rpm for 15 minutes.

In Stirred Tank Bioreactor (STB) with 2 L capacity (Bioengineering AG CH-8636 Wald), the same concentration of conidia was inoculated in 1 L of the same culture medium, containing 1% (w/v) wheat bran added 10% (v/v) wheat straw autohydrolysis liquor. The culture was also incubated at 30 °C, under agitation of 300 rpm and 1 vvm aeration rate. The pH was only monitored. The filtrates were used as the source of crude extra-cellular xylanase and β-xylosidase.

Xylanase activity was assayed using 1% (w/v) of birchwood xylan as substrate. Reaction mixtures contained 0.2 mL enzyme and 0.2 mL 1% xylan solution in citrate-phosphate buffer, pH 6.0. The mixture was incubated at 60 ºC, and after predetermined periods the released reducing sugars were estimated with DNS, using xylose as standard (Miller, 1959). One unit of xylanase activity was defined as the amount of enzyme that released 1 μmol product per minute under the conditions of the assay.

β-Xylosidase activity was assayed using 0.25% (w/v) of *p*-nitrophenyl-β-D-xylopyranoside (PNP-xyl) as substrate. Reaction mixtures containing 0.2 mL enzyme, 0.15 mL citrate-phosphate buffer, pH 4.5, and 0.05 mL 0.25% PNP-xyl in distilled water. The mixture was incubated at 70 ºC, and after predetermined periods the released *p*-nitrophenolate were estimated with saturated sodium tetraborate solution, using *p*-nitrophenol as standard (Kersters-Hilderson et al., 1982). One unit of β-xylosidase was defined as the amount of enzyme that released 1 μmol of product per minute under the conditions of the assay.

RESULTS AND DISCUSSION

It is well known that hemicelluloses, especially xylan from various sources, are excellent inductors for xylanase. Wheat (straw) bran is an inexpensive byproduct, which contains a lot of xylan. Therefore, it is one of the most popular components of complex media for xylanase production (Siedenberg et al., 1997). A variety of microorganisms, including bacteria, yeast and filamentous fungi, have been reported to produce xylanase, of which the most potent producers are fungi. At an industrial scale, xylanases are produced mainly by *Aspergillus* and *Trichoderma* spp (Haltrich et al., 1996; Bhat 2000).

Previous studies showed that wheat bran was the most favorable agricultural residue to xylanase production. Thus, in order to improve the production of xylanolytic enzymes, wheat straw autohydrolysis liquor was added to the cultivation medium to test, once the hydrothermal treatment of xylan-containing LCM breaks the hemicellulosic chain by the hydrolytic action of hydronium ions (generated from water autoionization and from *in situ* generated organic acids), yielding soluble products (mainly oligosaccharides), which can be assimilated more easily for the microorganism.

According to Figure 1, the best conditions for xylanase and β-xylosidase production were observed when the microorganism was cultivated with a mixture of 1% (w/v) wheat bran and 10% (v/v) wheat straw autohydrolysis liquor. This production was around 20% more than the one obtained with birchwood xylan, thus making this process very attractive for industrial application, because of the high cost of the xylan. The isolated use of wheat bran as inducer for xylanase production was around 70% of the production obtained with xylan and for β-xylosidase this value was around 30%, while the use of 100% wheat straw autohydrolysis liquor as carbon source corresponded to 50% of the xylanase activity observed with xylan. For β-xylosidase production, the use of the liquor as the only carbon source was not suitable (around 5% of the activity verified with xylan). Besides, the fermentation time with the mixture of wheat bran and wheat straw liquor made the process more viable once, in the industry, large production times also signify high production costs. High production of xylanase and β-xylosidase was observed after 48 h of fermentation with this mixture, obtaining a maximal production with 120 h of incubation, while, when xylan was used as substrate, the significant induction of xylanolytic enzymes was observed only after 144 – 168 h of fermentation.

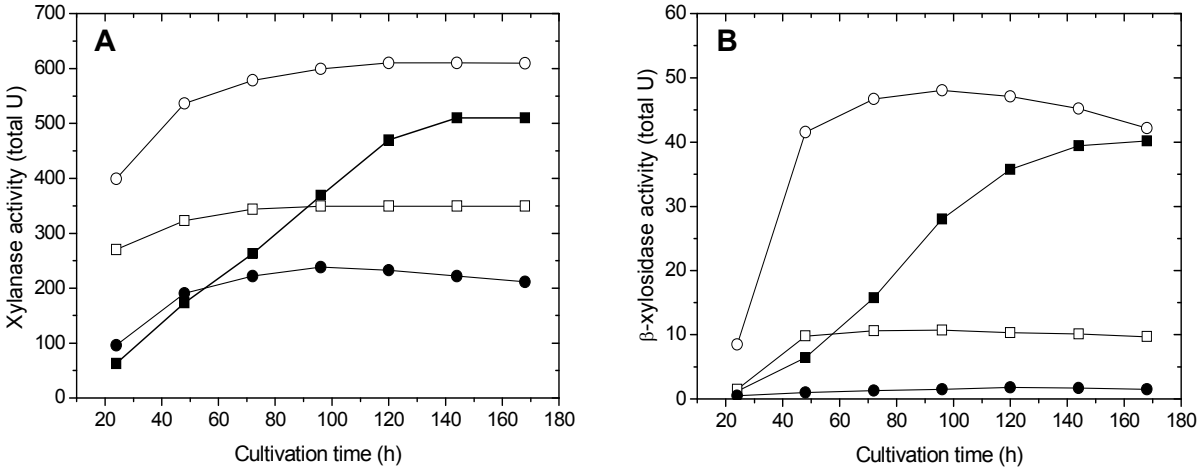

Figure 1. Performance of *A. ochraceus* during fermentation in Erlenmeyer flasks for xylanase (**A**) and β-xylosidase (**B**) production using different carbon sources: -■- 1% (w/v) birchwood xylan; -□- 1% (w/v) wheat bran; -●- 100% (v/v) wheat straw autohydrolysis liquor; -○- mixture of 1% (w/v) wheat bran and 10% (v/v) wheat straw autohydrolysis liquor. The microorganism was cultivated at 30ºC, 100 rpm.

Another tentative of production was performed in a Stirred Tank Bioreactor. Figure 2 shows the fermentation course and it was verified that the xylanase production was not maximal after 168 h of fermentation, while the maximal β-xylosidase production was verified with 144-168 h of fermentation. Therefore, in STB, the time of enzymatic production was higher than observed in Erlenmeyer flasks with the mixture of wheat bran and wheat straw autohydrolysis liquor; besides, the production rate ($U.mL^{-1}$) was lower.

In submerged culture, xylanase production by filamentous fungi may be affected by shear stress, which is related to the agitation rate. The high viscosities and non-Newtonian behavior of culture broths of filamentous fungi often force the use of high agitation rates to provide adequate mixing and oxygen transfer. However, micelial damage due to high shear stress limits the practicable range of stirrer speed and consequently the volumetric biomass and enzyme productivity of the culture. Several papers reported on the effects of agitation rate in combination with the aeration rate and dissolved oxygen tension (Hoq et al., 1994; Singh et al., 2000; Techapun et al., 2003; Chipeta et al., 2008). These results suggest the complexity of the fermentation process in STB and the necessity to optimize some conditions, as agitation and aeration rates. Recently, alternative bioreactors, such as the air-lift or bubble-column, which have a lower shear stress, have begun to find application in xylanase production.

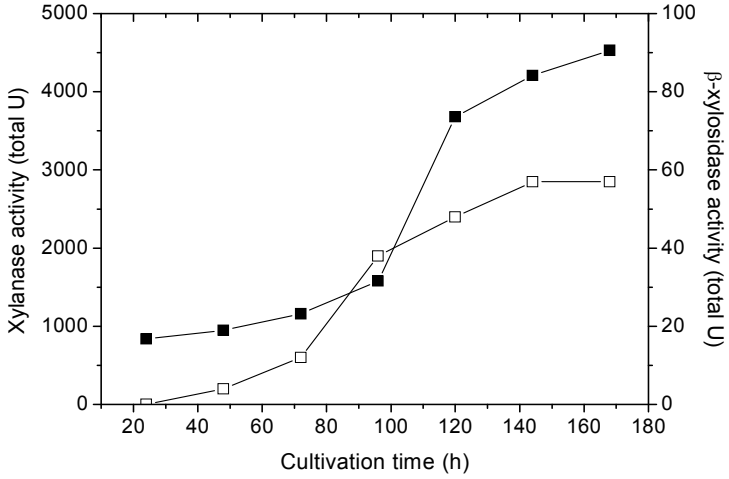

Figure 2. Performance of *A. ochraceus* during fermentation in STB for xylanase (-■-) and β-xylosidase (-□-) production using the mixture of 1% (w/v) wheat bran and 10% (v/v) wheat straw autohydrolysis liquor. The microorganism was cultivated at 30ºC, 300 rpm, 1 vvm.

CONCLUSION

The use of wheat straw autohydrolysis liquor as adjunct in xylanase and β-xylosidase production was very promising, since the use of residues adds value to final product due to its low cost. Besides of the fact of the enzymatic production with residues has been higher than with xylan, an expensive substrate which is considered the "model" for xylanase production. Application of these residues to xylanolytic production in STB showed that other experiments must be performed in order to optimize the process.

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