Proceedings of the 10th International Chemical and
Biological Engineering Conference - CHEMPOR 2008
Braga, Portugal, September 4-6, 2008
E.C. Ferreira and M. Mota (Eds.)

A new strategy for xylanase production using wheat straw autohydrolysis liquor as substrate

Michele Michelin[1,2], Maria de Lourdes T. M. Polizeli[2], Denise S. Ruzene[1], Daniel P. Silva[1], António A. Vicente[1], João A. Jorge[2], Héctor F. Terenzi[2], José A. Teixeira[1*]

[1] IBB - Institute for Biotechnology and Bioengineering, Centre of Biological Engineering, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal.
[2] Department of Biology, Faculty of Philosophy, Sciences and Letters of Ribeirão Preto, University of São Paulo, 14040-901 Ribeirão Preto-SP, Brazil.

Keywords: Xylanase, Wheat straw, Wheat bran, Autohydrolysis liquor, Fermentation
Topic: Multi-scale and/or multi-disciplinary approach to process-product innovation

Abstract

Agro-industrial residues are lignocellulosic materials with a high content of cellulose, hemicellulose and lignin. If such residues can be produced in bioprocesses (e.g. xylanase production) there is an attractive possibility of their integral use in biotechnological processes. In general, xylanase biosynthesis is induced by its substrate – xylan, but the high xylan content of some wastes such as corn cobs and wheat bran makes them an accessible and cheap source of inducers. Another alternative to improve the xylanase production, which is the main goal of this work, is the treatment of lignocellulosic materials in autohydrolysis processes which, under optimized conditions, lead to the solubilization of hemicelluloses (liquid phase, liquor) that may be favorable to xylanase production. The inclusion of these components in the nutrient medium composition can be a strategy to optimize the microbial xylanase biosynthesis. The best conditions for xylanase production were observed when the microorganism was cultivated in birchwood xylan for 6 days; however, satisfactory results were obtained using a combination of 1% wheat bran with 2% or 10% autohydrolysis liquor, for 5 days fermentation, once the xylanase production was around 86-87% of production with xylan. Besides, the obtained production with 100% wheat straw autohydrolysis liquor was also interesting, once after 7 days of cultivation, the xylanase production was higher than the ones obtained with wheat bran or by the combination of wheat bran and liquor.

1 Introduction

The hemicelluloses are a large group of high molecular polysaccharides, insoluble in water but soluble in alkaline solutions. These polysaccharides are associated with cellulose and lignin and play an important structurally-supportive role in building up of plant cell walls (Nakamura, 2003). The composition of hemicelluloses, includes branched heteropolymers of pentoses (xylose, arabinose), hexoses (mannose, galactose, glucose), and uronic acids. Xylan is the major constituent of hemicellulose and the second most abundant renewable resource with a high potential for degradation to useful end-products. Microbial xylanases (1,4-β-D-xylan xylanohydrolase, EC 3.2.1.8) are the preferred catalysts for xylan hydrolysis due to their high specificity, negligible substrate loss and side product generation. During the last decades, xylanases have attracted considerable research interest because of their potential industrial applications (Chen et al., 1997; Yuan et al., 2004).

* Corresponding author. Tel +351-253-604406. E-mail: jateixeira@deb.uminho.pt

The enzyme hydrolysis of xylan lies in the basis of its utilization as an energy source in animal feed or in different biotechnological processes (Coughlan and Hazlewood, 1993; Kulkarni et al., 1999; Subramanyan and Prema, 2002). The partial enzyme hydrolysis of xylan changes its physical and chemical properties, which concerns the quality of different products of the food and flavour industry. Xylanase finds applications in fruit juices and wines clarifying (Colins et al., 2005; Coughlan and Hazlewood, 1993). In brewing, xylanase is applied in filtering improvement (Yin et al., 2005). The utilization of xylanase in bread-making significantly improves the desirable texture, loaf volume and shelf life of bread (Courtin and Delcour, 2002). Xylanase enzyme preparations are also widely used in bio-bleaching in paper industry. They facilitate the delignification of the plant pulp in the production of high-quality paper. In that way, the chlorine-containing bleaching agents, that are a serious ecological problem, can be reduced (Gupta et al., 2000).

The industrial production of xylanase preparations is based on a microbial biosynthesis. Most often industrial xylanase producing strains moulds of the species *Aspergillus sp.* and *Trichoderma sp.*, as well as bacterial strains of the species *Bacillus sp.*, are used. There are two possibilities for cultivation of microbial enzyme producing strains: solid-state and submerged cultivation (Gawande and Kamat, 1999). At the present moment, the submerged cultivation is more widely used, allowing a higher degree of processes intensification and a better level of automation. Xylanases from moulds are extracellular, inducible enzymes. That determines the great significance of nutrient medium selection. The xylanase biosynthesis is induced by its substrate – xylan (Subramanyan and Prema, 2002; Kulkarni et al., 1999). The high xylan content of some of the wastes such as corn cobs and wheat bran makes them an accessible and cheap source of inducers. Annually about seven million tonnes of wastes are deposited from the member-countries of the EU, in the form of corn and wheat bran (Bonnin et al., 2001). The inclusion of these components in the nutrient media composition is the main strategy in microbial xylanase biosynthesis (Davidov and Atev, 1996).

Besides, treatments of lignocellulosic materials in aqueous media (autohydrolysis or hydrothermal treatments) under optimized conditions lead to the solubilisation of hemicelluloses, leaving a solid phase enriched in both cellulose and lignin (Garrote et al., 1999). This solid phase can be subjected to further processing to obtain a variety of commercial products, e.g. enzymatic hydrolysis and further fermentation of hydrolyzates (Rivas et al., 2004), allowing an integrated use of the raw material; in turn, the liquid phase can be used to optimize the microbial biosynthesis of enzymes. Thus, in this work, the performance of wheat straw autohydrolysis liquor as adjunct in medium containing wheat bran for xylanase production in submerged cultivation of the strain *Aspergillus terricola* was evaluated.

2 Materials and methods

Material

Wheat residues (wheat bran and wheat straw) 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).

Liquor obtaining: wheat straw autohydrolysis treatment

Wheat straw samples and water were mixed in a closed and pressurized vessel in order to obtain a liquid/solid ratio of 10:1 w/w. The system was heated to 200 ºC during 30 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.

Microorganism and fermentation runs

The microorganism used in this work was the fungal strain *Aspergillus terricola* Marchal. This fungus was colleted on *campus* Ribeirão Preto of São Paulo University (Brazil), in an area near to laboratory of Microbioly and Cellular Biology, on Biology department of Faculty of Philosophy, Sciences and Letters for Michele Michelin, and identified by the fungal 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^{7} cells.mL^{-1}, were inoculated into 250 ml Erlenmeyer flasks containing 50 ml of the liquid medium described by Vogel et al. (Vogel et al., 1964), pH 6.0, containing the carbon source: 1% (w/v) birchwood xylan; 1% (w/v) wheat bran; 100% (v/v) wheat straw autohydrolysis liquor; combination of 1% (w/v) wheat bran and 2% (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, a maximum of seven days. During fermentations, samples were taken in specified intervals and both the mycelia and residues were removed by centrifugation at 10000 g for 15 minutes. The filtrates were used as the source of crude extra-cellular xylanase and β-xylosidase.

Figure 1 shows a scheme for the production of wheat straw autohydrolysis liquor, extraction of hemicelluloses from ground wheat straw.

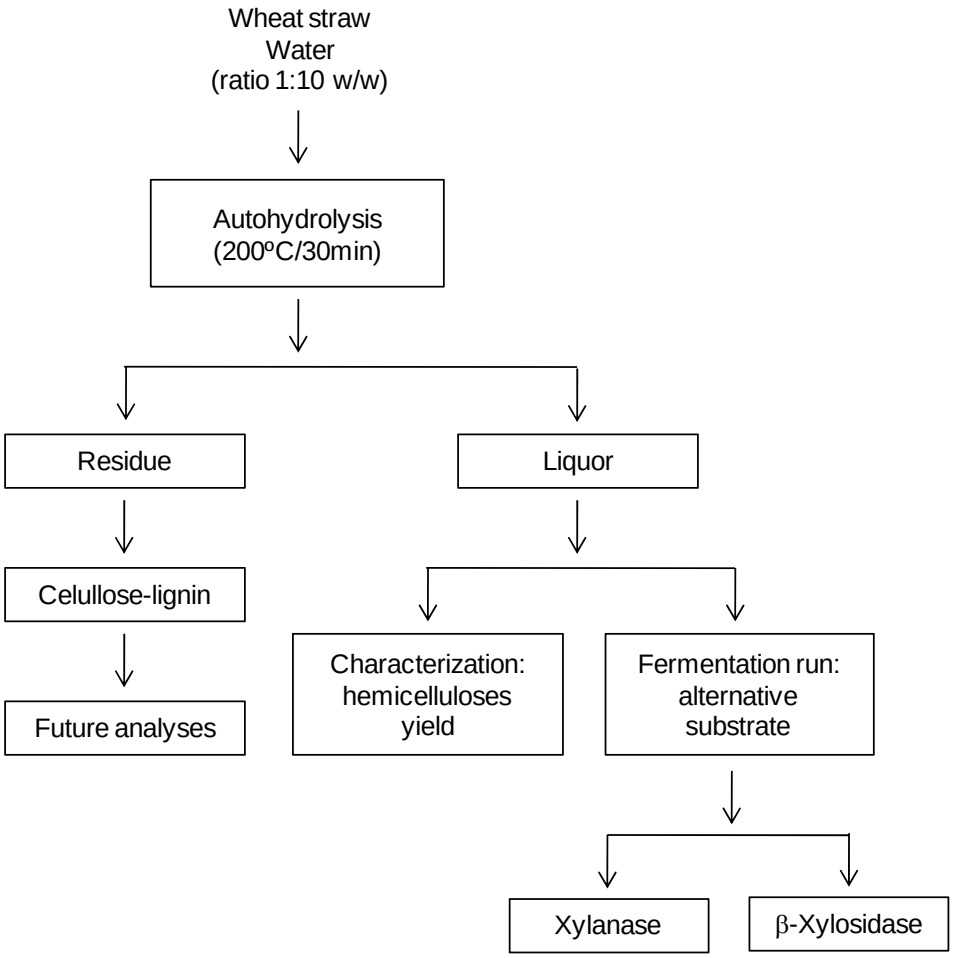

Figure 1. Scheme for extraction of hemicelluloses from ground wheat straw

Activity determinations

Xylanase activity was assayed using 1% (w/v) of birchwood xylan (Sigma, St. Louis, MO, USA) as substrate. Reaction mixtures contained 0.2 ml enzyme and 0.2 ml 1% xylan solution in citrate-phosphate buffer, pH 6.0 (Vilela et al.,1973). The mixture was incubated at 60ºC, and after predetermined periods the released reducing sugars were estimated with 3,5-dinitrosalicylic acid (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 (Vilela et al., 1973) and 0.05 ml 0.25% PNP-xyl in distilled water. The mixture was incubated at 70ºC, and after predetermined periods the released *p*-nitrophenolate were estimated with saturated sodium tetraborate solution, using *p*-nitrophenol as standard. 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.

3 Results and discussion

Several fungal industrial production processes use complex media, consisting of agricultural byproducts as corn steep liquor, peanut flour, sugar beat molasses, pharma medium, etc. These media are inexpensive and yield much higher productivity than synthetic ones. The particular polymer (cellulose, hemicellulose, starch, protein), which often forms a suspension in the complex cultivation medium, serves as an inducer for the formation of hydrolyzing enzymes. This is the case for the production of xylanase, which is induced by xylan, an integral component of wheat bran (Adolph et al., 1996).

In our work, and according to Figure 2, the best conditions for xylanase production were observed when the microorganism was cultivated in birchwood xylan during 6 days of incubation. However, when on industrial level, this substrate become impracticable because of its high cost. In this context, the use of residues as well as its subproducts become very atractive. Besides, the use of wheat bran as inducer for xylanase production was very good (around 82% of production verified with xylan). However, the use of combinations of wheat bran and wheat straw autohydrolysis liquor, as well as only autohydrolysis liquor was superior that obtained with only wheat bran, once that with 5 days of fermentation the production obtained with combinations of wheat straw autohydrolysis liquor was of 86-87% when compared with birchwood xylan; and the prodction of xylanolytic enzymes using 100% liquor as adjunt on fermentation during seven days was 88% of production obtained with xylan

The fermentation process was conducted during 7 days; however in this period the xylanase production was still being induced by the wheat straw autohydrolysis liquor. Thus, with this result two conclusions were taken: (i) xylanase production can be improved in periods longer than 7 days when using autohydrolysis liquor; and (ii) it is possible that the induction of xylanases using wheat straw autohydrolysis liquor, produced under different conditions of time and temperature, can be more favorable for xylanase production, once the enzymic production started with 5 days of incubation (Figure 2). These results suggest the presence of short xylooligosaccharides on wheat straw autohydrolysis liquor, being that the use of wheat straw autohydrolysis liquor that posses xylooligosaccharides higher perhaps can be more favorable to xylanase induction.

Thus, by temperature control and reaction time it is possible to influence characteristics of the xylo-oligosaccharides such as the acetyl content and the molar mass distribution (Nabarlatz et al., 2004, 2005), but the nature of the raw material has also a significant role.

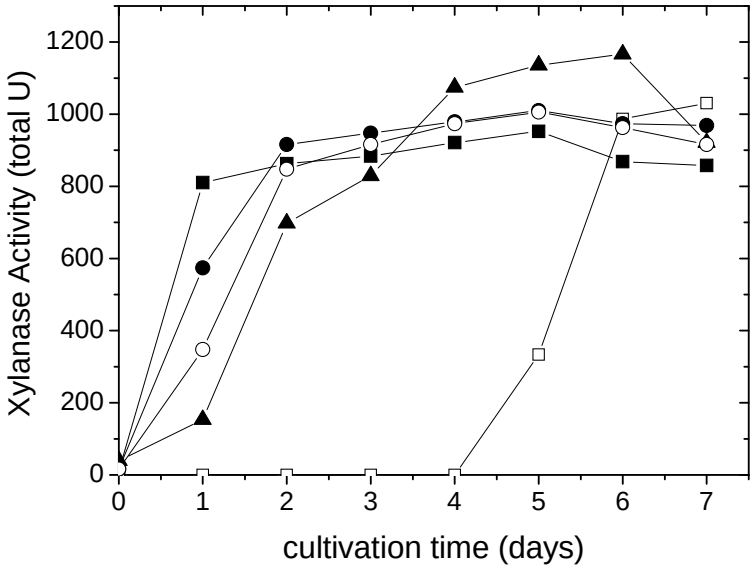

Figure 2. Performance of *A. ochraceus* during fermentation for xylanase production using different carbon sources: 1% (w/v) birchwood xylan (-▲-), 1% (w/v) wheat bran (-■-), 100% (v/v) wheat straw autohydrolysis liquor (-□-), combination of 1% (w/v) wheat bran and 2% (v/v) wheat straw autohydrolysis liquor (-●-), and combination of 1% (w/v) wheat bran and 10% (v/v) wheat straw autohydrolysis liquor (-○-)as adjunct. The microorganism was cultivated at 30ºC, 100 rpm.

4 Conclusion

Wheat straw autohydrolysis liquor showed to be promising as adjunct in xylanase and β-xylosidase production processes by filamentous fungi. This suggests that studies of liquor preparation under more convenient times and temperatures can result in a liquor more appropriated to the production of xylanolytic enzymes. This process is very attractive due to the use of residues (such as wheat straw) and also due to its low cost, therefore adding value to final product.

Acknowledgements

Biota FAPESP (Brazil); FCT (Portugal).

References

Adolph, S., Muller, S., Siedenberg, d., Jager, H.L., Schugerl, K., Giuseppin, M. (1996). Cultivation of *Aspergillus awamori* on wheat bran: Ultrastructural description of growth and localization of xylanase by immunogold labeling. Journal of Biotechnology, 46:221-234.

Chen, C., Chen, J.L., Lin, T.Y. (1997). Purification and characterization of a xylanase from *Trichoderma longibrachiatum* for xylooligosaccharide production. Enzyme and Microbial Technology, 21:91–96.

Bonnin, E., Brunel, M., Gouy, Y., Lesage-Meesen, L., Asther, M., Tribault, J.F. (2001). *Aspergillus niger* I-1472 and *Pycnoporus cinnabarinus* MUCL 39533, selected for the biotranformation of ferulic acid to vanilin, are also able to produce cell wall polysaccharidedegrading enzymes and feruloyl esterases. Enzyme and Microbial Technology, 28:70–80.

Colins, T., Gerday, C., Feller, G. (2005). Xylanases, xylanase families and extermophilic xylanases. FEMS Microbiology Letters, 29:3–23.

Coughlan, M., Hazlewood, G. (1993). β-1,4-D-xylan-degrading enzyme systems: biochemistry, molecular biology and applications. Biotechnology and Applied. Biochemistry, 17:259–289.
Courtin, C.M., Delcour, J.A. (2002). Arabinoxylans and endoxylanases in wheat flour bread-making. Journal of Cereal Science, 35:225–243.
Davidov, E.R., Atev, A. (1996). Method for production of enzyme preparation. Patent number Ru 2057179 C1.
Garrote, G., Domínguez, H., Parajó, J.C. (1999). Hydrothermal processing of lignocellulosic materials. Holz Roh Werk., 57:191–202.
Gawande, P., Kamat, M. (1999). Production of *Aspergillus* xylanase by lignocellulosic waste fermentation and its application. Journal of Applied Microbiology, 87:511–519.
Gupta, S., Bhushan, B., Hoondai, G.S. (2000). Isolation and characterization of xylanase from *Staphylococcus sp.* SG-13 and its application in biobleaching of kraft pulp. Journal of Applied Microbiology, 88:325–334.
Kersters-Hilderson, H., Claeyssens, M., Doorslaer, E.V., Saman, E., Bruyne, C.K (1982). β-D-xylosidase from *Bacillus pumilus*. Methods in Enzymology, 83: 631-639.
Kulkarni, N., Shendye, A., Rao, M. (1999). Molecular and biotechnological aspects of xylanases. FEMS Microbiology Letters, 23:411–456.
Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J. (1951). Protein measurement with the folin phenol reagent. Journal of Biological Chemistry, 193:265-275.
Miller, G. L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. Analytical Chemistry, 31:426-428.
Nakamura, S. (2003). Structure and function of a multiplidomain alkaline xylanase from alkaliphilic *Bacillus sp.* strain 41M-1. Catalysis Surveys from Asia, 7:157–164.
Nabarlatz, D., Farriol, X., Montané, D. (2004). Kinetic modeling of the autohydrolysis of lignocellulosic biomass for the production of hemicellulose-derived oligosaccharides. Industrial and Engineering Chemistry Research, 43:4124-4131.
Nabarlatz, D., Farriol, X., Montané, D. (2005). Autohydrolysis of almond shells for the production of xylooligosaccharides: product characteristics and reaction kinetics. Industrial and Engineering Chemistry Research, 44:7746–7755.
Rivas, B., Moldes, A.B., Domínguez, J.M., Parajó, J.C. (2004). Lactic acid production from corn cobs by simultaneous saccharification and fermentation: a mathematical interpretation. Enzyme and Microbial Technology, 34:627–634.
Subramanyan, S., Prema, P. (2002). Biotechnology of microbial xylanases: Enzymology, molecular biology and application. Critical Reviews in Biotechnology, 22:33–64.
Villela, G.G., Bacila, M., Tastaldi, H. (1973). Técnica e experimentos de bioquímica. Ed. Guanabara Koogan, p.523.
Vogel, H.F. (1964). Distribution of lysine pathways among fungi: evolutionary implications. American Naturalist, 98:435-446.
Yin, L., Jian, L., Guoxian, G., Zhonping, S., Zhongui, M. (2005). Studies on water-extractable arabinoxylans during malting and brewing. Food Chemistry, 93:33–38.
Yuan, Q.P., Zhang, H., Qian, Z.M., Yang, X.J. (2004). Pilot-plant production of xylo-oligosaccharides from corncob by steaming, enzymatic hydrolysis and nonofiltration. Journal of Chemistry and Technology Biot., 79:1073–1079.