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COMPARISON BETWEEN CONTINUOUS AND BATCH PROCESSING TO PRODUCE XYLANASE BY *Penicillium canescens* 10-10c

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Abstract - *Penicillium canescens* 10-10c strain was cultivated on barley straw hydrolysate as a soluble nutrient source and as inducer for xylanase production. Barley straw hydrolysate was obtained by treatment of barley straw with NaOH or hot water. In shake flask cultures, NaOH treatment was found to increase the biomass production, but was not accompanied by an increase in xylanase production. The best xylanase production (54 U/ml) was observed on hydrolyzed extract from barley straw treated with hot water (100 ºC) for 3 hours. Enzyme production was further improved by scaling up the cultivation process to a 3-L stirred tank bioreactor. For batch cultivations in the bioreactor, the maximum xylanase productivity reached 1.31 and 0.46 U/ml/h, respectively, after 96 and 168 hours of cultivation. However, xylanase productivity reached 3.46 U/ml/h in the continuous culture. These results suggest that xylanase can be produced efficiently by *Penicillium canescens* 10-10c in continuous culture from an inexpensive source such as barley straw hydrolysate.
Keywords: Batch culture; Continuous culture; *Penicillium canescens*; Xylanase.

INTRODUCTION

Xylanolytic enzymes are a group of enzymes that hydrolyze xylan and arabinoxylan polymers. This enzyme group includes endo-β-1, 4-xylanase, β-xylosidase, arabinofuranosidase and acetylxylan esterase (Biely, 1993). These enzymes are produced by a wide variety of microorganisms, among which the filamentous fungi are especially interesting as they secrete these enzymes into the medium and their xylanase activities are much higher than those found in yeast and bacteria (Guimarães *et al*., 2006). Endo-β-1,4-xylanase plays an important role in animal feed, increasing the body weight gains of the animals (Medel *et al*., 2002). In the pulp and paper industry, xylanases are employed in the prebleaching process to reduce the use of toxic chlorine chemicals (Wong *et al*., 2000). In the bread and bakery industry, xylanases are used to increase the dough viscosity, bread volume, and shelf life (Romanowska *et al*., 2003). Other potential applications include the conversion of xylan in wastes from the agriculture and food industries into xylose and the production of fuel and chemical feedstocks (Sunna and Antranikian, 1997).

Continuous operation could be more efficient compared to other operating conditions, because the process does not have any dead-times (time to clean, load, unload, prepare, etc.) (Da-Costa *et al*., 1997; Baptisa-Neto *et al*., 2005). Therefore, continuous culture systems have been widely used to cultivate microbes for industrial and research purposes (Zangirolami *et al*., 2002; Viegas *et al*., 2002; Kim *et al*., 2004; Neto *et al*., 2005; Dominguez *et al*., 2009). Few studies have been reported on the

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continuous production of xylanases with bacteria and fungi, mainly because of the problems associated with media containing insoluble xylan substrates (Tangnu *et al*., 1981; Rőthlisberger *et al*., 1992). Lignocellulosic materials have often been used as carbon sources for xylanase production in submerged and solid fermentation processes (Gaspar *et al*., 1997; Bakri *et al*., 2003; Assamoi *et al*., 2008). Solid substrates are, however, hard to handle, particularly if continuous or periodic feeding and broth removal are desired. To overcome these problems, natural solid raw materials are replaced by soluble substrates that promote good growth and induction of enzyme production. Several examples of the extraction of xylooligosacharides by thermal treatment or with the aid of acidic or alkaline solutions have been reported (Pessoa Jr. *et al*., 1997; Sun *et al.*, 2000; Lavarack *et al.*, 2002; Kim *et al.*, 2002; Abril *et al*., 2012). Therefore, in this study, barley straw hydrolysate was utilized as a soluble inducer for xylanase production from *Penicillium canescens* in batch and continuous culture.

MATERIALS AND METHODS

Microorganism

The *Penicillium canescens* 10-10c strain was supplied by G.I. Kvesidatse, Institute of Plant Biotechnology, Academy of Science, Tbilisi, Georgia. The strain was chosen for its ability to produce high cellulase-free xylanase activity (Gaspar *et al*., 1997; Bakri *et al*., 2003).

Culture Medium

The culture medium used for xylanase production was composed of barley straw hydrolysate, mineral salts and yeast extract. The mineral salt medium contains (g/L): $Na_2HPO_4.2H_2O$ 10, KCl 0.5, Yeast extract 5, and $Mg_2SO_4.7H_2O$ 0.15**.** The pH was adjusted to 6.5 before sterilization. Barley straw hydrolysate was prepared as follows, 100 g of milled barley straw were treated with 1000 mL of distilled water or a solution of 1 M NaOH at 25, 60, 80, 100 ºC for 3 hours and at 121 ºC for 30 minutes. The liquid fraction was separated by centrifugation and used as the carbon source solution.

Shake Flask Culture

The experiments were conducted in 250 mL Erlenmeyer flasks containing 50 mL of culture medium. The flasks were sterilized at 121 °C for 20 min. 0.5 mL fresh spore suspension (containing around 10^8 spores/mL) was added to the sterilized medium Fresh fungal spores were used as inocula and the flasks were incubated in an orbital shaker at 30 °C and 150 rpm for 5 days.

Bioreactor Cultivation Conditions

A 3-L Electro-lab fermenter (Electro-lab limited, UK) was used as the base mechanical vessel for xylanase production from *Penicillium canescens* 10-10c in batch and continuous culture. The agitation system is made of two Rushton DT6 turbines. The diameter of these turbines was d = 50 mm. The clearance above the base of the bioreactor and the distance between the two impellers were equal to the turbine diameter (d). Fermentation was carried out on barley straw hydrolysate obtained from milled barley straw treated with 1000 mL of distilled water at 100 ºC for 3 hours and the medium was supplemented with mineral salts and yeast extract as described above.

In batch culture, the bioreactor was filled with 1.5 L of culture medium and then sterilized at 121 °C for 20 min. After sterilization, the bioreactor was inoculated with a spore suspension to give a spore concentration of 10^6 spores/mL. A regulation system was used to control the temperature at 30 °C throughout the experiment. The sterile air flow rate was kept at 1 vvm. The impeller speed was maintained at 200 rpm for batch and continuous cultures. Foaming was controlled by the addition of 0.05% (v/v) Tego KS911 antifoam (Goldschmidt, Essen, Germany).

In continuous culture, the medium was pumped into the bioreactor by a peristaltic pump. The pump speed was regulated to pump 75 ml/hour of culture medium in order to reach a dilution rate of $D=0.05\ h^{-1}$. The effluent was continuously discharged through a pump which had the same speed to allow the retention of 1.5 liter of culture medium in the bioreactor.

Enzyme Activity and Protein Determination

Xylanase activity was determined according to Bailey *et al*. (1992) using 1.0% birchwood xylan in 0.05 M citrate buffer (pH 5) as substrate. Xylan solution and the enzyme at appropriate dilutions were incubated at 55 ºC for 5 min and the reducing sugars were determined by the dinitrosalicylic acid method, with xylose as a standard (Miller, 1959). The released xylose was measured spectrophotometrically

at 550 nm. One unit of xylanase is defined as the amount of enzyme required to release 1 micromole of reducing sugar as xylose equivalent per min under the above assay conditions. The amount of total protein was determined according to the method of Lowry *et al*. (1951) using bovine serum albumin (BSA) as a protein concentration standard.

Biomass

Mycelial dry weight was determined by filtering the culture medium through pre-weighed Whatman No. 44 filter paper, washing twice with distilled water, and finally drying to constant weight at 80 ºC and reweighing. The difference in weight denoted the mycelial growth of fungus.

RESULTS AND DISCUSSION

Xylanase Production by *Penicillium canescens* 10-10c on Barley Straw Hydrolysate

Shake-flask culture experiments were performed for testing xylanase production on barley straw hyrolysates obtained from treatment of barley straw with distilled water or with 1.0 M NaOH solution at different temperatures. Xylanase production from *P. canescens* 10-10c strain using barley straw hyrolysates after 120 h of culture is shown in Table 1. The best xylanase production (54 U/ml) obtained in an Erlenmeyer flask was after treatment with warm distilled water at 100 ºC for 3 hours. Thus, this condition was selected for subsequent batch and continuous cultures in a bioreactor. The NaOH treatment led to higher biomass formation compared to the treatment with water. However, this was not followed by an increased xylanase production. Many researchers have used lignocellulosic hydrolysates for enzyme production and have indicated that the oligosaccharides formed during the lignocellulose hydrolysis are believed to play important roles in enzyme induction (Palma *et al*., 1996; Xiong *et al*., 2005; Lo *et al*., 2010). Deejing and Ketkorn (2009) found that hyrolysates obtained from pretreatment of lignocellulosic materials with 1.0 M NaOH showed high reducing sugar concentration. Results obtained from preliminary studies on xylanase production by *P. canescens* showed that xylanase expression is subject to regulation by a metabolic repression. In submerged fermentation, the accumulation of reducing sugars had a negative effect on the production of xylanase. Similarly, the expression of xylanase by *P. canescens* was repressed by glucose, xylose, and lactose (Gaspar *et al*., 1997). The addition of 1% glucose to 1% birch wood xylan caused severe catabolic repression and decreased xylanase production by about 61% compared with the medium containing only 1% birch wood xylan (Bakri *et al*., 2003).

Table 1: Biomass and xylanase production by *Penicillium canescens* after 120 hours of culture on barley straw hydrolysate obtained by treatment with distilled water or NaOH (40g/L) at different temperatures.

Treatment	Dry biomass (mg/mL)	Xylanase (U/mL)	Specific activity (U/mg of dry biomass)
NaOH + (25 ºC 3 hours)	4.17 (± 0.28)	13.14 (± 1.82)	3.15 (± 0.21)
NaOH + (60 ºC 3 hours)	13.12 (± 0.99)	33.46 (± 3.51)	2.55 (± 0.46)
NaOH + (80 ºC 3 hours)	15.87 (± 1.06)	27.21 (± 2.28)	1.71 (± 0.12)
NaOH + (100 ºC 3 hours)	7.63 (± 0.37)	16.37 (± 1.83)	2.14 (± 0.09)
NaOH + (121 ºC 0.5 hours)	7.27 (± 0.44)	8.78 (± 1.51)	1.21 (± 0.11)
H2O + (25 ºC 3 hours)	2.09 (± 0.21)	25.15 (± 1.79)	12.03 (± 1.47)
H2O + (60 ºC 3 hours)	2.58 (± 0.36)	31.41 (± 2.58)	12.17 (± 0.79)
H2O + (80 ºC 3 hours)	2.45 (± 0.31)	35.74 (± 3.67)	14.59 (± 1.97)
H2O + (100 ºC 3 hours)	2.86 (± 0.31)	54.19 (± 3.39)	18.95 (± 0.32)
H2O + (121 ºC 0.5 hours)	2. 81 (± 0.18)	23.52 (± 1.88)	8.37 (± 0.46)

Values represent means and standard deviations (in parentheses) of three experiments

Batch Culture

The cultivation of *P. canescens* was carried out on barley straw hydrolysate in batch bioreactor. The results obtained from the batch culture are presented in Fig. 1.

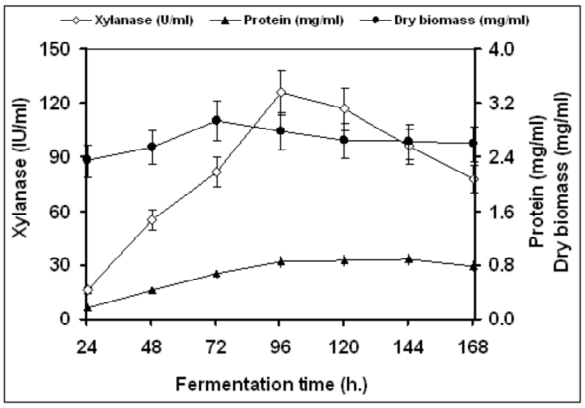

Figure 1: Xylanase production (U/mL) from *P. canescens* in batch culture in the 3 L bioreactor using barley straw hydrolysate obtained by treatment with water at 100 ºC for 3 hours. The error bars are the lower and higher ranges of the data.

Initially, the fungus produced low concentrations of the studied enzyme, but with the passage of time there was gradual increase in xylanase synthesis up to 96 h. At the fourth day of incubation, the fungus efficiently synthesized the enzyme and gave 126 U/mL of xylanase, which decreased to 78 U/mL after six days of incubation. The dissolved oxygen change during cultivation of *P. canescens* is shown in Fig. 2.

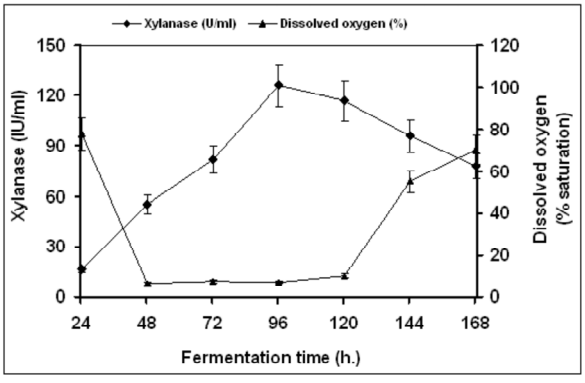

Figure 2: Evolution of xylanase production (U/mL) and dissolved oxygen (%) during growth of *P. canescens* in batch culture in the 3 L bioreactor using barley straw hydrolysate obtained by treatment with water at 100 ºC for 3 hours.

The DO decreased to about 7% of saturation after about 48 hours of cultivation. Previous studies demonstrated that the decrease in DO can be attributed to biomass progressively increasing during the growth phase. In the second phase, the substrate, which contains enzyme inducers, was progressively depleted and shear rates can cause morphological as well as physiological changes in filamentous fungi, resulting in decreased biomass and xylanase production (Gaspar *et al*., 1997; Gaspar *et al*., 1998; Bakri *et al*., 2002).

Continuous Culture

One of the main drawbacks in industrial applications of enzyme catalysis is the relatively high cost of the biocatalyst. Therefore, the production of high amounts of biocatalyst at a low cost is critical. Thus, it would be extremely interesting to develop the technology for continuous production (Dominguez *et al.,* 2009). However, there are no reports dealing with xylanase enzyme production by *P. canescens* in continuous mode. With this aim, an experiment in continuous mode was carried out in a bioreactor with a barley straw hydrolysate medium. The continuous feeding was started 48 h after inoculation and xylanase production was determined during the culture process. Figure 3 shows the profiles of extracellular xylanase, soluble protein and biomass obtained during growth of *Penicilliun canescens* in a continuous culture. Moreover, the feeding enabled a continuous xylanase production and the stability of enzyme activity obtained after 60 hours of culture. Xylanase production during the steady state was about 69 U/mL using a dilution rate of 0.05 h^{-1}, as shown in Figure 3. The measured extracellular protein content showed a linear relationship to xylanase activity, indicating that the amount of xylanases in proportion to the total extracellular protein remained unchanged. The comparison between batch and continuous processing to produce xylanase from *P. canscens* is shown in Figure 4. The experiment in continuous culture confirmed the benefit of this type of culture for xylanase production. Although the xylanase concentration in the batch culture (126 U/ml) after 96 h of culture was higher than that in continuous culture (69 U/ml), the enzyme productivity obtained in continuous culture (3.46 U/ml/h) was approximately 2.64 and 7.47 times higher than that in batch culture (1.31 and 0.46 U/ml/h) after 96 and 168 h. of fermentation, respectively. This benefit of continuous culture in increasing enzyme productivity as a result of the more efficient use of the substrate has been reported by many investigators (Fencl and Pazlarová, 1982;

Dominguez *et al.,* 2009). Alpha-amylase productivity in continuous cultivation was four to five times higher as compared with batch cultivation (Fencl and Pazlarová, 1982). Similar behavior has been reported for lipolytic enzyme production in continuous culture of *Thermus thermophilus* HB27. The lipolytic enzyme was around 2-fold higher in continuous culture than in batch culture (Dominguez *et al.,* 2009).

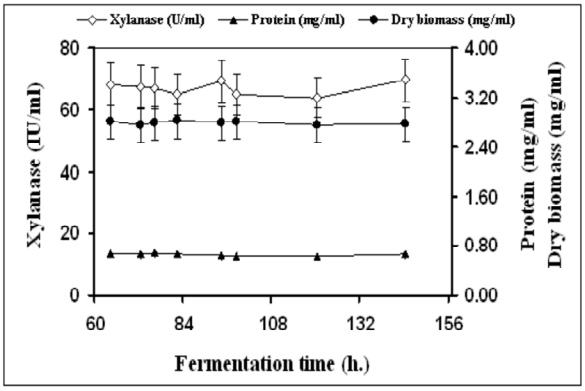

Figure 3: Xylanase production (U/mL) from *P. canescens* in continuous culture in the 3 L bioreactor using barley straw hydrolysate obtained by treatment with water at 100 ºC for 3 hours. The error bars are the lower and higher ranges of the data.

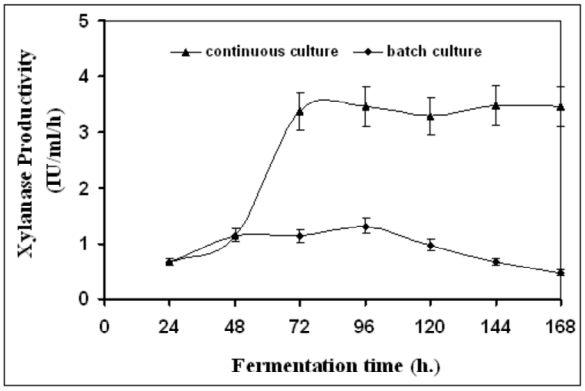

Figure 4: Xylanase productivity (U/mL) from *P. canescens* in batch and continuous culture in the 3 L bioreactor using barley straw hydrolysate obtained by treatment with water at 100 ºC for 3 hours. The error bars are the lower and higher ranges of the data.

CONCLUSIONS

For economic reasons, it is important to maximize enzyme production by microbial process development. Improvements in enzyme productivity will have a direct impact on the process performance, economics and feasibility. Batch and continuous cultures of *P. canescens* have been studied for the production of xylanase from barley straw hydrolysate as the carbon source. A cost-effective medium of lignocellulosic material hydrolysate can be used as an attractive substitute for solid substrates, which have both mixing and mass transfer problems. Although the maximum xylanase concentratrion was higher in batch culture, the highest xylanase productivity was obtained in continuous culture. The increase in xylanase productivity was 2.64- and 7.47-fold when compared to batch culture after 96 and 168 h of fermentation. The results reported in the present work show that continuous culture was the best operation mode to produce xylanase enzyme from *P. canescens*.

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REFERENCES

Abril, D., Meduna, M. and Abril, A., Sugar cane bagasse prehydrolysis using hot water. Brazilian Journal of Chemical Engineering, 29, pp. 31-38 (2012).

Assamoi, A. A., Delvigne, F., Aldric J. M., Destain, J. and Thonart, P., Improvement of xylanase production by *Penicillium canescens* 10-10c in solid state fermentation. Biotechnology, Agronomy, Society and Environment, 12, 111-118 (2008).

Bailey, M. J., Biely, P. and Poutanen, K., Interlaboratory testing of methods for assay of xylanase activity. Journal of Biotechnology, 23, pp. 257-270 (1992).

Bakri, Y., Jacques, P., Shi, L. K. and Thonart, P., Influence of a new axial impeller on K_La and xylanase production by *Penicillium canescens* 10-10c. Applied Biochemistry and Biotechnology, 98/100, pp. 1037-1048 (2002).

Bakri, Y., Jacques, P. and Thonart, P., Xylanase production by *Penicillium canescens* 10-10c in solid state fermentation. Applied Biochemistry and Biotechnology, 105/108, pp. 737-748 (2003).

Baptista-Neto, A., Teodoro, J. C., Filho, L. C. M. C., Badino, A. C. and Hokka, C. O., Comparisons between continuous and batch processing to produce clavulanic acid by *Streptomyces*

clavuligerus. Brazilian Archives of Biology and Technology, 48, pp. 97-104 (2005).

Biely, P., Biochemical aspects of the production of microbial hemicellulases. pp. 29-51. In: Coughlan, M. P. and Hazlewood, G. P., (Eds.) Hemicellulose and Hemicellulases. Portland Press, London (1993).

Da-Costa, A. C., Lima, E. L. and Alves, T. L. M., Start-up strategy for continuous bioreactors. Brazilian Journal of Chemical Engineering, 14, pp. 167-177 (1997).

Deejing, S. and Ketkorn, W., Comparison of hydrolysis conditions to recover reducing sugar from various lignocellulosic materials. Chiang Mai Journal of Science, 36, 384-394 (2009).

Dominguez, A., Deive, F. J., Pastrana, L., Rúa, M. L., Longo, M. A. and Sanroman, M. A., Thermostable lipolytic enzymes production in batch and continuous cultures of *Thermophilus* HB27. Bioprocess and Biosystems Engineering, 33, pp. 347-354 (2009).

Fencl, Z. and Pazlarová, J., Production of extracellular enzymes in continuous culture. Folia Microbiologica, 27, pp. 340-349 (1982).

Gaspar, A., Cosson, T., Roques, C. and Thonart, P., Study on the production of a xylanolytic complex from *Penicillium canescens* 10-10c. Applied Biochemistry and Biotechnology, 67, pp. 57-70 (1997).

Gaspar, A., Strodiot, L. and Thonart, P., Improvement of oxygen transfer coefficient during *Penicillium canescens* culture: Influence of turbine design, agitation speed, and air flow rate on xylanase production. Applied Biochemistry and Biotech., 70/72, pp. 535-545 (1998).

Guimarães, L. H. S., Nogueira, S. C. P., Michelin, M., Rizzatti, A. C. S., Sandrim, V. C., Zanoelo, F. F., Aquino, A. C. M. M., Junior, A. B. and Polizeli, M. L. T. M., Screening of filamentous fungi for production of enzymes of biotechnological interest. Brazilian Journal of Microbiology, 37, pp. 474-480 (2006).

Kim, K. H., Tucker, M. P. and Nguyen, Q. A., Effects of operating parameters on countercurrent extraction of hemicellulosic sugars from pretreated softwood. Applied Biochemistry and Biotechnology, 98/100, pp. 147-159 (2002).

Kim, D. Y., Yim, S. C., Lee, P. C., Lee, W. G., Lee, S. Y. and Chang, H. N., Batch and continuous fermentation of succinic acid from wood hydrolysate by *Mannheimia succiniciproducens* MBEL55E. Enzyme and Microbial Technology, 35, pp. 648-653 (2004).

Lavarack, B. P., Griffin, G. J. and Rodman, D., The acid hydrolysis of sugarcane bagasse hemicellulose to produce xylose, arabinose, glucose and other products. Biomass and Bioenergy, 23, pp. 367-380 (2002).

Lo, C. M., Zhang, Q., Callow, N. V. and Ju, L. K., Cellulase production by continuous of *Trichoderma reesei* Rut C30 using acid hydrolysate prepared to retain more oligosaccharides for induction. Bioresource Technology, 101, pp. 717-723 (2010).

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J., Protein measurement with the Folin phenol reagent. The Journal of Biological Chemistry, 193, pp. 265-275 (1951).

Medel, P., Baucells, F., Gracia, M. I., De Blas, C. and Mateos, G. G., Processing of barley and enzyme supplementation in diets for young pigs. Animal Feed Science and Technology, 95, pp. 113-122 (2002).

Miller, G. L., Use of dinitrosalicylic acid reagent for determination of reducing sugars. Analytical Chemistry, 31, pp. 426-428 (1959).

Neto, A. B., Hirata, D. B., Cassiano Filho, L. C. M., Bellão, C., Badino Júnior, A. C., Hokka, C. O. A., A study on clavulanic acid production by *Streptomyces clavuligerus* in batch, fed-batch and continuous processes. Brazilian Journal of Chemical Engineering, 22, pp. 557-563 (2005).

Palma, M. B., Milagres, A. M. F., Prata, A. M. R. and Mancilha, I. M., Influence of agitation and agitation on the xylanase activity from *Penicillium janthinellum*. Process Biochemistry, 31, pp. 141-145 (1996).

Perssoa Jr. A., Mancilha, I. M. and Sato, S., Acid hydrolysis of hemicellulose from sugarcane bagasse. Brazilian Journal of Chemical Engineering, 14, (3), pp. 291-297 (1997)

Romanowska, I., Polak, J., Jonowska, K. and Bielecki, S., The application of fungal endoxylanase in bread-making. Communications in Agricultural and Applied Biological Sciences, 68, pp. 317-320 (2003).

Rőthlisberger, P., Fiechter, A. and Switzerland, W., Produation of thermostable xylanases in batch and continuous culture by *Thermomonospora fusca* KW3. Applied Microbiology and Biotechnology, 37, pp. 416-419 (1992).

Sunna, A. and Antranikian, G., Xylanolytic enzymes from fungi and bacteria. Critical Reviews in Biotechnology, 17, pp. 39-67 (1997).

Sun, R. C., Tomkinson, J., Wang, Y. X. and Xiao, B., Physico-chemical and structural characterization of hemicelluloses from wheat straw by alkaline

peroxide extraction. Polymer, 41, pp. 2647-2656 (2000).

Tangnu, S. K., Blanch, H. W., Wilke, C. R., Production of xylanase by *Streptomyces xylophagus* nov. sp. Acta Biotechnologica, 1, pp. 31-40 (1981).

Wong, K. K. Y., James, C. S. and Campion, S. H., Xylanase pre- and post- treatments of bleached pulps decrease absorption coefficient. Journal of Pulp and Paper Science, 26, pp. 377-383 (2000).

Viegas, M. C., Andrietta, S. R. and Andrietta, M. G. S., Use of tower reactors for continuous ethanol production. Brazilian Journal of Chemical Engineering, 19, pp. 167-173 (2002).

Xiong, H., von Weymarn, N., Turunen, O., Leisola, M., Pastinen, O., Xylanase production by *Trichoderma reesei* Rut C-30 grown on L-arabinose-rich plant hydrolysates. Bioresource Technology, 96, pp. 753-759 (2005).

Zangirolami, T. C., Carlsen, M., Nielsen, J. and Jørgensen, S. B., Growth and enzyme production during continuous cultrures of a high amylase producing variant of *Aspergillus oryzae*. Brazilian Journal of Chemical Engineering, 19, pp. 55-68 (2002).