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ORIGINAL PAPER

Production of xylanolytic enzymes by *Aspergillus terricola* in stirred tank and airlift tower loop bioreactors

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Abstract Fungi producing high xylanase levels have attracted considerable attention because of their potential industrial applications. Batch cultivations of *Aspergillus terricola* fungus were evaluated in stirred tank and airlift bioreactors, by using wheat bran particles suspended in the cultivation medium as substrate for xylanase and β-xylosidase production. In the stirred tank bioreactor, in physical conditions of 30°C, 300 rpm, and aeration of 1 vvm (1 l min^{-1}), with direct inoculation of fungal spores, 7,475 U l^{-1} xylanase was obtained after 36 h of operation, remaining constant after 24 h. In the absence of air injection in the stirred tank reactor, limited xylanase production was observed (final concentration 740 U l^{-1}). When the fermentation process was realized in the airlift bioreactor, xylanase production was higher than that observed in the stirred tank bioreactor, being 9,265 U l^{-1} at 0.07 vvm (0.4 l min^{-1}) and 12,845 U l^{-1} at 0.17 vvm (1 l min^{-1}) aeration rate.

Keywords Xylanase · Bioreactors · *Aspergillus* · Wheat bran

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Introduction

Biomass from plant material is the most abundant and widespread renewable raw material for sustainable production of clean and affordable biofuels, biopower, and high-value bioproducts. Thus, lignocellulosic materials from forest, agriculture, set-aside lands or industry, mainly made up of lignin, cellulose, and hemicelluloses, are potential feedstocks for bioprocess utilization [9]. Xylans, the most abundant of the hemicelluloses of plant cell walls, are composed of 1,4-β-D-xylopyranose residues with different substituent groups in the side-chain [3, 7, 24].

Xylanases (EC 3.2.1.8), which cleave the backbone and initiate depolymerization of xylan, have received most attention, mainly because of their potential use in prebleaching of cellulose pulp to minimize use of active chlorine [21]. Apart from use in pulp and paper industry, xylanases are also used in food and foodstuff processing industry; as food additives in animal feed for poultry, swine, and ruminants; in manufacture of bread to increase volume and water adsorption; in juice and wine industries for extraction, clearing, and stabilization of fruit pulp; and in processing of plant fibers in textile industry. Xylose, the hydrolytic product of xylan, can be also converted by fermentation into combustible liquids (ethanol), xylitol, furfural, and solvents [23].

Successful application of xylanase requires production of large amounts of this enzyme. However, in many of the works published on this subject, fermentations were carried out in small flasks [6, 9, 26], which are not viable for mass production. On the other hand, Kim et al. [19] found that xylanase production by *Aspergillus niger* decreased considerably when the fungus was grown in a stirred tank bioreactor (STB), presumably because of damage to mycelia caused by shear stress. Thus, to achieve optimal production,

selection of an appropriate bioreactor configuration is critical. Airlift bioreactors (ALB) provide a low-shear environment for enzyme production. They do not have mechanical stirrers, and so the risk of contamination is reduced, as is energy demand. In addition, they are uncomplicated, reliable, and low cost. The aim of this work is to evaluate the performance of different bioreactors (stirred tank and airlift) and the influence of aeration for xylanase production by *Aspergillus terricola* using wheat bran as carbon source.

Materials and methods

Material

Wheat residues (wheat bran) were kindly supplied by a local farmer (Portugal), and stored at room temperature until use.

Microorganism

Aspergillus terricola was isolated from tree trunk surface (*Hovenia dulcis*) in the Faculty of Philosophy, Sciences and Letters of Ribeirão Preto, University of São Paulo, Brazil. The strain was classified as *Aspergillus terricola* and deposited at the Mycology Culture Collection URM at the Federal University of Pernambuco, Brazil. Stock cultures were propagated on potato dextrose agar (PDA) medium at 30°C for 1 week, and stored at 4°C. This microorganism was selected from among 35 fungi that were good producers of xylanases.

Inoculum preparation

The microorganism was inoculated in Vogel medium [29] containing 0.5% (w/v) wheat bran as carbon source. The spore concentration in the suspension was determined in a Neubauer counting chamber. Approximately 3×10^8 spores ml^{-1} were inoculated directly into the bioreactor.

Bioreactor configuration and operating conditions

Initially, enzyme production was performed in a 2-l stirred tank bioreactor (STB; Bioengineering AG CH-8636, Wald, Switzerland) equipped with automatic monitoring and control facilities for temperature, pH, agitation, and aeration. The working volume was 1 l, the temperature was kept at 30°C and agitation at 300 rpm, and the aeration rate was either adjusted to 1 vvm (1 l min^{-1}) or was absent.

An airlift bioreactor (ALB), made of polymethylmethacrylate (Perspex; Department of Biological Engineering, University of Minho/Portugal) and equipped with automatic monitoring and control facilities for temperature, pH, and aeration, was also used for enzyme production. In this case, the working volume was 6 l. A full description of the ALB and a diagram are shown in Fig. 1. The bioreactor was programmed to work at 30°C, and filter-sterilized air was supplied at 0.07 vvm (0.4 l min^{-1}) or 0.17 vvm (1 l min^{-1}) by means of a perforated plate at the bottom of the bioreactor, thus promoting efficient air diffusion throughout the bioreactor.

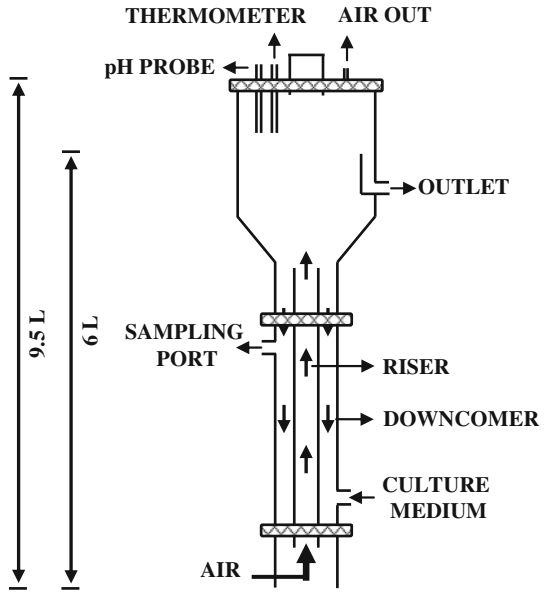

Fig. 1 Schematic representation of the airlift tower loop bioreactor

In the cultivation medium, wheat bran at 0.5% (w/v) was the only carbon source used. In all experiments the initial pH was adjusted to 6.0 and monitored during the process. Antifoam solution was used when necessary. Samples were collected every 12 or 24 h, filtered, and analyzed in triplicate.

Enzymatic assays

Xylanase activity was determined as described by Miller [22] using 1% (w/v) birchwood xylan as substrate. The reaction mixture contained 0.2 ml of the respective substrate suspended in McIlvaine buffer at pH 6.5, and 0.2 ml of the enzyme. The samples were incubated at 60°C. β-Xylosidase activity was determined as described in Kersters-Hilderson et al. [17] using 0.25% (w/v) *p*-nitrophenyl-β-D-xylopyranoside (PNP-xyl) as substrate. The reaction mixture contained 0.2 ml enzyme, 0.15 ml McIlvaine buffer at pH 4.0, and 0.05 ml PNP-xyl suspended in distilled water. The samples were incubated at 70°C. One unit of enzyme activity was defined as the amount of enzyme which releases 1 µmol reducing sugar from the respective substrates per minute under the assay conditions. Total activity was defined as units per ml multiplied by the total enzyme volume of the filtrate. All samples were analyzed in triplicate.

Results and discussion

Cultivation in stirred tank bioreactor (STB)

The influence of aeration was investigated under the following conditions: The main cultures were performed with 3×10^8 spores ml^{-1}, at stirrer speed of 300 rpm, temperature of 30°C, initial pH of 6.0, and aeration rate of 1 vvm. In the cultivation medium, wheat bran at 0.5% (w/v) was the only carbon source used.

It is well known that hemicelluloses, especially xylan from various sources, are excellent inducers for xylanase (EC 3.2.1.8). The cost of production and low yields of these enzymes are the major obstacles to industrial application. Therefore, investigations on the ability of cellulose- and hemicellulose-hydrolyzing microbial strains to utilize inexpensive substrate have been carried out. Much work has been directed to develop hyperproducing microbial strains while also focusing on improvement of the fermentation processes [12, 16]. Among the various biomass materials, lignocellulosic biomass has been considered as a promising feedstock because of its abundance, low cost, and huge potential availability.

Wheat bran is an inexpensive byproduct containing a lot of xylan, which makes it one of the most popular components of complex media for xylanase production. It consists of 28% hemicellulose, 9% starch, 8.7% cellulose, 3.2% lignin, and 3% pectin [27]. However, wheat bran particles must be suspended in the cultivation medium so that adequate contact occurs between particles and the biomass.

Stirring speed of 300 rpm was the lowest speed required to maintain the wheat bran circulating homogeneously throughout the bioreactor. Besides, Siedenberg et al. [27] observed that levels of xylanase activity were the highest at 300 rpm, decreasing with increasing stirrer speed. The lower xylanase activity at higher stirrer speeds can be explained by the less intimate contact between the fungal mycelium and the wheat bran, since the attachment of the fungus to the wheat bran surface is reduced at higher stirrer speeds.

In submerged culture, xylanase production by filamentous fungi may also be affected by shear stress, which is related to the agitation rate. The high viscosity and Newtonian behavior of culture broths of filamentous fungi often require the use of high agitation rates to provide adequate mixing and oxygen transfer. However, mycelial damage due to high shear stress limits the practicable range of stirrer speed values and consequently the volumetric biomass and enzyme productivity of the culture [5].

Induction of xylanase formation by *A. terricola* started after 12 h of cultivation, and a plateau of activity was observed between 36 and 60 h of fermentation (7,475 U l^{-1}), while induction of β-xylosidase activity started only after 48 h of cultivation with a plateau of activity observed in the period 120–168 h (10.1 U l^{-1}) (Fig. 2).

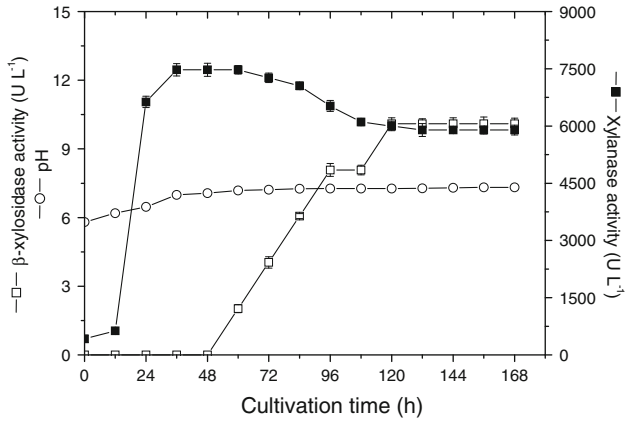

Fig. 2 Cultivation in the stirred tank bioreactor at 30°C, 300 rpm, initial pH of 6.0, and aeration rate of 1 vvm (1 l min^{-1}). Main cultures were performed with inoculum of 3×10^8 spores ml^{-1}

Fenice et al. [10] reported that laccase and Mn-peroxidase (MnP) productions were strongly affected by the impeller speed in STR. The activity of laccase was highest (4,600 U l^{-1} after 13 days of fermentation) at 250 rpm, while that of MnP was highest at 500 rpm (370 U l^{-1} on day 9). The aeration rate also greatly affected both enzyme activities, which were highest (3,900 and 360 U l^{-1} on day 9 for laccase and MnP, respectively) at 1.0 vvm. In fact, stirrer speeds above 500 rpm led to reduced levels of enzyme activity. Negative effects on production of both enzymes were also observed at 1.5 vvm. Probably, the microorganism suffered a certain amount of shear stress.

Reddy et al. [25] reported the highest xylanase activity (13,500 nKat ml^{-1}) by *T. lanuginosus* SSBP at 1 vvm, while aeration rates greater than 1 vvm adversely affected production of xylanase and accessory enzymes. In that work, aeration rates were found to have a strong influence on xylanase production. However, authors speculated that production of accessory enzymes (β-xylosidase and β-glucosidase) is dependent on neither agitation nor aeration, and may be inhibited at genetic level. Hoq et al. [14] reported that increasing aeration rates from 0.5 to 1 vvm favored xylanase production by *T. lanuginosus* RT9 (41,630 U l^{-1} h^{-1}), and for aeration rates above 1 vvm, reduced levels of xylanase activity were also observed.

However, if very high air flow rates are used in conjunction with low stirring speeds, air envelopes the impeller without dispersion and the air flow pattern in the vessel is dominated by air flowing up the stirrer shaft. This phenomenon, known as impeller "flooding," should be avoided, because an impeller surrounded by air no longer contacts the liquid properly, resulting in poor mixing, reduced air dispersion, and diminished oxygen transfer rates [8].

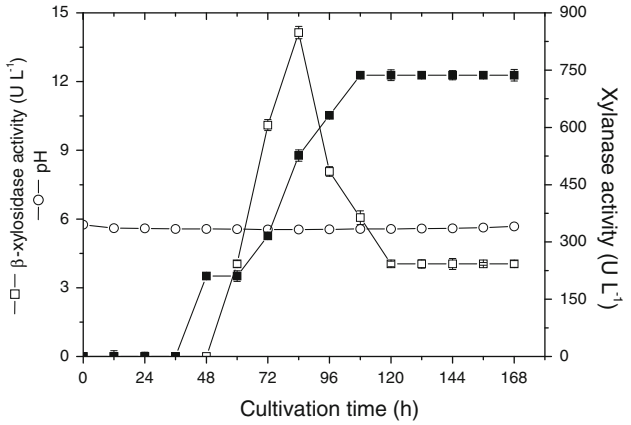

Fig. 3 Cultivation in the stirred tank bioreactor at 30°C, 300 rpm, initial pH of 6.0, and without aeration. Main cultures were performed with inoculum of 3×10^8 spores ml^{-1}

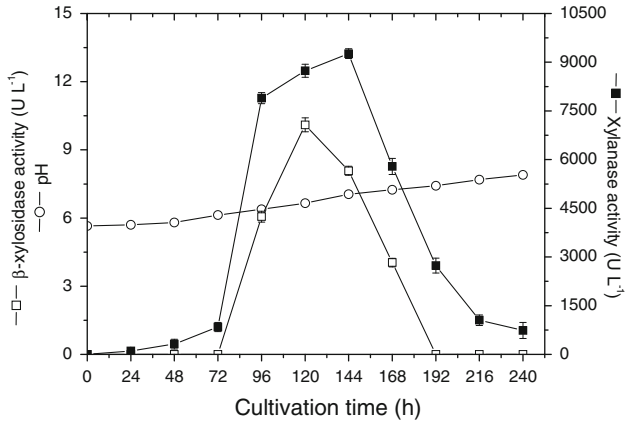

Fig. 4 Cultivation in the airlift tower loop bioreactor at 30°C, initial pH at 6.0, and aeration rate of 0.07 vvm (0.4 l min^{-1}). Main cultures were performed with inoculum of 3×10^8 spores ml^{-1}

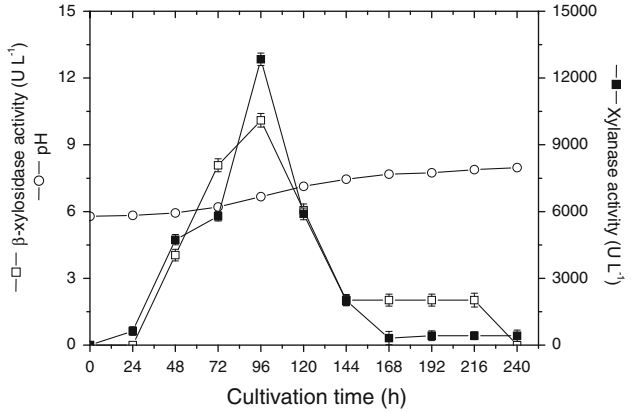

Fig. 5 Cultivation in the airlift tower loop bioreactor at 30°C, initial pH of 6.0, and aeration rate of 0.17 vvm (1 l min^{-1}). Main cultures were performed with inoculum of 3×10^8 spores ml^{-1}

Batch cultivation under the same conditions but without aeration led to limitation of xylanase production (Fig. 3). The genus *Aspergillus* includes aerobic microorganisms, and the fact that pH practically did not change proves the difficulty of spore germination under those conditions. β-Xylosidase activity was at levels similar to those of the aerobic fermentation. At this stage it is important to stress that β-xylosidases are often intracellular, but they can sometimes be associated to the membrane (as indicated by Kulkarni et al.) [20], thus justifying the observed low levels of extracellular β-xylosidase.

Although aeration seems to have been highly influential in xylanase production in STB by *A. terricola*, it is important to note the difficulties of working with solid substrates, since with aeration a large fraction of the wheat bran was impelled to the bioreactor surface (outside of the fermentation medium). Fontana et al. [11] defined a culture medium based on wheat bran aqueous extract (WBE) (without solids), which resulted in polygalacturonase (PG) activities similar to those obtained in media containing wheat bran (WB). After 96 h of cultivation, the highest endo- and exo-PG activity levels were obtained in the WB medium—40.3 and 18.6 U ml^{-1}, respectively—whereas with the WBE medium, lower enzymatic activities were achieved: 33.1 U ml^{-1} for endo-PG, and 16.1 U ml^{-1} for exo-PG.

Cultivation in airlift bioreactor (ALB)

The runs in the ALB were performed under initial inoculation of 3×10^8 spores ml^{-1}, at 30°C, initial pH of 6.0, and aeration of 0.07 vvm or 0.17 vvm. Under both aeration conditions, xylanase production occurred, being higher at the highest aeration rate.

When 0.07 vvm of aeration was introduced in the ALB, xylanase and β-xylosidase synthesis occurred practically during the same period, i.e., mainly between 96 and 168 h of fermentation, with peak activity at 144 h (9,265 U l^{-1}) for xylanase and 120 h (10.1 U l^{-1}) for β-xylosidase (Fig. 4). However, with 0.17 vvm of aeration, both xylanase and β-xylosidase production were faster, occurring between 48 and 144 h of fermentation, with maxima for xylanase (12,845 U l^{-1}) and β-xylosidase (10.1 U l^{-1}) production observed at 96 h (Fig. 5).

Regarding the production period, Fenice et al. [10] observed that the lag phase of laccase production in ALB was markedly reduced with respect to that in STR. In fact, the onset of enzyme activity occurred after 2 days from inoculation and the maximal production (4,300 U l^{-1} on day 7) occurred earlier. Thereafter, activity levels remained nearly constant until the end of fermentation (14 days). The time required to reach the maximal MnP activity (410 U l^{-1}) was also reduced (7 days). A negative effect of agitation (mechanical stress) on production of fungal peroxidases [4, 28] and laccases [13, 30] has been observed

in other studies. In fact, with a pneumatically agitated system, both the lag phase and the time required to reach maximal enzyme activities were considerably reduced with respect to STR.

Aeration was very important to obtain good productivity values for xylanolytic enzymes, while also contributing to foster the growth of the microorganism. Fungal biomass determination was hindered by the presence of solid residues (wheat bran), and also because of adherence of the fungus to the wheat bran and by physical adsorption of fungal cells to wheat bran [1], making it impossible to determine the fungal cell mass concentration in the culture. Furthermore, aeration influenced the time of production of those enzymes, thus contributing to improve the viability of the process.

A high aeration rate was important to maintain circulation of the medium inside the ALB, particularly due to filamentous mycelium formation. This filamentous network increased the medium viscosity considerably, causing a reduction of medium circulation.

The glucoamylase level of the free cell culture reported by Kilonzo et al. [18] in the ALB was approximately 20% higher than that in the in STB due to high cell density (cell dry weight/volume of bioreactor).

The specific power input and shear stress on the cells are described to be lower in ALB, in comparison with STB. Kim et al. [19] and Siedenberg et al. [27] also observed that xylanase production was higher in ALB than in STB. ALBs can only be operated at low specific power inputs at which oxygen transfer occurs with high efficiency with respect to the power input. However, their optimal operation range differs considerably from that of STBs. When using ALBs, diluted cultivation medium has to be used to reduce the cell mass concentration and the viscosity of the medium. The solid substrate concentration (e.g., peanut flour) must be reduced as well to achieve the necessary reduction in the viscosity of the cultivation medium. With consumption of the solid substrate and the change of the morphology of the fungus, the viscosity of the cultivation medium decreased and the mass transfer into the medium and the supply of oxygen and nutrient to cells improved [27].

Aleksieva and Peeva [2] observed in *Humicola lutea* that proteolytic activity obtained in batch cultivation in ALB and STB were similar, reaching maximum activity of around 1,100–1,200 U ml^{-1} after 60 h.

In contrast, Fontana et al. [11] reported that, when *Aspergillus oryzae* IPT301 was cultivated in STR and airlift bioreactors, slightly higher polygalacturonase production was achieved for the STR, in terms of both yield and productivity. Levels of 91.3 U ml^{-1} of endo-PG and 65.2 U ml^{-1} of exo-PG were reached in the STR bioreactor and 86.2 U ml^{-1} of endo-PG and 60.6 U ml^{-1} of exo-PG in the airlift bioreactor in approximately 96 h of cultivation. According to Kahar et al. [15], mycelium growth and morphology, and formation of products are related to the type of bioreactor used. For *A. oryzae* IPT301, apparently, the expected shearing effect of the STR turbines is not so critical. However, the similar results for the final endo- and exo-polygalacturonase activities suggest that the airlift bioreactor has potential for use in production of these enzymes in larger scale operation, with lower installation and operation costs in comparison with STR.

Conclusions

The results obtained suggest that variables such as aeration and bioreactor configuration are key to the definition of a strategy to optimize biosynthesis and production of microbial enzymes. Xylanase production rates by *Aspergillus terricola* in ALB were considerably higher than in STB. Other researchers reported that production of some enzymes decreased considerably when the fungus was grown in STB, presumably because of damage to mycelia caused by shear stress. This type of bioreactor, which is widely used for xylanase production, is known to induce enough shear to rupture mycelial cells and deactivate xylanase. Recently, alternative bioreactors, such as ALB or bubble columns, which induce lower shear stress, have started to find application in xylanase production.

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References

1. Adolph S, Muller SR, Siedenberg D, Jager K, Lehmann H, Schugerl K, Giuseppin M (1996) Cultivation of *Aspergillus awamori* on wheat bran: ultrastructural description of growth and localization of xylanase by immunogold labeling. J Biotechnol 46:221–234
2. Aleksieva P, Peeva L (2000) Investigation of acid proteinase biosynthesis by the fungus Humicola lutea 120–5 in an airlift bioreactor. Enz Microbial Technol 26:402–405
3. Biely P (1985) Microbial xylanolytic system. Trends Biotechnol 3:286–290
4. Bonnarme P, Delattre M, Drouet H, Corrieu G, Odier E (1993) Toward a control of lignin and manganese peroxidase hypersecretion by *Phanerochaete chrysosporium* in agitated vessels: evidence of the superiority of pneumatic bioreactors on mechanically agitated bioreactors. Biotechnol Bioeng 41:440–445
5. Chipeta ZA, Du Preez JC, Christopher L (2008) Effect of cultivation pH and agitation rate on growth and xylanase production by *Aspergillus oryzae* in spent sulphite liquor. J Ind Microbiol Biotechnol 35:587–594
6. Coelho GD, Carmona EC (2003) Xylanolytic complex from *Aspergillus giganteus*: production and characterization. J Basic Microbiol 43:269–277

7. Coughlan MP, Hazlewood GP (1993) β-1, 4-D-xylan-degrading enzyme system: biochemistry, molecular biology and applications. Biotechnol Appl Biochem 17:259–289
8. Doran PM (1995) Bioprocess engineering principles. Academic, London
9. Fang HY, Chang SM, Hsieh MC, Fang TJ (2007) Production, optimization growth conditions and properties of the xylanase from *Aspergillus carneus* M34. J Mol Catalysis B: Enzymatic 49:36–42
10. Fenice M, Sermanni GG, Federici F, D'Annibale A (2003) Submerged and solid-state production of laccase and Mn-peroxidase by *Panus tigrinus* on olive mill wastewater-based media. J Biotechnol 100:77–85
11. Fontana RC, Polidoro TA, Silveira MM (2009) Comparison of stirred tank and airlift bioreactors in the production of polygalacturonases by *Aspergillus oryzae*. Bioresour Technol 100:4493–4498
12. Haltrich D, Nidetzky B, Kulbe KD, Steiner W, Zupancic S (1996) Production of fungal xylanases. Bioresour Technol 58:137–161
13. Hess J, Leitner C, Galhaup C, Kulbe KD, Hinterstoisser B, Steinwender M, Haltrich D (2002) Enhanced formation of extracellular laccase activity by the white-rot fungus *Trametes multicolor*. Appl Biochem Biotechnol 98:229–241
14. Hoq MM, Hempel C, Deckwer D-W (1994) Cellulase-free xylanase by *Thermomyces lanuginosus* RT9: effect of agitation, aeration, and medium components on production. J Biotechnol 37:49–58
15. Kahar P, Kobayashi K, Iwata T, Hiraki J, Kojima M, Okabe M (2002) Production of polylysine in an airlift bioreactor (ABR). J Biosci Bioeng 93:274–280
16. Kang SW, Park YS, Lee JS, Hong SI, Kim SW (2004) Production of cellulase and hemicellulases by *Aspergillus niger* KK2 from lignocellulosic biomass. Bioresour Technol 91:153–156
17. Kersters-Hilderson H, Claeyssens M, Doorslaer EV, Saman E, Bruyne CK (1982) β-D xilosidase from *Bacillus pumilus*. Methods Enzymol 83:631–639
18. Kilonzo P, Margaritis A, Bergougnou M (2009) Airlift-driven fibrous-bed bioreactor for continuous production of glucoamylase using immobilized recombinant yeast cells. J Biotechnol 143:60–68
19. Kim SW, Kang SW, Lee JS (1997) Cellulase and xylanase production by *Aspergillus niger* KKS in various bioreactors. Bioresour Technol 59:63–67
20. Kulkarni N, Shendye A, Rao M (1999) Molecular and biotechnological aspects of xylanases. FEMS Microbiol Rev 23:411–456
21. Michelin M, Peixoto-Nogueira SC, Betini JHA, Silva TM, Jorge JA, Terenzi HF, Polizeli MLTM (2010) Production and properties of xylanases from *Aspergillus terricola* Marchal and *Aspergillus ochraceus* and their use in cellulose pulp bleaching. Bioprocess Biosyst Eng 33:813–821
22. Miller GH (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem 31:426–429
23. Polizeli MLTM (2009) Properties and commercial applications of xylanases from fungi. In: Rai M (ed) Advances in fungal biotechnology. IK International, New Delhi, Bangalore, pp 82–108
24. Pulls J, Poutane K (1989) Mechanism of enzymatic hydrolysis of hemicelluloses (xylans) and procedures for determination of the enzyme activities involved. In: Coughlan MP (ed) Enzyme systems for lignocellulose degradation. Elsevier Applied Science, London, pp 151–165
25. Reddy V, Reddy P, Pillay B, Singh S (2002) Effect of aeration on the production of hemicellulases by *T. lanuginosus* SSBP in a 30 l bioreactor. Process Biochem 37:1221–1228
26. Shah AR, Madamwar D (2005) Xylanase production by a newly isolated *Aspergillus foetidus* strain and its characterization. Process Biochem 40:1763–1771
27. Siedenberg D, Gerlach SR, Czwalinna A, Schugerl K, Giuseppin MLF, Hunik J (1997) Production of xylanase by *Aspergillus awamori* on complex medium in stirred tank and airlift tower loop reactors. J Biotechnol 56:205–216
28. Venkatadri R, Irvine RL (1990) Effect of agitation on ligninase activity and ligninase production by *Phanerochaete chrysosporium*. Appl Environ Microbiol 56:2684–2691
29. Vogel HJ (1964) Distribution of lysine pathways among fungi: evolutionary implications. Am Naturalist 98:435–446
30. Yoshiyama M, Ito Y (1994) Polyphenol oxidase production in a jar fermentor by *Coriolus versicolor*. J Ferment Bioeng 78:188–190