



Full Length Article

Effect of Wheat Bran Concentration on Xylanase Biosynthesis by *Aspergillus niger*

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ABSTRACT

Wheat bran an indigenous carbon source was tested as substrate by *Aspergillus niger* for optimum synthesis of xylanase using the submerged fermentation technique. The trials for xylanase production were conducted at three concentration levels (2.5, 3.0 & 3.5%) of wheat bran, four different fermentation temperatures (i.e., 25.0, 27.5, 30.0 & 32.5°C) and four various initial pH levels (5.0, 5.5, 6.0 & 6.5) of the culture medium for a period of 96 h. It is deduced from the study, that the organism exhibited maximum enzyme activity (78.03±2.73 IU/mL) at 3.0% wheat bran concentration followed by 66.07±1.94 and 64.47±2.75 IU/mL at 3.5 and 2.5% concentration, respectively at 30°C, 5.5 pH after a period 72 h of incubation. The comparison of the effect of various pH levels of culture medium exhibited that pH 5.5 had enhanced role in xylanase synthesis as compared to other pH levels investigated in the study. Time scale analysis revealed that a fermentation period of 72 h was best suitable for obtaining maximum yields of xylanase. Moreover a temperature of 30°C was found to be optimum for higher yields of xylanase exhibiting the mesophilic behavior of the organism.

Key Words: Xylanase; *Aspergillus niger*; Wheat bran; Mesophilic; Biosynthesis; Enzyme

INTRODUCTION

Wheat bran is one of the important by-products of food industry. The efficient manipulation of this by-product is its utilization for the production of value added products through fermentation biotechnology. Xylan is the substrate used by *Aspergillus niger* for the synthesis of xylanase. Xylan is the second most abundant polysaccharide and major component in plant cell wall that consists of β-1,4-linked xylopyranosyl residues (Puls, 1997). The structure of xylans found in cell walls of plants can differ greatly depending on their origin and different structures attached to the xylan backbone. Although most of the xylans have branched structures, however some linear polysaccharides have been isolated (De Vries & Visser, 2001). The complete enzymatic hydrolysis of xylan into its constituent monosaccharides requires the synergistic action of a group of xylanolytic enzymes. This is due to the fact that xylans from different sources exhibit a significant variation in composition and structure (Hazlewood & Gilbert, 1993; Cesar & Mrsa, 1996; Latif *et al.*, 2006). The most important enzyme is endo-1,4-xylanase (EC 3.2.1.8) that initiates the conversion of xylan into xylooligosaccharides. Xylosidase, debranching enzymes (L-arabinofuranosidase & glucuronidase) and esterases (acetyl xylan esterase, feruloyl esterase) allow the complete degradation of the xylooligosaccharides to their monomeric constituents

(Jeffries, 1996; Biely *et al.*, 1997; Subramaniyan & Prema, 1998). The various biotechnological techniques like submerged and solid state fermentation are employed for xylanase biosynthesis (Cai *et al.*, 1998; Gawande & Kamat, 1999; Kansoh & Gammel, 2001). The submerged fermentation is most beneficial as compared to other techniques due to more nutrients availability, sufficient oxygen supply and less time required for the fermentation (Hoq *et al.*, 1994; Gomes *et al.*, 1994; Veluz *et al.*, 1999; Gouda, 2000). The production of microbial xylanases is preferred over plant and animal sources, because of their availability, structural stability and easy genetic manipulation (Bilgrami & Pandey, 1992). Agricultural waste materials/by products like corn cobs, sugar cane bagasse, rice husk, rice straw and oat straw have been used by many scientists for xylanase synthesis (Siedenberg *et al.*, 1998; Christov *et al.*, 1999; Gawande & Kamat, 1999; Haq *et al.*, 2002). In the present project wheat bran at 2.5, 3.0 and 3.5% concentration was tested to investigate its potential to enhance xylanase synthesis by the organism.

MATERIALS AND METHODS

Substrate. Indigenous carbon source like wheat bran was utilized as substrate for the biosynthesis of xylanase enzyme. The wheat bran was procured from Taj Flour Mills, Faisalabad. The substrates was dried, ground to 40

mm mesh and treated with 2.0% NaOH. The prepared sample was stored in air tight container, for further utilization in the xylanase synthesis.

Fermentative organism. The pre-isolated and purified culture of the fungus *Aspergillus niger* was obtained from the Biotechnology Laboratory of NIFSAT, University of Agriculture, Faisalabad for xylanase biosynthesis.

Growth on PDA for sporulation. *Aspergillus niger* culture was cultivated on the Potato dextrose Agar as the spores were to be stored for longer period for the utilization of organism in different trials. The sporulation medium was prepared and pH 6.0 was maintained with 1 M HCl and 1 M NaOH. The prepared medium was autoclaved at 121°C for 15 min under 1.1 kg/cm² pressure. After cooling, the medium was transferred aseptically to pre-sterilized cotton plugged test tubes. The tubes were inoculated with the mother culture of the organism and incubated at 30°C for 3 days to allow the spores to germinate (Asghar, 2000).

Preparation of inoculum. The medium for inoculation was prepared; pH 5.5 was maintained and sterilized by autoclaving. The culture from the sporulation medium was transferred to the inoculation medium in 500 mL conical flask by using inoculation loop under aseptic conditions. The inoculated medium was incubated at 37°C in an orbital shaker at 130 rpm for 3 days.

Enzyme production. After 72 h of incubation, 3% of the inoculum was added to each fermentation flask (250 mL) for xylanase synthesis. The optimization of various culture conditions like pH, temperature of incubation and period of fermentation was carried out during the study.

Optimization

Carbon source. Substrate of the local origin wheat bran was used at 2.5, 3.0 and 3.5% concentrations.

pH. Xylanase biosynthesis was carried out at different pH values (5.0, 5.5, 6.0 & 6.5) using mentioned carbon source to find out the optimum pH level for enzyme production.

Temperature. For optimization of temperature, the production of xylanase was performed at different temperatures (25, 27.5, 30 & 32.5°C).

Incubation time. To find out the optimum time required for maximum xylanase activity, samples were harvested at different time intervals i.e., 24, 48, 72 and 96 h.

Sample harvesting. After specific interval of incubation, the biomass from the experimental flasks was filtered through Whatman filter paper No. 1. The filtrate was centrifuged at 10,000 rpm for 15 min at 10°C in the centrifuge [Sigma Laborzentrifugen (3K30) D-37520, Osterode-am-Harz, Germany] to remove the spores and mycelia of the organism. The supernatant was carefully collected and stored at refrigerated temperature in sterilized glass bottles.

Enzyme activity. Xylanase hydrolyzes the polymer xylan into the xylose monomers. The free xylose units produced as a result of xylanase activity; react with 3-5 dinitrosalicylic acid (DNS) reagent and form a colored complex that is measured by spectrophotometer at

wavelength 550 nm. Greater the amount of xylose produced, darker will be the color of the enzyme-xylose complex and more will be light absorbed.

Enzyme assay. The filtrate was assayed for xylanase activity; determined at 55°C using 0.6% (w/v) oat spelt xylan (sigma) at pH 6.0. Reducing sugars were measured using DNS method (Miller, 1959; Carmona, 1998). Enzyme activity was expressed as IU/mL.

Unit of activity. According to the International Union of Biochemistry, one international unit of Xylanase (1 IU) corresponds to the amount of enzyme required to release 1 micromole of reducing sugar (xylose) in 1 min.

Estimation of activity. For the estimation of enzyme activity, 1.0 mL of enzyme filtrate was added in a test tube followed by 0.5 mL xylan (0.6%) along with 0.5 mL of distilled water. The test tube was incubated at 30°C for 30 min. Later, DNS reagent was added (2.0 mL) to the test tube, kept in boiling water for 5 min and cooled in ice water. A blank was also prepared in the same way as mentioned earlier but with out xylanase. The color intensity was estimated at 550 nm using spectrophotometer (CECIL CE 7200).

Statistical analysis. The data obtained were analyzed by Complete Randomized Design (CRD) and level of significance was determined by analysis of variance technique as described by Steel *et al.* (1997).

RESULTS

Xylanase was synthesized by *Aspergillus niger* on different wheat bran concentrations using four different pH levels (5.0, 5.5, 6.0 & 6.5) and four incubation temperatures (25.0, 27.5, 30.0 & 32.5°C) over a period of 96 h.

The mean values in Fig. 1 depict the effects of different pH levels on xylanase biosynthesis at 2.5% wheat bran concentration and 25°C over an incubation period of 96 h. It is obvious from the graphical illustration that in the beginning of study, the organism produced small amount of enzyme at all pH levels, but as the time of incubation proceeded, enzyme synthesis increased gradually up to 72 h and then a decreasing trend was observed. The organism produced maximum enzyme (45.93±1.19 IU/mL) at pH 5.5 after 72 h of incubation that decreased to 44.67±1.02 IU/mL at 96 h.

In an other trial (Fig. 2) at the initiation of the trial, lower activities of xylanase were observed at all pH levels, because organism was at acclimatizing stage however as the study was prolonged, the fungus showed increasing trend in enzyme synthesis up to 72 h then showed a decline. At pH 5.5, the organism produced maximum enzyme (55.43±1.08 IU/mL) after 72 h of incubation followed by 44.63±1.22 and 36.67±1.02 IU/mL at pH 5.0 and 6.0, respectively. However at pH 6.5, the fungus showed least enzymatic activities as manifested from the value 23.57±1.87 IU/mL at 96 h of fermentation.

The synthesis of xylanase was carried out by *A. niger*

Table I. Mean squares for xylanase production at various concentrations of wheat bran

SOV	df	2.5%	3%	3.5%
Temp	3	832.53**	2235.88**	1410.93**
pH	3	1855.71**	2397.36**	2136.00**
Time	3	11287.65**	16536.27**	12262.13**
Temp x pH	9	108.32589**	60.8443**	42.4054**
Temp x Time	9	139.37**	177.67**	106.47**
pH x Time	9	156.77**	139.13**	139.36**
Temp x pH x Time	27	35.65**	31.072**	31.168**
Error	128	16.374531	0.061406	0.064583
Total	191			

** Highly significant

Fig. 1. Xylanase production by *Aspergillus niger* during 96 h fermentation at 25°C and 2.5% wheat bran in culture medium at different pH levels

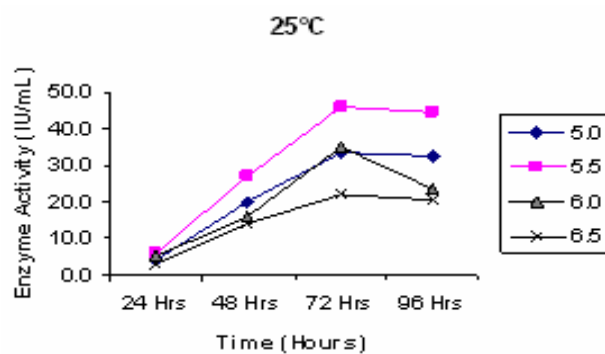
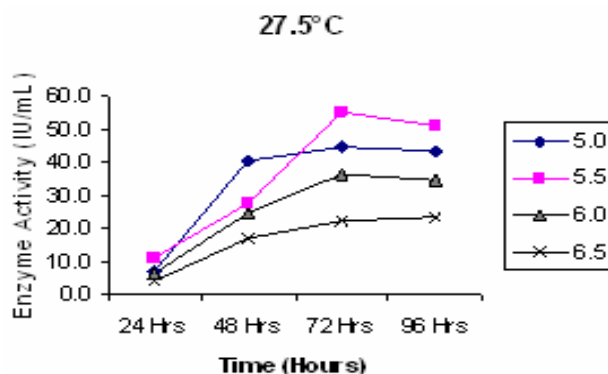


Fig. 2. Xylanase production by *Aspergillus niger* during 96 h fermentation at 27.5°C and 2.5% wheat bran in culture medium at different pH levels



at 30°C, various pH levels of the medium and 2.5% concentration of wheat bran as carbon source (Fig. 3). Initially, the fungus produced low activities of the enzyme but with the passage of time, there was gradual increase in enzyme synthesis up to 72 h. At 3rd day of incubation and pH 5.5, the fungus efficiently synthesized enzyme and gave 64.47±2.75 IU/mL of xylanase that reduced to 45.53±1.36 IU/mL at 96 h of incubation.

The graphical representation (Fig. 4) elucidated that the activity of enzyme was minimum at all pH levels up to 24 h of incubation. The means explicated an increasing

Fig. 3. Xylanase production by *Aspergillus niger* during 96 h fermentation at 30°C and 2.5% wheat bran in culture medium at different pH levels

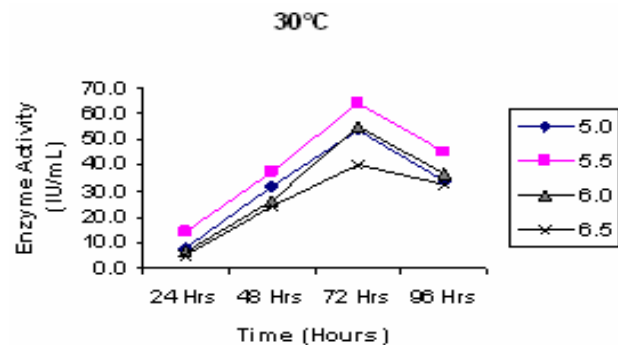


Fig. 4. Xylanase production by *Aspergillus niger* during 96 h fermentation at 32.5°C and 2.5% wheat bran in culture medium at different pH levels

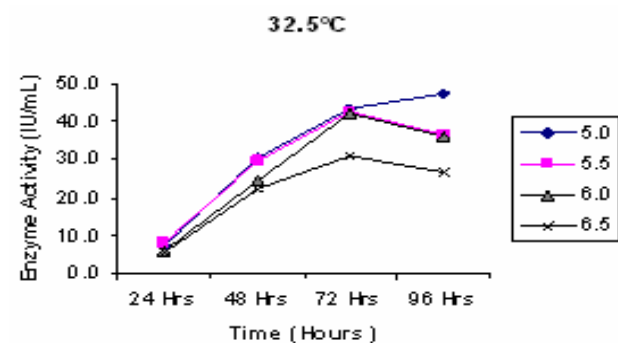
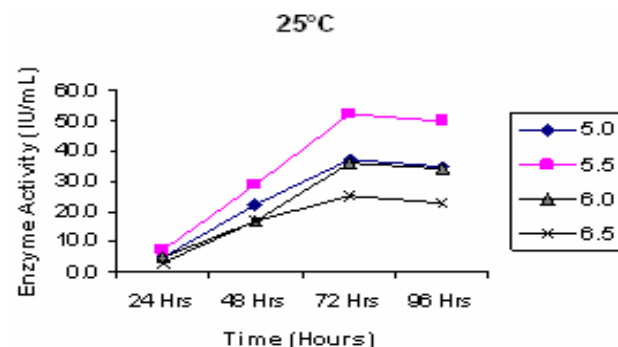


Fig. 5. Xylanase production by *Aspergillus niger* during 96 h fermentation at 25°C and 3.0% wheat bran in culture medium at different pH levels



trend in enzyme activity with the passage of time up to a certain period and after that it began to decrease. The organism showed varying behavior at pH 5.0 as the enzyme production showed linear correlation with fermentation time till the end of trial i.e., 96 h however, in all other cases enzyme activity increased up to the 3rd day of fermentation but showed a declining trend afterwards. Maximum xylanase activity (47.20±2.13 IU/mL) was observed at 96 h of incubation when the initial pH of the medium was adjusted

Fig. 6. Xylanase production by *Aspergillus niger* during 96 h fermentation at 27.5°C and 3.0% wheat bran in culture medium at different pH levels

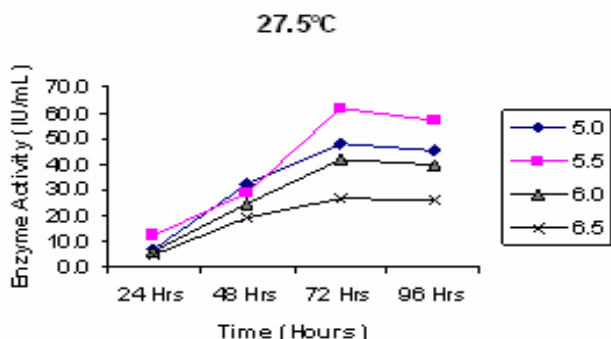


Fig. 7. Xylanase production by *Aspergillus niger* during 96 h fermentation at 30°C and 3.0% wheat bran in culture medium at different pH levels

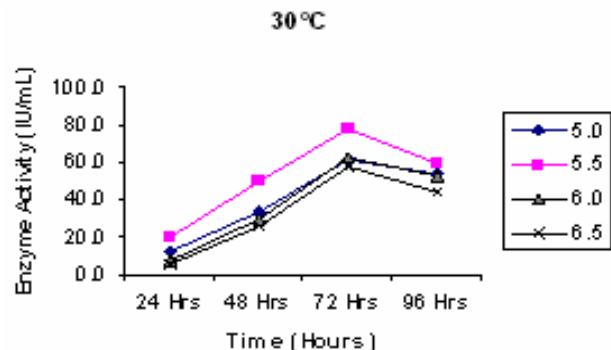
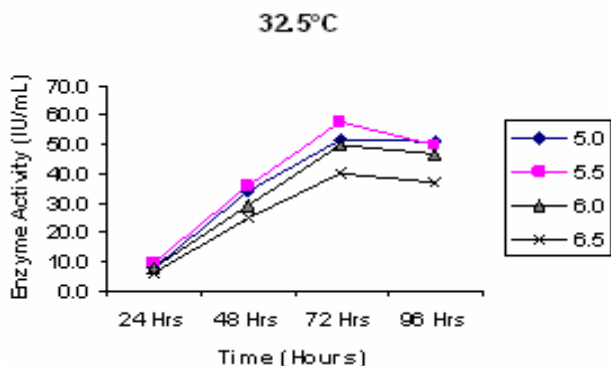


Fig. 8. Xylanase production by *Aspergillus niger* during 96 h fermentation at 32.5°C and 3.0% wheat bran in culture medium at different pH levels



to 5.0. When fermentation was carried out at pH 6.5, the fungus produced only 31.07±1.09 IU/mL of the xylanase after 72 h that decreased to 26.57±1.08 IU/mL at 96 h.

The mean values in Fig. 5 depict that the fungus could not produce considerable amounts of xylanase during the first 24 h of incubation as it was the acclimatizing period for the organism. Later on, on the 3rd day of incubation at pH 5.5, the fungus produced maximum xylanase activity

52.00±1.09 IU/mL followed by 36.97±1.13 IU/mL and 36.03±1.12 IU/mL at pH levels 5.0 and 6.0, respectively. While at pH 6.5, the fungus could produce only 25.03±1.09 IU/mL of the enzyme activity at 72 h.

The graphical representation (Fig. 6) explicit that after 72 h of incubation the organism produced maximum enzyme activity (62.07±1.92 IU/mL) at pH 5.5 that decreased to 57.00±1.98 IU/mL when the study was extended at 96 h. The decline in the enzyme activity after 72 h may be due to action of the proteolytic enzyme present in the culture medium. In contrast to pH 5.5, the organism produced least activities of the enzyme at pH 6.5 i.e., 27.03±1.54 and 26.07±1.03 IU/mL at 72 and 96 h of fermentation.

The mean values plotted in Fig. 7 indicated that at pH 5.5, the fungus produced maximum enzyme activity (78.03±2.73 IU/mL) over 72 h of incubation that decreased to 59.97±2.06 IU/mL at 96 h of fermentation. The organism showed lower potential for the enzyme production at pH 6.5 and released only 58.03±1.85 and 44.00±2.53 IU/mL of the enzyme at 72 and 96 h of incubation, respectively.

The time scale production of the xylanase by *A. niger* at 32.5°C, four different pH levels of the culture medium, 3.0% wheat bran concentration is represented in Fig. 8. The mean values explicated that the organism produced minute quantity of enzyme up to 24 h but afterwards, the enzyme activity increased at all pH levels. At 48 h of incubation, the fungus produced maximum activity (35.97±1.25 IU/mL) at pH 5.5, while in case of pH 5.0 the organism showed 34.03±1.83 IU/mL xylanolytic activity followed by 29.03±1.09 IU/mL at pH 6.0 and 25.07±1.08 IU/mL at pH 6.5.

The Fig. 9 explained the time course production of xylanase by *A. niger* at 25°C, selected pH values and 3.5% wheat bran concentration. The fungus exhibited maximum enzyme activity (49.07±1.85 IU/mL) after 72 h when the fermentation was carried out at pH 5.5 that decreased to 46.97±2.09 IU/mL at 96 h. In contrast, pH 6.5 proved to be the least effective to promote enzyme synthesis and the fungus could produce 20.07±1.86 and 19.97±1.02 IU/mL enzyme activity at 72 and 96 h, respectively. In another experiment (Fig. 10), the fungus produced maximum enzyme activity 58.07±1.34 IU/mL at pH 5.5 when the incubation was carried out for 72 h. There was observed that pH 6.5 was the least effective to promote xylanase production indicating maximum enzyme activity of 26.13±1.02 IU/mL at 72 h of fermentation.

In an other study (Fig. 11) the organism produced 66.07±1.94 IU/mL enzyme activity at pH 5.5 after 72 h that became 48.07±2.05 IU/mL after 96 h of fermentation. However, the organism produced only 50.03±2.04 IU/mL of xylanase after 72 h, when the fermentation was carried out at pH 6.0. Likewise comparing the enzyme synthesis at all pH levels it was found that least activity 36.07±1.37 IU/mL was at pH 6.5 on the 4th day of incubation.

Fig. 12 illustrates that Xylanase production showed

Fig. 9. Xylanase production by *Aspergillus niger* during 96 h fermentation at 25°C and 3.5% wheat bran in culture medium at different pH levels

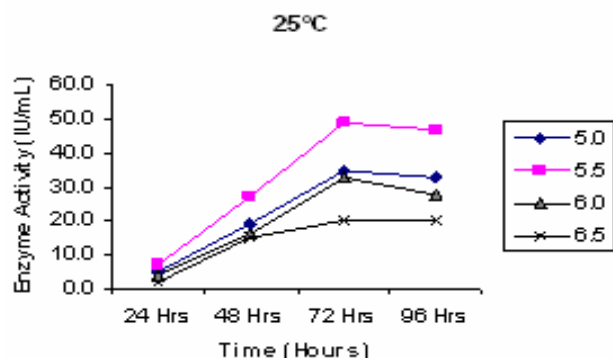
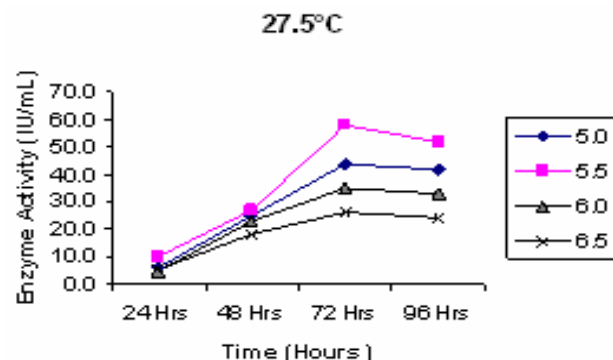


Fig. 10. Xylanase production by *Aspergillus niger* during 96 h fermentation at 27.5°C and 3.5% wheat bran in culture medium at different pH levels



positive correlation with time up to 72 h. The maximum enzyme activity 52.03 ± 2.53 IU/mL was observed at pH 5.5 on 3rd day of incubation, whereas incubation of organism up to 96 h resulted in the enzymatic activity as 47.97 ± 1.83 and 40.07 ± 2.04 IU/mL at pH 5.5 and 6.0, respectively. Minimum xylanolytic activity (32.03 ± 1.09 IU/mL) was recorded at pH 6.5 and 96 h.

DISCUSSION

Aspergillus niger is mesophilic in nature therefore, it performed best at 30°C, while higher temperatures exerted negative impact on the growth of organism resulting in reduced xylanase synthesis. Moreover the lower concentration (2.5%) of wheat bran resulted in lesser xylanase activities due to less availability of nutrients by the substrate, while the higher concentrations of the substrate (3.5%) also gave lower xylanase activities due to poorer oxygen availability in the thick culture medium. On the other hand the wheat bran concentration (3.0%) played a better role due to the provision of sufficient nutrients and oxygen supply. The impact of time of fermentation on the xylanase activities revealed that at the start of trials the organism was in acclimatizing stage, so could not produce sufficient activities of the enzyme. At 72 h the culture was most

Fig. 11. Xylanase production by *Aspergillus niger* during 96 h fermentation at 30°C and 3.5% wheat bran in culture medium at different pH levels

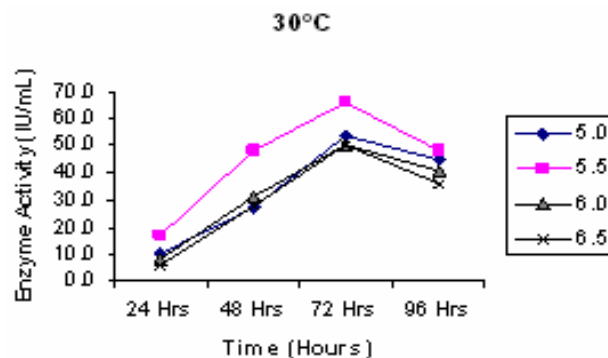
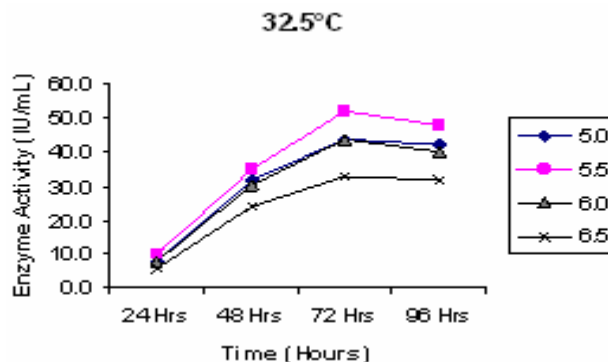


Fig. 12. Xylanase production by *Aspergillus niger* during 96 h fermentation at 32.5°C and 3.5% wheat bran in culture medium at different pH levels



vigorous and secreted maximum xylanase activities, while after 72 h, the xylanase activities decreased due to depletion of the nutrients from the culture medium and loss of xylanase activities by the proteolytic enzyme present in the culture medium.

The findings of the present study are in line with those of Duenas (1995) that 30°C as the best temperature for *Aspergillus* to produce maximum xylanase activity. In another investigation conducted by Chen *et al.* (1999) *A. niger* showed maximum activities 357.2 IU/mL for the enzyme when cultivated in shake flask at 28-32°C for 60 h. Furthermore, Ilieva *et al.* (1995) also calculated highest activities of xylanase at 30°C and 3.5-4.0 pH. During the present study, *A. niger* produced maximum enzyme at 72 h of fermentation. Xylanase yield increased gradually with time however, after 72 h the depletion of nutrients from the culture medium caused negative impact on fungal growth thus resulted in reduced enzyme synthesis. The findings of the present study are supported by the results reported by Camacho and Aguilar (2003) that when wheat bran was used as carbon source for the growth of *Aspergillus sp.*, maximum enzyme activity was observed at 72 h of fermentation. Likewise Cai *et al.* (1997) and Senthilkumar *et al.* (2005) observed maximum xylanase activities at 72 h of incubation. However present results showed some

variations with the work of Palma *et al.* (1996); they got maximum (98.5 U/mL) xylanase activity at 96 h of incubation of *Aspergillus niger*. Chen *et al.* (1999) reported maximum enzyme recovery (357.2 U/mL) at 60 h, while Gawande and Kamat (1999) reported maximum xylanase activity (26.7 IU/mL) after 48 h of incubation. During the study, the fungus produced maximum enzyme activity at 3% wheat bran concentration that is supported by Gokhale *et al.* (1986); they obtained higher yields of the enzyme when *A. niger* NCIM 1207 was grown on wheat bran. The results are also in conformity with those of Haq *et al.* (2002) who reported highest enzyme activity (1845U/g) on wheat bran when used mutant strain *A. niger* GCBMX-45 for enhanced xylanase production. Cai *et al.* (1998) studied the potential of *A. niger* A3 for the production of xylanase in solid state fermentation, initial pH 4.6, temperature 28°C, ratio of wheat bran to bagasse 1: 1.5 and fermentation for 72 h; specific activity of xylanase (514.7 IU/g) was observed. Likewise, Couri *et al.* (2000) synthesized a mixture of polygalacturonase, cellulase, xylanase and protease by using *A. niger* 3T5B8 when grown on various agricultural waste materials in solid state fermentation. Wheat bran as carbon source resulted in highest concentrations of mixture of enzymes (xylanase 30.62 U/mL). It is concluded from the outcomes of the present study that the fungus (*A. niger*) synthesized highest enzyme activities at 72 h of fermentation. The enzyme production increased gradually with the advancement in incubation time. However, at 96 h a decrease in enzyme synthesis was observed that may be due to the exhaustion of nutrients from the medium that affected the organism's growth.

CONCLUSION

The highest xylanase activity 78.03±2.73 IU/mL was observed at 30°C, pH 5.5, 3.0% concentration of wheat bran and incubation period of 72 h.

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