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Full Length Research Paper

Effect of corn cobs concentration on xylanase biosynthesis by *Aspergillus niger*

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Corn cobs, an indigenous carbon source, were 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 and 3.5%) of corn cobs, four different fermentation temperatures (25.0, 27.5, 30.0 and 32.5°C) and four various initial pH levels (5.0, 5.5, 6.0 and 6.5) of the culture medium for a period of 96 h. It was deduced from the study that the organism exhibited maximum enzyme activity (60.03 ± 1.83) at 3.0% corn cobs concentration, followed by 52.03 ± 1.21 and 50.07 ± 2.14 at 2.5 and 3.5% concentration, respectively at 30°C and 5.5 pH after a period 72 h of incubation. The comparison of the effect of various pH levels of culture medium showed that pH 5.5 had enhanced role in xylanase synthesis, as compared to other pH levels investigated in the study. Time scale analysis also 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 mesophillic behavior of the organism.

Key words: Xylanase, *Aspergillus niger,* corn cobs, mesophillic, biosynthesis, enzyme.

INTRODUCTION

Corn cobs are 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. It is the second most abundant polysaccharide and major component in plant cell wall, consisting 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, some linear polysaccharides have however been isolated (De Vries and Visser, 2001). The complete enzymatic hydrolysis of xylan into its constituent monosaccharide 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 and Gilbert, 1993; Cesar and 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 and glucuronidase) and esterases (acetyl xylan esterase and feruloyl esterase) allow the complete degradation of the xylooligosaccharides to their monomeric constituents (Jeffries, 1996; Biely et al., 1997; Subramaniyan and Prema, 1998).

Various biotechnological techniques like submerged and solid state fermentation are employed for xylanase

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biosynthesis (Cai et al., 1998; Gawande and Kamat, 1999; Kansoh and Gammel, 2001). The submerged fermentation is most beneficial as compared to other techniques due to the 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; Bim and Franco, 2000; 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 and Pandy, 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 and Kamat, 1999; Haq et al., 2002). In the present project, corn cobs at 2.5, 3.0 and 3.5% concentration were tested to investigate its potential to enhance xylanase synthesis by the organism.

MATERIALS AND METHODS

Substrate

Indigenous carbon source like corn cobs were utilized as substrate for the biosynthesis of xylanase enzyme. The wheat bran was procured from Rafhan Maize Products (Pvt) Limited Faisalabad. The substrates were 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 *A. niger* was obtained from the Biotechnology Laboratory of NIFSAT, University of Agriculture, Faisalabad, for xylanase biosynthesis.

Growth on PDA for sporulation

A. niger culture was cultivated on the Potato dextrose Agar (PDA) 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/cm^2 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, maintained at pH 5.5 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 inoculums 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 corn cobs were 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 and 6.5) using the aforementioned 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 and 32.5°C).

Incubation time

To find out the optimum time required for maximum xylanase activity, samples were harvested at different time intervals; 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 then carefully collected and stored at a 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. The greater the amount of xylose produced, the darker the color of the enzyme-xylose complex and the more the 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.

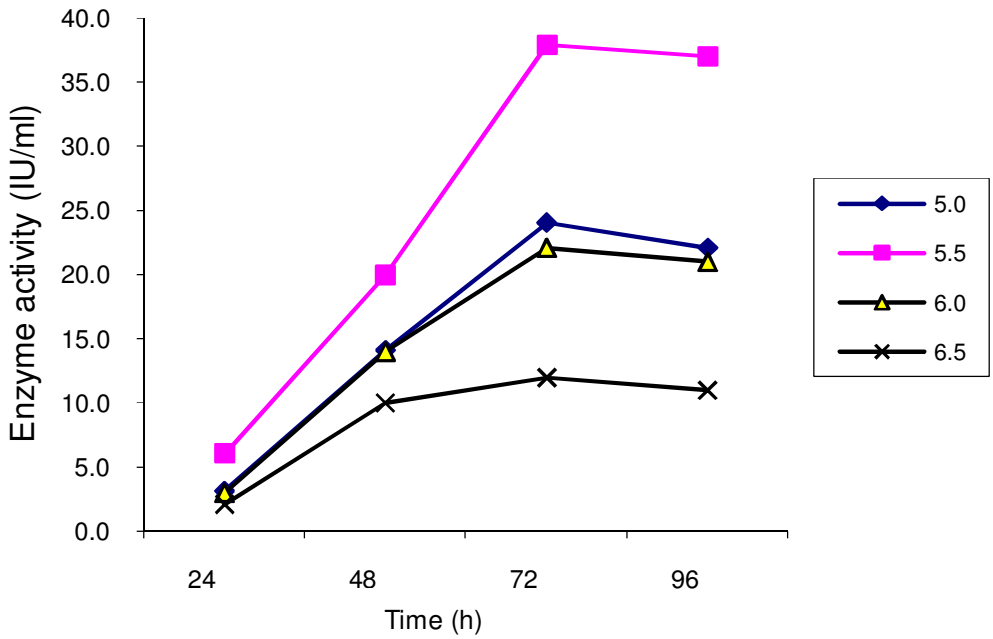

Figure 1. Xylanase production by A*spergillus niger* during 96 h fermentation at 25°C and 2.5% corn cobs concentration in culture medium at different pH levels.

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 aforementioned, but without 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 *A. niger* on different corn cobs concentrations using four different pH levels (5.0, 5.5, 6.0 and 6.5) and four incubation temperatures (25.0, 27.5, 30.0 and 32.5°C) over a period of 96 h.

During the experiment, xylanase synthesis was carried out by *A. niger* at four different pH values using 2.5% corn cobs as carbon source, at a temperature of 25°C (Figure 1, Table 1). The graphical illustration indicated that the fungus produced small activities of the enzyme at 24 h of incubation. Later, the enzyme synthesis continued to increase up to 72 h, and thereafter showed a declining trend that might be due to both the depletion of nutrients in the culture medium and break down of secreted xylanase by the proteolytic enzymes present in the medium. Maximum xylanase activity (37.93 ± 1.02 IU/ml) was achieved when the fermentation was carried out at pH 5.5 for 3 days, but was reduced to 37.03 ± 1.02 IU/ml on 4th day of incubation. It is also evident from the graphical depiction that the organism exhibited greater activities of the enzyme at pH 5.5 as compared to other pH levels on all incubation periods. The fungus produced least xylanase activities (11.97 ± 0.09 IU/ml) at pH 6.5 and 72 h, whereas; 10.97 ± 0.08 IU/ml activity was observed at 96 h of incubation.

The mean values from Figure 2 depict the effect of various pH levels and time of fermentation on the xylanase synthesis by *A. niger* using 2.5% corn cobs at temperature 27.5°C. At the initiation of trial (24 h), small amounts of the enzyme were produced, but later the enzyme synthesis increased gradually up to 72 h and started to decrease with further prolongation of fermentation period. It was observed that temperature 27.5°C, pH 5.5 and incubation period of 72 h were the factors to attain maximum xylanolytic activity.

Furthermore, (Figure 3) at 72 h, the enzyme activity (36.03 ± 1.09 IU/ml) was minimum at 6.5 pH, while at 96 h, the least activity (31.97 ± 0.09 IU/ml) was observed at pH 5.0. The xylanase biosynthesis by *A. niger* is presented in Figure 4 when incubated at 32.5°C and 2.5% concentration of corn cobs. The mean values explicated a linear correlation between various pH levels and time intervals during three days of incubation. The organism exhibited maximum enzyme activity (39.97 ± 1.08 IU/ml) at pH 5.5 and 72 h, which decreased to 38.07

Table 1. Mean squares for xylanase production at various concentrations of corn cobs.

SOV	df	2.5%	3%	3.5%
Temperature	3	1391.200**	2603.65**	2414.76**
pH	3	1336.449**	1481.551**	3259.01**
Time	3	7227.898**	9155.60**	8368.8**
Temperature × pH	9	28.4871**	31.21**	414.859^{ns}
Temperature × time	9	125.65**	208.647**	638.507^{ns}
pH x time	9	108.341**	123.332**	712.600^{ns}
Temperature × pH × time	27	21.1199**	20.125**	410.183^{ns}
Error	128	0.068	0.07989	469.61
Total	191			

** Highly significant; ns = non-significant.

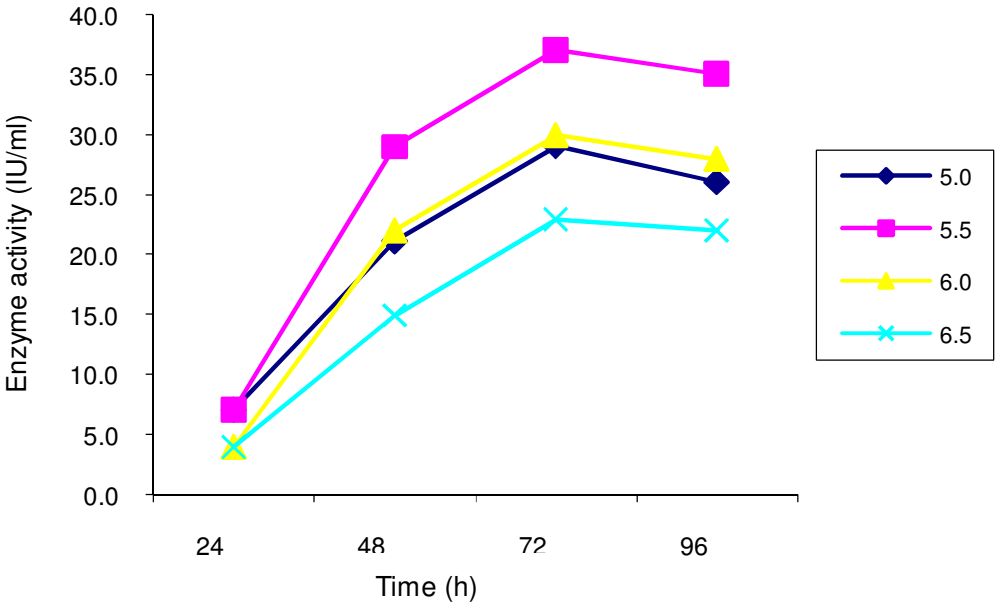

Figure 2. Xylanase production by *A. niger* during 96 h fermentation at 27.5°C and 2.5% corn cobs concentration in culture medium at different pH levels.

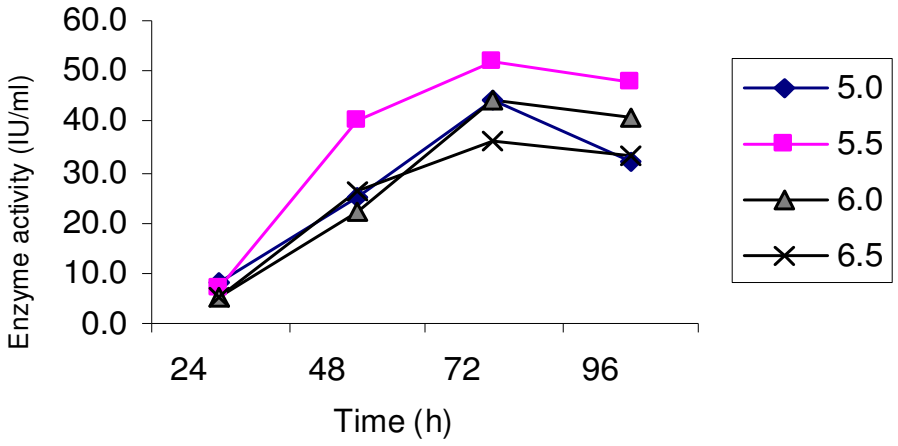

Figure 3. Xylanase production by *A. niger* during 96 h fermentation at 30°C and 2.5% corn cobs concentration in culture medium at different pH levels.

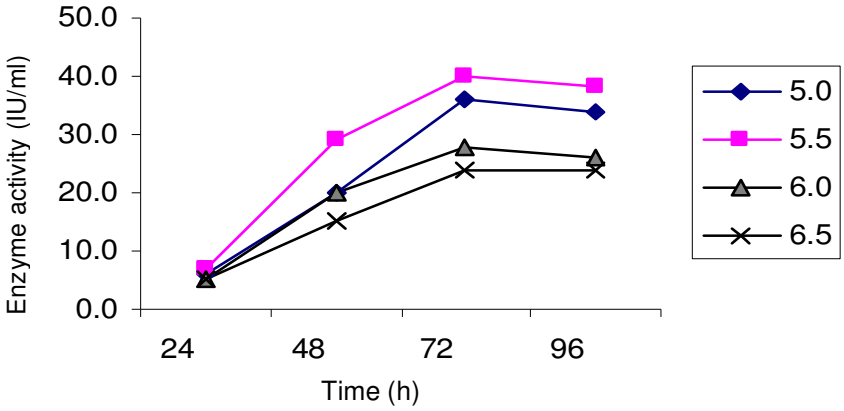

Figure 4. Xylanase production by *A. niger* during 96 h fermentation at 32.5°C and 2.5% corn cobs concentration in culture medium at different pH levels.

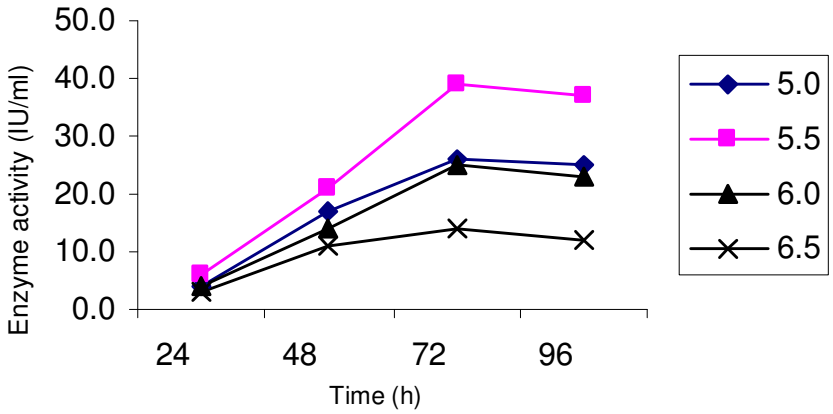

Figure 5. Xylanase production by *A. niger* during 96 h fermentation at 25°C and 3.0% corn cobs in culture medium at different pH levels.

± 1.04 IU/ml at 96 h.

In an experiment, the effect of various pH levels and time of fermentation period for xylanase synthesis by *A. niger* at 25°C and 3.0% corn cobs concentration, was investigated as represented in Figure 5. It is clear that in the beginning of the fermentation process (up to 24 h) the organism produced low activities of enzyme. Nevertheless, enzyme synthesis went on increasing up to 72 h and thereafter it exhibited a declining trend. When the trial was carried out at pH 5.5, the fungus produced maximum enzymatic activity 39.00 ± 1.09 IU/ml at 72 h that decreased to 37.00 ± 1.23 IU/ml at 96 h of incubation. In contrary, at pH 6.5, the organism produced least enzyme activities at all fermentation periods and produced 14.03 ± 0.64 and 11.97 ± 0.89 IU/ml at 72 and 96 h of fermentation, respectively.

The mean values in Figure 6 reflect the effect of incubation time and media pH on xylanase synthesis by *A. niger* at 27.5°C and 3.0% corn cobs concentration. When the fermentation was carried out at pH 5.5, the organism produced maximum enzyme activity (45.07 ± 1.09 IU/ml) at 72 h of incubation, which declined to 42.00 ± 0.84 IU/ml at 96 h of fermentation. The xylanase synthesis by *A. niger* is presented in Figure 7 when incubated at 30°C and 3.0% corn cobs concentration. The mean values explicated that the organism produced minute quantity of enzyme up to 24 h, but afterwards, the enzyme activity increased up to 72 h at all pH levels.

Figure 8 shows the mean values for the enzyme synthesis by *A. niger* when the experiment was carried out at various pH levels at 32.5°C and 3.0% corn cobs concentration in the culture medium. It is obvious that at 72 h of incubation, the fungus produced maximum xylanolytic activity (44.07 ± 1.12 IU/ml) at pH 5.5 that dwindled to 40.03 ± 1.06 IU/ml at 96 h of study. The graphical explanation of the data (Figure 9) elaborated that the fungus produced maximum enzymatic activity 35.03 ± 1.12 IU/ml at pH 5.5 over a period of 72 h that decreased to 33.97 ± 1.06 IU/ml at 96 h of incubation.

It is obvious (Figure 10) from the means that at pH 5.5 of the culture medium, the fungus produced highest xylanase activity 42.97 ± 1.21 IU/ml at 72 h of incubation

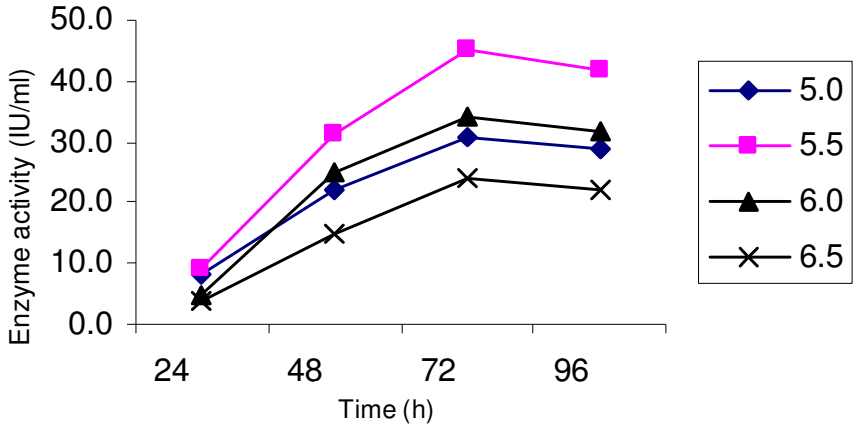

Figure 6. Xylanase production by *A. niger* during 96 h fermentation at 27.5°C and 3.0% corn cobs in culture medium at different pH levels.

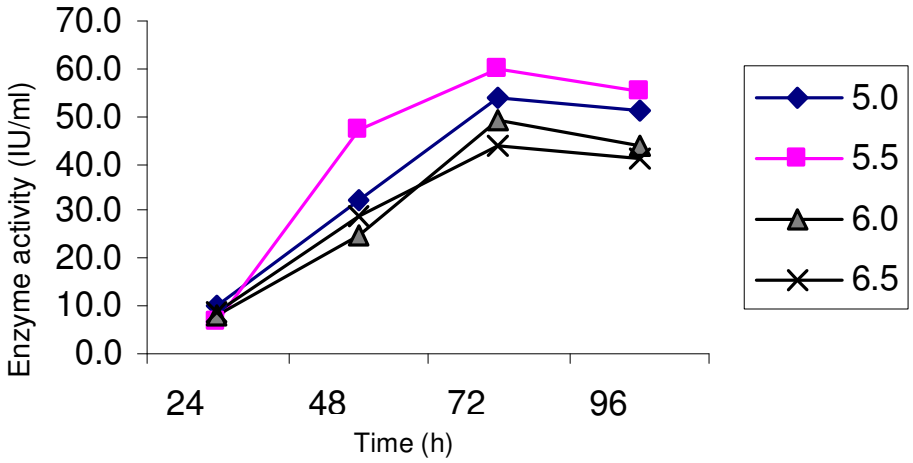

Figure 7. Xylanase production by *A. niger* during 96 h fermentation at 30°C and 3.0% corn cobs in culture medium at different pH levels.

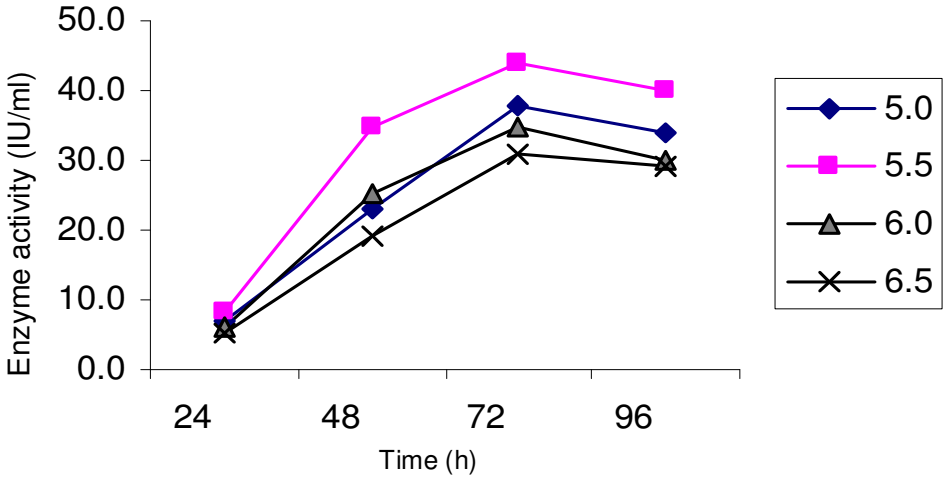

Figure 8. Xylanase production by *A. niger* during 96 h fermentation at 32.5°C and 3.0% corn cobs in culture medium at different pH levels.

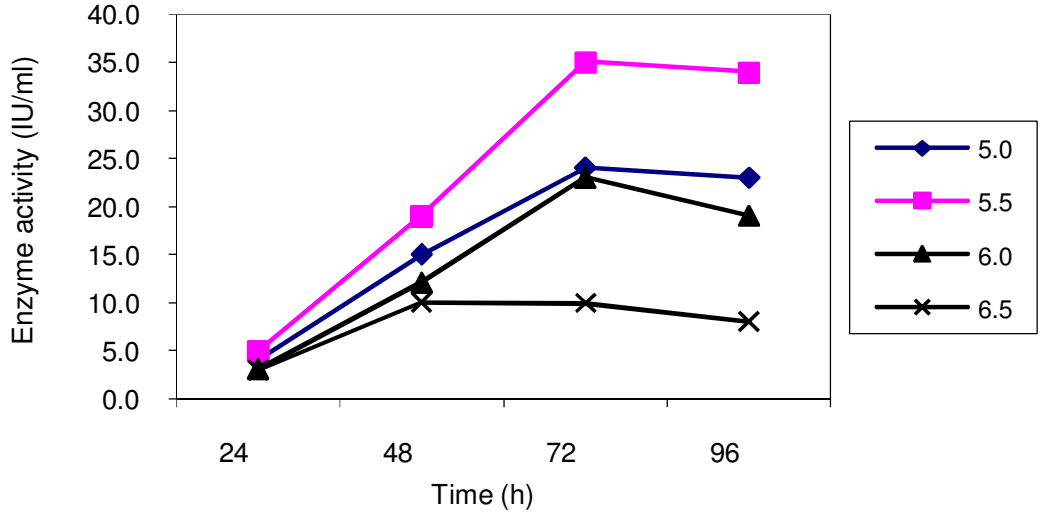

Figure 9. Xylanase production by *A. niger* during 96 h fermentation at 25°C and 3.5% corn cobs in culture medium at different pH levels.

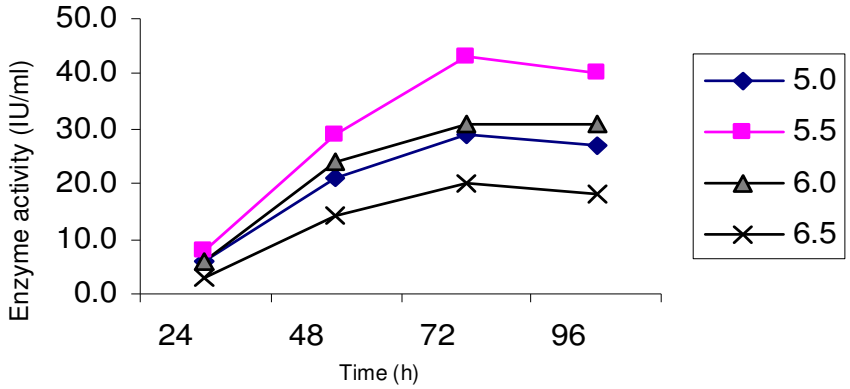

Figure 10. Xylanase production by *A. niger* during 96 h fermentation at 27.5°C and 3.5% corn cobs in culture medium at different pH levels.

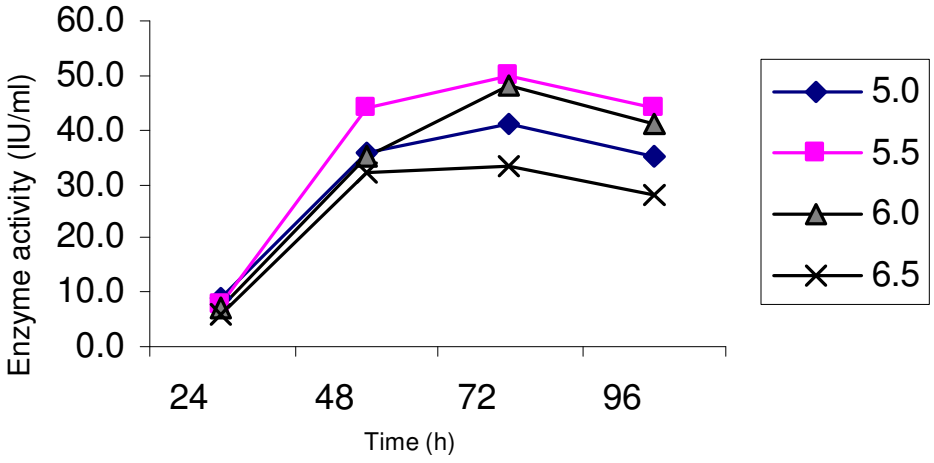

Figure 11. Xylanase production by *A. niger* during 96 h fermentation at 30°C and 3.5% corn cobs in culture medium at different pH levels.

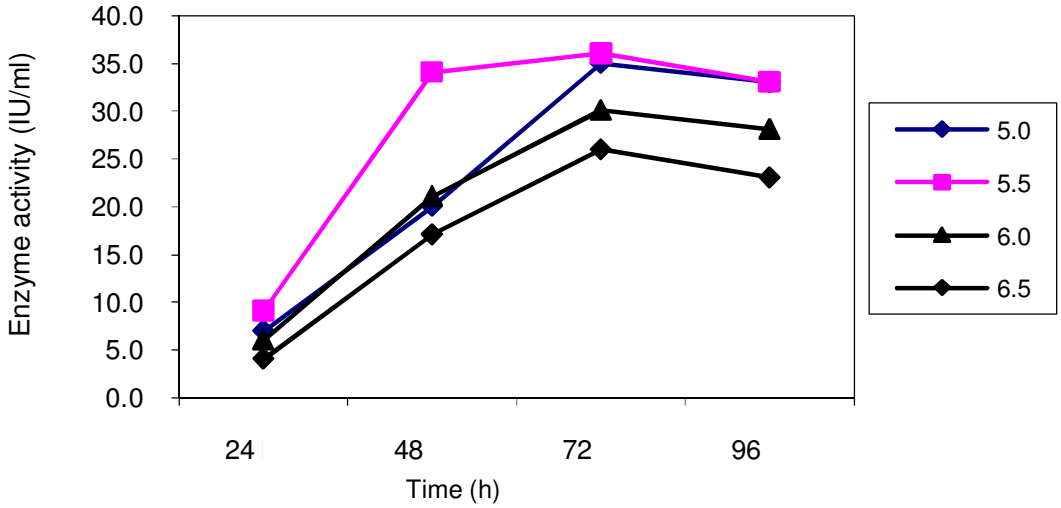

Figure 12. Xylanase production by A. *niger* during 96 h fermentation at 32.5°C and 3.5% corn cobs in culture medium at different pH levels.

that decreased to 40.03 ± 1.09 IU/ml at 96 h of fermentation. The pH 6.5 was least supportive to the fungus for xylanase synthesis; 20.07 ± 0.98 and 17.97 ± 1.07 IU/ml of the enzyme was produced at 72 and 96 h of incubation, respectively. It was also observed (Figure 11) that the fungus could produce little activities of the enzyme during the initial stages of incubation but with advancement in time there was a linear association of enzyme production with incubation time. It is obvious from the depiction (Figure 12) that fermentation at medium pH of 5.5 over a period of 72 h resulted in maximum enzymatic activity (36.03 ± 1.07 IU/ml) that decreased to 33.03 ± 0.98 IU/ml at 96 h.

The data for xylanase production by *A. niger* using corn cobs as substrate therefore indicated maximum xylanase activity 60.03 ± 1.83 IU/ml at 30°C, pH 5.5, 3% concentration and 72 h of incubation.

DISCUSSION

A. niger is mesophillic in nature and thus 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 corn cobs 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 corn cobs concentration (3.0%) played a better role due to the provision of sufficient nutrients and oxygen supply. Also, the impact of time of fermentation on the xylanase activities revealed that at the start of trials the organism was in acclimatizing stage, and so could not produce sufficient activities of the enzyme. At 72 h, the culture was most vigorous and secreted maximum xylanase activities, while after 72 h, the xylanase activities decreased due to both 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) and Ali et al. (2002) that 30°C is 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 to 32°C for 60 h. Furthermore, Ilieva et al. (1995) also calculated highest activities of xylanase at 30°C and pH 3.5 to 4.0. During the present study, *A. niger* produced maximum enzyme at 72 h of fermentation and 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 resulting in reduced enzyme synthesis. 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 IU/ml) xylanase activity at 96 h of incubation of *A. niger*. Chen et al. (1999) reported maximum enzyme recovery (357.2 IU/ml) at 60 h, while Gawande and Kamat (1999) reported maximum xylanase activity (26.7 IU/ml) after 48 h of incubation. 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, but 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.

The present results are in line with the findings of

Senthilkumar et al. (2005), who observed optimum xylanase recovery at 72 h of incubation. The findings are also supported by the results found by Camacho and Aguillar (2003), that when corn cob was used as carbon source for the growth of *Aspergillus.* sp, it synthesized 37.0 and 39.5 IU/ml xylanase activity at an incubation period of 48 and 72 h, respectively. However, Palma et al. (1996) and Kohli et al. (2001) observed maximum enzyme production after 96 h, whereas Kansoh and Gammal (2001) and Park et al. (2002) reported that the optimum xylanase production could be obtained after 5 days fermentation period. The contradiction may be due to the difference in fermentation conditions.

Conclusion

The highest xylanase activity (60.03 ± 1.83 IU/ml) was observed at 30°C, pH 5.5, 3.0% concentration of corn cobs and an incubation period of 72 h.

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