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SELECTION OF *ASPERGILLUS NIGER* MUTANT USING ANTIMETABOLITE 2-DEOXY D-GLUCOSE AFTER N-METHYL N-NITRO N-NITROSO GUANIDINE (MNNG) TREATMENT

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Abstract

A xylan-degrading enzyme (endo ß-1,4 xylanase, EC 3.2.1.8) cleaves ß-1,4 glycosidic bond to produce xylose and is useful mainly in biobleaching paper pulp, pharmaceutical and food industries. The present investigation deals with the selection of derepressed mutant of *Aspergillus niger* GCBT-35 using antimetabolite 2-deoxy D-glucose (2DG) after N-methyl N-nitro N-nitroso guanidine (MNNG) treatment and optimization of cultural conditions for the enhanced xylanase production. Medium containing (g/l) wheat bran 20.0, $NaNO_3$ 1.0, NH_4Cl 1.0, KH_2PO_4 1.0, $CaCl_2$ 1.0, $MgSO_4$. $7H_2O$ 0.3, meat extract 5.0 and Tween 80 1.5 ml was found to be best for xylanase production. The optimal production of xylanase (289.86 U/ml/min) was achieved 72 h after the conidial inoculation, when 1.0% (w/v) meat extract was used as a nitrogen source in the culture medium at an initial medium of 5.5 pH. This enhancement in xylanolytic activity is about 2.7 fold higher than that of wild culture.

Introduction

Xylan is the most abundant hemicellulose found mostly in the secondary cell wall of a plant cell. The basic molecular structure of xylan is a linear backbone comprised of β-1,4 linked D-xylopyranose units. The endo β-1,4 xylanase (EC 3.2.1.8) cleaves β-1,4 glycosidic bond to produce xylose (Maciel *et al*., 2008). There is a great interest in the enzymatic hydrolysis of xylan because of possible applications in ruminal digestion, waste treatment, fuel, chemical production and paper manufacture. These uses have placed a greater stress on increasing xylanase production (Lindberg *et al*., 2007; Tapingkae *et al*., 2008; Bakri *et al*., 2008).

Among the microorganisms that have xylanolytic activity, *Aspergillus niger* is one of the well known producer (Record *et al*., 2003; Raana *et al*., 2004; Maciel *et al*., 2008). Random mutagenesis can enhance enzyme productivity several fold than the parental strain. N-methyl N-nitro N-nitroso guanidine (MNNG), an alkylating agent, has found a potential routine application for inducing mutagenesis in the genome of microorganisms. The use of 2-Deoxy-D-glucose (DG), a toxic glucose analogue, as an antimetabolite has been routinely practiced for selection of the hyper-producing mutants (Steiner *et al*., 1998; Chadha *et al*., 1999; Rajoka & Khan, 2005; Azin & Noroozi, 2001, Loera & Cordova, 2003). The same approach of MNNG treatment and mutant selection was used in the present study.

The optimization of the fermentation medium plays an important role in enzyme production (Monica *et al*., 2002). The organisms need essential elements such as carbon, nitrogen, phosphorus and sulphur for growth and subsequent xylanase production. The concentration of these elements has a profound effect on the yield of xylanase (Bakri *et al*., 2003). Pakistan is an agricultural country and produces tons of agricultural by-products annually. These by-products are available for their commercial exploitation as cheap raw material for the synthesis of xylanase through fermentation. Xylanase production by *A. niger* has been found maximum when wheat bran was used as substrate compared to sugar cane bagasse or rice straw (Haq *et al*., 2002; Yao-xing *et al*., 2008).

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Time course study is one of the critical factors, which determines the efficacy of the process along with product formation. Many workers obtained maximum xylanase production 96 h after the inoculation. The production of xylanase is also known to be influenced by the medium pH. The purified extracellular xylanase produced by *Aspergillus* strain has optimal pH 5.5 (Kohli *et al.*, 2001; Camacho & Aguilar, 2003). Bakri *et al.,* (2003) found yeast extract as the best nitrogen source among the different nitrogen sources investigated such as, peptone, ammonium nitrate, sodium nitrate, ammonium chloride and ammonium sulfate.

The present study deals with the screening of 2-deoxy D-glucose resistant mutant strains of *A. niger* developed after N-methyl N-nitro N-nitroso guanidine treatment for utilization of agricultural by-product as inexpensive substrate.

Materials and Methods

Organism: *Aspergillus niger* strain GCBT-35 was taken from the available stock culture of *Institute of industrial Biotechnology, Government College University, Lahore, Pakistan.* The culture was maintained on xylan agar slants containing (g/l, w/v): KH_2PO_4 1.0, KCl 0.5, $(NH_4)_2$ SO_4 0.5, $MgSO_4.7H_2O$ 0.2, $CaCl_2$ 0.1, yeast extract 0.5, xylan, 5.0 and agar 20.0, (pH 5.5). It was stored at 4°C in a cold lab (Model: MPR-1410, SANYO, Japan) for culture maintenance.

2-deoxy-D-glucose resistance: Different concentration of 2-deoxy-D-glucose (0-1.0%, w/v; Sigma, France), were sterilized by passing through 0.45 µm pore sized cellulose acetate membrane filters (Sartorius, Germany) and added to Petri plates containing xylan agar medium. One hundred µl of the conidial suspension was transferred to the individual plates and incubated at 30±1°C. Growth was observed and 0.8% (w/v) concentration of 2-deoxy-D-glucose was shown to be inhibitory for the growth of parent strain.

Inoculum preparation: A volume of 45.0 ml of Vogel's medium containing (%, w/v): trisodium citrate 0.25, KH_2PO_4 0.5, NH_4NO_3 0.2, $(NH_4)_2SO_4$ 0.4, $MgSO_4$ 0.02, peptone 0.1 and yeast extract 0.2 (pH 5.5) was dispensed in 250 ml conical flask followed by the addition of approximately 20-25 glass beads (2.0-5.0 mm, dia.) to break mycelial pellets. The flask was sterilized at 15.0 lbs/in^2 pressure (121°C) for 15 min. Then 2.0 ml of the sterilized 50.0% (w/v) glucose stock solution was aseptically added into the autoclaved vogel's medium as an additional carbon source (Das & Ponty 1980). The flasks were inoculated with a loopful of *A. niger* conidia under aseptic conditions. Inoculum was allowed to grow at 30±1°C in a rotary shaking incubator (200 rpm) for 24 h. The cells were harvested, centrifuged at 6,000 rpm for 15 min, washed twice and re-suspended in saline water. This mycelial suspension was used for improvement through mutation by MNNG.

Mutation technique: Washed vegetative mycelial suspension was centrifuged at 6000 rpm for 10 min., and the pellet thus obtained was suspended in 15.0 ml of citrate buffer (0.1 M, pH 5.0) containing MNNG (3.0 - 7.0 mg/ml). Samples were drawn periodically (15-50 min) and reaction was stopped by addition of cysteine (100 mg/ml, w/v). Three consecutive washes followed by centrifugation were made with citrate buffer to get pellets free of MNNG. A control was run parallel replacing the MNNG with sterile distilled water. After appropriate dilution of sample in citrate buffer, they were transferred to the individual plates

containing xylan agar medium, 0.5% triton X-100 and 0.8% (w/v) 2-deoxy-D-glucose. The Petri plates were incubated at 30ºC and the number of colonies in each plate was counted with colony counter (Model: GM-16, Griffin, Germany).

The young colonies were selected on the basis of a larger transparent zone of xylan hydrolysis with 90% death rate and transferred to the xylan agar slants. The cultures were incubated at 30ºC for 5-7 days, for maximum sporulation. The selected strains were further screened for xylanase production by submerged fermentation.

Preparation of conidial suspension: The conidial suspension was prepared by adding 10.0 ml of sterilized 0.005 % (w/v) diocetyl ester of Sodium sulfo succinic acid (Monoxal O.T.) to a 5-7 day old xylan agar slant culture having profuse conidial growth. A sterile wire-loop was gently used to break the conidial clumps. The tube was shaken vigorously to make homogeneous suspension. The conidial count was made with Haemacytometer.

Fermentation technique: Twenty-five millilitre of the fermentation medium was added into the individual 250 ml cotton plugged conical flasks. The flasks were autoclaved at 15.0 lbs/in^2 pressure (121°C) for 15 min. After cooling to an ambient temperature, the flasks were inoculated with 1.0 ml of the conidial suspension containing 1.2×10^6 conidia and incubated at 30°C in a rotary shaking incubator (200 rpm) for 72 h. The ingredients of the flasks were filtered and the filtrate was used for the estimation of xylanase. All the experiments were run parallel in triplicates.

Culture media: Following six culture media were tested for xylanase production by the mutant strain of *A. niger*. The composition of the culture media (g/l) is as follows:-

M1: Peptone 10.0, meat extract 10.0, NaCl 10.0, KH_2PO_4 1.0, wheat bran 20.0, pH 5.0 (Monica *et al*., 2002).
M2: Yeast extract 15.0, KH_2PO_4 5.0, wheat bran 20.0, pH 6.5 (Johnson *et al*., 1999).
M3: $NaNO_3$ 1.0, NH_4Cl 1.0, KH_2PO_4 1.0, $CaCl_2$ 1.0, $MgSO_4.7H_2O$ 0.3, meat extract 5.0, Tween-80 1.5 ml, wheat bran 20.0, pH 5.0.
M4: $NaNO_3$ 6.0, KH_2PO_4 1.5, KCl 0.5, $MgSO_4.7H_2O$, 0.5, $ZnSO_4$ 0.001, $FeSO_4$ 0.001, $MnSO_4$ 0.001, $CuSO_4$ 0.001, wheat bran 20.0, pH 6.0 (Partricia *et al.*, 2001).
M5: KH_2PO_4 9.64, $CO(NH_2)_2$ 1.46, $CaCl_2.6H_20$ 0.30, $MgSO_4.7H_20$ 0.56, $FeSO_4$ 0.00978, $CoSO_4$ 0.00054, $MnSO_4$ 0.00052, $ZnSO_4.7H_2O$ 0.0018, $CuSO_4.5H_2O$ 0.00086, wheat bran 20.0, pH 3.5 (Rita *et al*., 2003).
M6: $NaNO_3$ 3.0, KCl 2.0, KH_2PO_4 1.0, $MgSO_4.7H_2O$ 0.5, $FeSO_4.7H_2O$ 0.01, wheat bran 20.0, pH 6.5 (Marui *et al*., 2002).

Enzyme assay: Xylanase was assayed by taking 1.0 ml xylan solution (1.0%, w/v) as substrate, 0.5 ml of acetate buffer (0.1 M), pH 4.5 and 0.5 ml of appropriate enzyme dilution. Reaction mixture was incubated at 50^{o}C for 30 min. Total reducing sugars were determined by using dinitrosalicylic acid (DNS) after Miller (1959).

One unit of xylanase is defined as "the amount of enzyme that releases one µmol of reducing sugar (as xylose equivalent) per minute under the defined assay conditions".

Table 1. Screening of *A. niger* developed through N-methyl N-nitro N-nitroso guanidine (MNNG) treatment for xylanase production.

MNNG-treated mutant strains	Xylanase saccharifying activity (U/ml/min)
Parental	104.59±2.9
RH_{MNNG}^{1}	128.21±1.7
RH_{MNNG}^{2}	115.36±3.18
RH_{MNNG}^{3}	140.24±1.53
RH_{MNNG}^{4}	152.31±1.68
RH_{MNNG}^{5}	161.03±1.44
RH_{MNNG}^{6}	132.51±2.62
RH_{MNNG}^{7}	153.36±1.95
RH_{MNNG}^{8}	150.33±2.11
RH_{MNNG}^{9}	109.48±1.33
RH_{MNNG}^{10}	144.31±2.27
RH_{MNNG}^{11}	155.79±2.6
RH_{MNNG}^{12}	172.31±2.16
RH_{MNNG}^{13}	168.12±1.33
RH_{MNNG}^{14}	160.49±2.6
RH_{MNNG}^{15}	122.29±3.71

The strains have been isolated after the MNNG exposure (15-50 min) of *A. niger* GCBT-35. At higher MNNG concentration other than 4.0 mg/ml, the fungal death rate became ≈ 100 %.

The ± indicates standard deviation among the three parallel replicates, which differ significantly at $p \leq 0.05$.

Statistical analysis: Treatment effects were compared by the protected least significant difference method (Costat, cs6204w. exe) after Snedecor & Cochran (1980). Significance difference among the replicates has been presented as Duncan's multiple ranges in the form of probability (<p>) values.

Results and Discussion

The data in the Table 1 shows the screening of 15 different mutant strains of *Aspergillus niger* for enhanced xylanase production in 250 ml shake flasks. Among all the cultures screened, *A. niger* RH_{MNNG}^{12} gave maximum xylanolytic activity (172.31 U/ml/min) which is about 1.65 fold higher than the wild culture GCBT-35. It might be due to the fact that MNNG causes alkylation of guanine residues that have produced permanent lesions within the DNA and therefore clusters of closely linked mutations produced (Drazic & Delac, 1970; Tasneem *et al*., 2003). In turn, this cluster of mutations might have enhanced the expression of the genes coding for xylanase production. So MNNG has been found an effective mutagen bringing about an over-expression of xylanase gene. Among the different fermentation media (M1, M2, M3, M4, M5 and M6) tested, the maximum enzyme production (175.04 U/ml/min) was obtained when the culture medium M3 was used (Fig. 1). M3 consisted of (g/l) $NaNO_3$ 1.0, NH_4Cl 1.0, KH_2PO_4 1.0, $CaCl_2$ 1.0, $MgSO_4.7H_2O$ 0.3, meat extract 5.0, Tween-80 1.5 ml, wheat bran 20.0 (pH 5.0) which shows that there is a good combination of all the nutritional requirements essential for the growth of fungus as well as enzyme synthesis. The other media gave comparatively less enzyme activity. Different agricultural by-products were

investigated for the synthesis of xylanase by *A. niger* RH_{MNNG}^{12} (Fig. 2). The enzyme production was optimal when wheat bran was used. It might be due to the fact that wheat bran provided adequate amount of nutrients (proteins 1.32%, carbohydrates 69.0%, fats 1.9%, fiber 2.6%, ash 1.8%, Ca 0.05%, Mg 0.17%, P 0.35%, K 0.45%, S 0.12% and various amino acids) to the organism. These nutrients are essential for the growth of microorganism and subsequent xylanase production (Park *et al*., 2002). Narayan (2004) also obtained maximum enzyme production by cultivation on wheat bran. Other substrates might be unable to meet all the requirements for enzyme synthesis and fungus growth. Different concentrations (0.5-5.0%, w/v) of wheat bran were also evaluated for xylanase biosynthesis (Fig. 3) and maximum production were obtained (176.82 U/ml/min) at 2.0% (w/v) wheat bran. Further increase in the concentration of wheat bran resulted in the decreased xylanase synthesis. It might be due to the thickening of the fermentation medium, which made hindrance in the proper agitation and resulted in the decreased air supply. The sufficient supply of air is essential for better growth of mycelia as well as secretion of enzyme in the fermented broth (Palma *et al.*, 1996). At low concentration, wheat bran might not complete nutritional requirements of the fungus and so resulting in less enzyme activity.

In the present study, the fermentation flasks were incubated for 24-168 h after conidial inoculation and gradual increase in xylanolytic activity was observed (Fig 4). Maximum production of enzyme (176.64 U/ml/min) was obtained 72 h after the inoculation. The incubation period beyond 72 h resulted in decreased xylanase production. It might be due to the decreased availability of nutrients in fermentation medium, age of fungus, inhibitors produced by fungus itself and rapid digestion of susceptible portion of xylan molecules (Reese *et al*., 1969). Loera & Cordova (2003) obtained the maximum xylanolytic activity by *A. niger* after 96 h of incubation. Therefore, present results are more encouraging than their results. Medium pH has a direct influence on mould metabolism during fermentation process. The effect of changing the initial pH of the medium on xylanase production by the mutant strain *A. niger* RH_{MNNG}^{12} was investigated (Fig. 5). Maximum xylanolytic activity (270.53 U/ml/min) was achieved when pH of the basal medium was adjusted at 5.5, which is over 1.5 fold higher than the value at pH 5.0. When the pH was decreased or increased other than 5.5, xylanase production was significantly reduced. It might be due to that the fungi require slightly acidic pH for growth and the enzyme production (Gomes *et al*., 1994). Similar kind of study has also been reported by Camacho & Aguilar (2003) for the production of xylanase.

The productivity of xylanase is greatly influenced by both the source and concentration of nitrogen in the culture medium (Kulkarni *et al*., 1999). In the present studies, different organic nitrogen sources such as urea, polypeptone, meat extract and yeast extract were added to the fermentation medium at 0.5% level (Fig. 6). Among all the nitrogen sources tested, meat extract gave maximum xylanolytic activity (271.69 U/ml/min). It might be due to the fact that meat extract provides more available nitrogen for the growth of mycelium hence, the production of xylanase also increases (Kansoh *et al*., 2001). The various concentrations ranging from 0.5 to 3.0% (w/v) were also evaluated (Fig. 7) and enzyme production was found to be maximum (289.86 U/ml/min) at 1.0% level of meat extract. As the level of meat extract was further increased in the fermentation medium, the production of enzyme was greatly inhibited. It might be due to the fact that high concentration of nitrogen source has some toxic effects on the fungal growth.

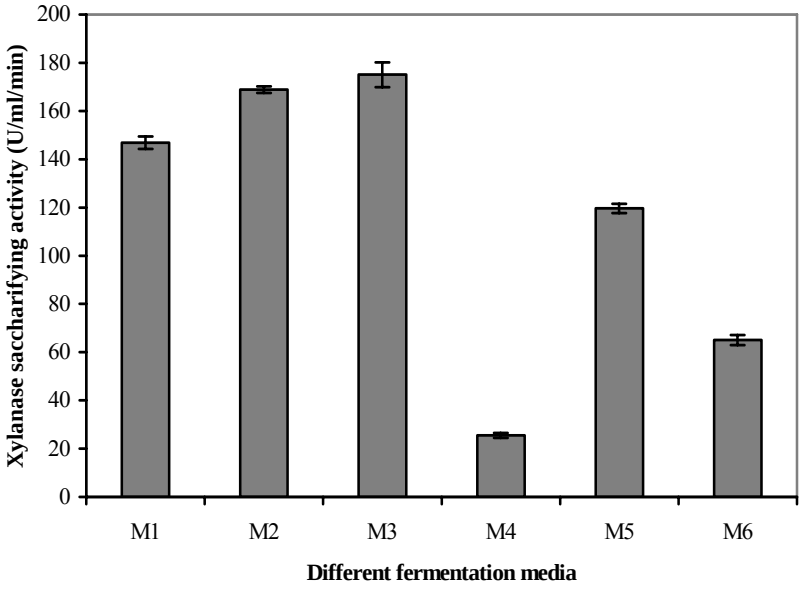

Fig. 1. Selection of fermentation medium for xylanase production by the mutant strain *A. niger* RH_{MNNG}^{12}
Y-error bars indicate standard deviation among the three parallel replicates.

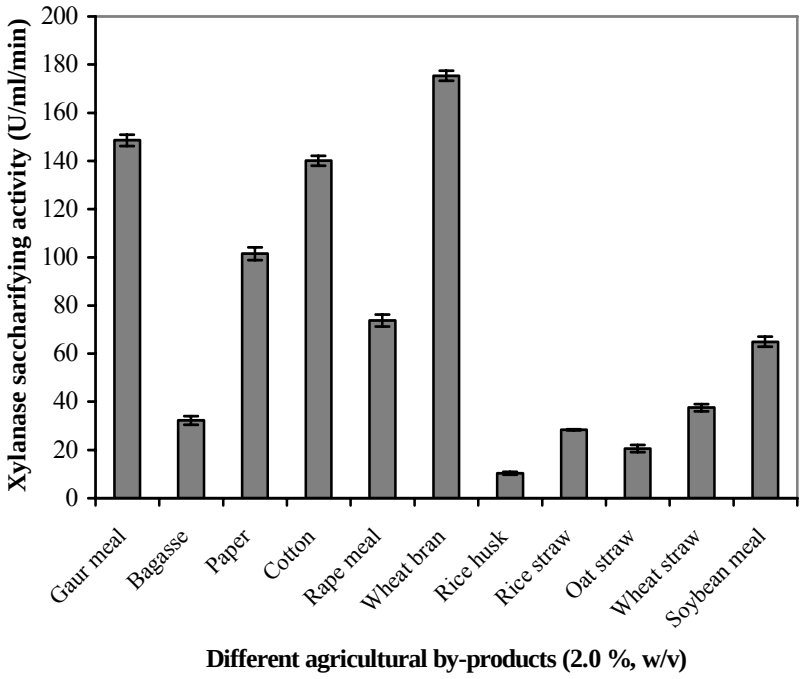

Fig. 2. Selection of agricultural by-product for xylanase production by the mutant strain *A. niger* RH_{MNNG}^{12}
Y-error bars indicate standard deviation among the three parallel replicates.

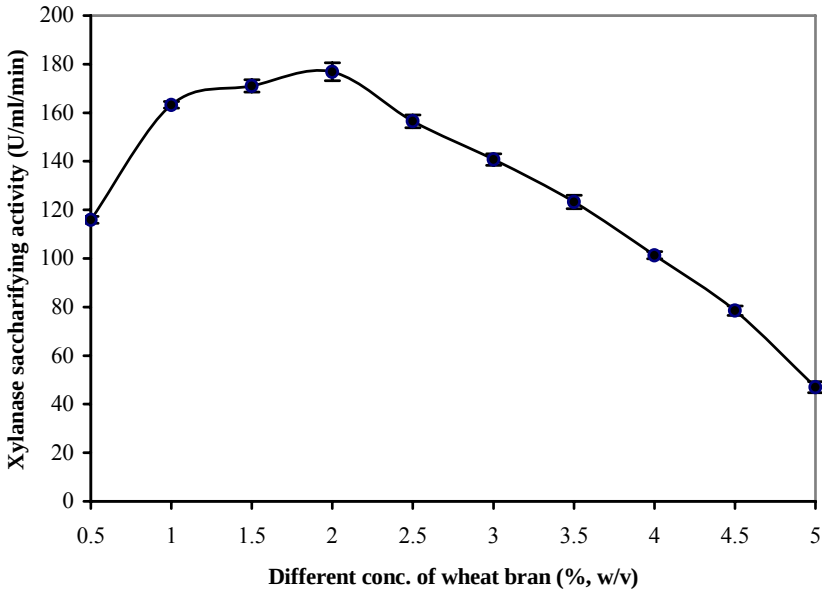

Fig. 3. Effect of different concentrations of wheat bran on production of xylanase by the mutant strain *A. niger* RH_{MNNG}^{12}
Y-error bars indicate standard deviation among the three parallel replicates

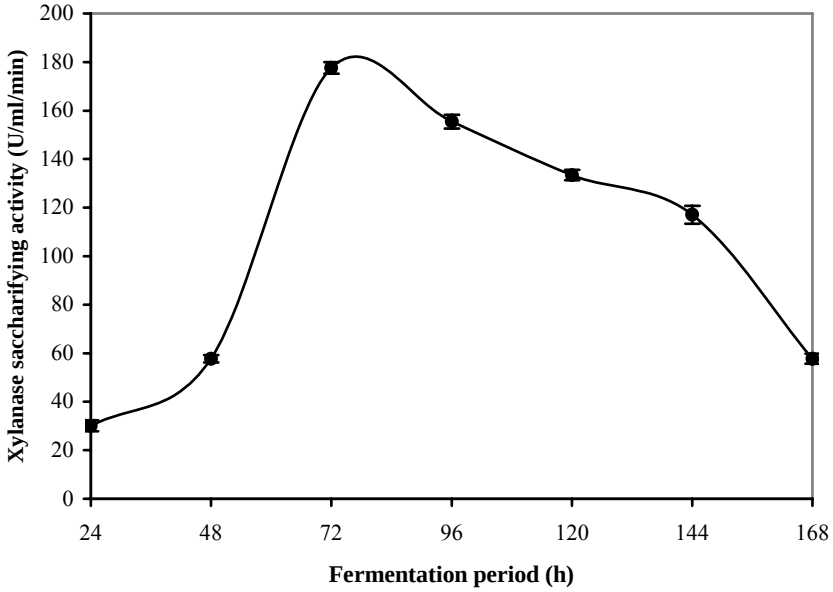

Fig. 4. Time course study for production of xylanase by the mutant strain *A. niger* RH_{MNNG}^{12}
Y-error bars indicate standard deviation among the three parallel replicates.

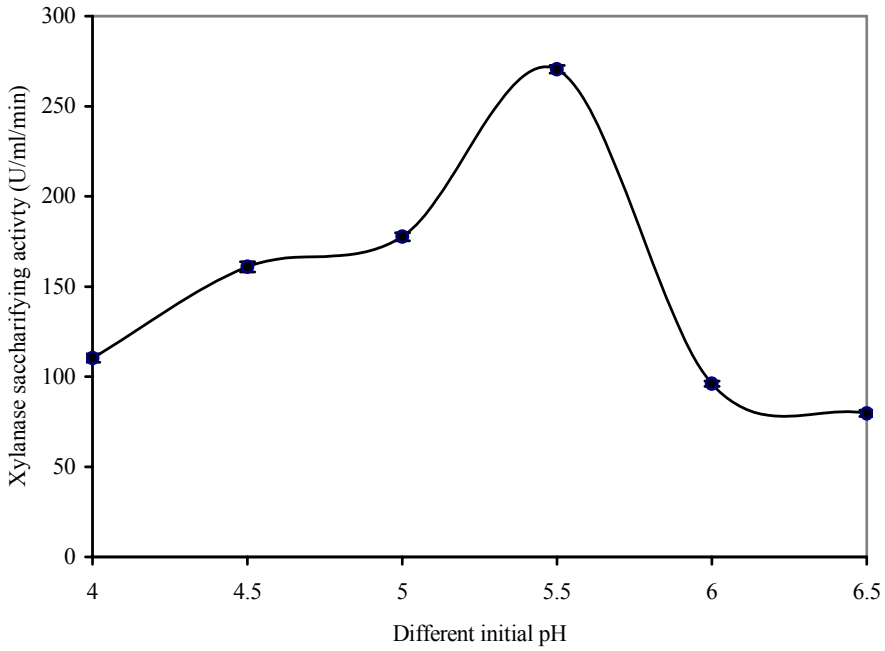

Fig. 5. Effect of different initial pH on production of xylanase by the mutant strain *A. niger* RH_{MNNG}^{12}
Y-error bars indicate standard deviation among the three parallel replicates.

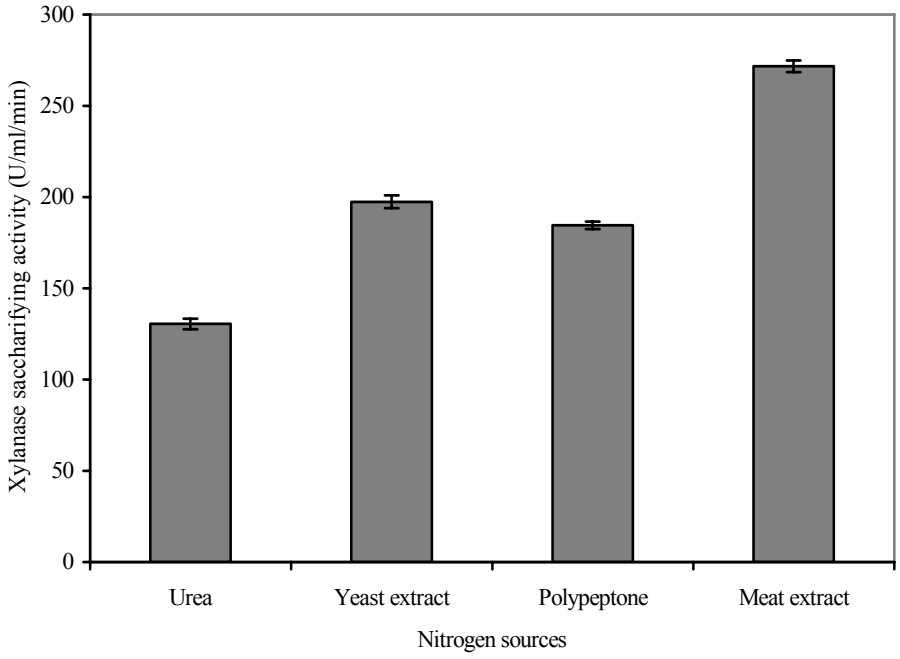

Fig. 6. Effect of different nitrogen sources on production of xylanase by the mutant strain *A. niger* RH_{MNNG}^{12}.
Y-error bars indicate standard deviation among the three parallel replicates.

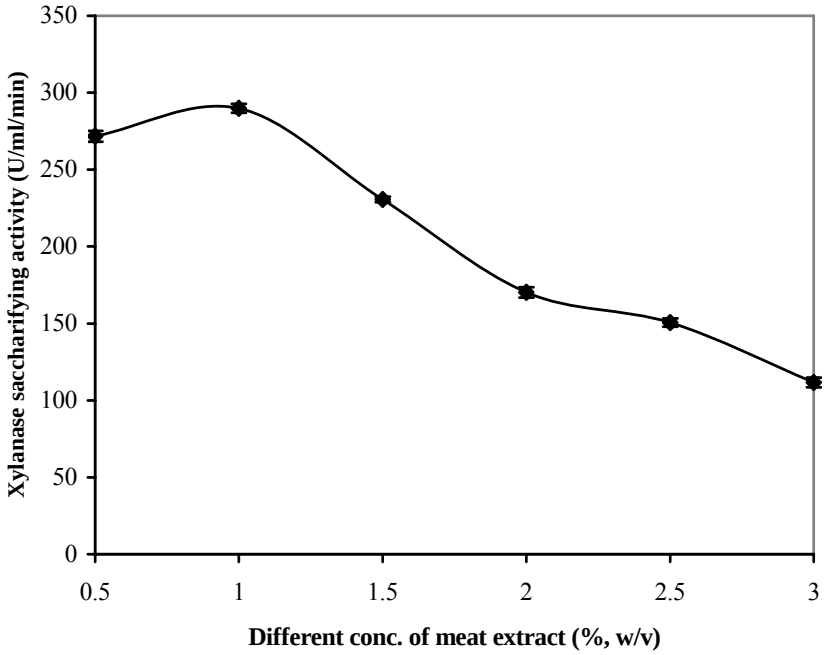

Fig. 7. Effect of different concentrations of meat extract on production of xylanase by the mutant strain *A. niger* RH_{MNNG}^{12}

Y-error bars indicate standard deviation among the three parallel replicates.

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