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Production and Properties of Thermostable Xylanase by *Thermomyces lanuginosus* NK-2 Grown on Lignocelluloses

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Abstract: Thermophilic *Thermomyces lanuginosus* NK-2, isolated from wheat straw compost, produced 1418 IU mL^{-1} of thermostable xylanase (EC 3.2.1.8) on Czapek's basal medium containing wheat bran (2% w/v). The specific activity of NK-2 xylanase was found to be 1107 IU mg^{-1} of total protein. The fungus could also utilize different low-value agricultural residues and produce xylanase on corncobs (1004 IU mL^{-1}), lentil bran (941 IU mL^{-1}) and groundnut shells (355 IU mL^{-1}). Supplementation of pure xylan (1% w/v) to wheat bran medium enhanced xylanase yield by 39% while other C-supplements repressed it. The xylanase was found to be active over a broad pH (4.0-9.0) and temperature range (25-85°C) and the pH and temperature optima were 5.0 and 60°C, respectively. It showed excellent thermal stability by retaining 83.4% activity after one hour of incubation at 60°C and its half-life at 70°C was 40 min. Presence of β-xylosidase (0.007 IU mg^{-1}) was also noticed while CMCase and α-L-arabinosidase were not detected.

Key words: *Thermomyces lanuginosus,* xylanase, thermostable, lignocelluloses

INTRODUCTION

Lignocellulosic biomass, a rapidly renewable bioresource, mainly consists of cellulose, hemicellulose and lignin. Hemicellulose is the second most abundant polysaccharide in nature and xylan constitutes its predominant fraction (Timmel, 1964). Enzymatic hydrolysis of xylan using microbial xylanases has been of special interest in prebleaching of pulp (Viikari *et al*., 1994) and bioconversion of lignocellulosic materials to produce fuel, SCP and other value added commodities (Woodward, 1984; Kango and Jain, 2005). Other potential applications of xylanases include clarification of fruit juices and wines and enhancement of the nutritional value of silage, fodder and poultry feed (Van Paridon *et al*., 1992; Wong and Saddler, 1992). Pertaining to industrial conditions the desirability of thermostable xylanases has been emphasized by many workers (Yu *et al*., 1987; Archana *et al*., 1999). Several strains of *Thermomyces lanuginosus* have been reported to produce high levels of cellulase-free, thermostable xylanase in different culture conditions (Hoq and Deckwer, 1995; Chadha *et al*., 1999; Singh *et al*., 2003). Cellulase-free thermostable xylanase preparations are most suitable for selective removal of xylan in biopulping and fibre upgradation where cellulosic component must be preserved and industrial processing is conventionally done under high temperature regimes (Alam *et al*., 1994). However, intraspecific variation in terms of xylanase production and properties among isolates of diverse geographical origin of this morphospecies are evident (Chadha *et al*., 1999; Singh *et al*., 2003). Several reports on utilization of abundant and renewable lignocellulosic residues for development of value added bioproduct indicate their judicious and successful utilization for biotechnological processes. We have explored the possibility of xylanase production on different low-value lignocellulosic residues that emerge from cash crops. Among these, very few reports are available on xylanase production on groundnut shells and lentil bran. In the present study an effort was made to explore lignocellulosic substrates for production of thermostable xylanase by a local thermophilic isolate *T. lanuginosus* NK-2. Alongwith wheat bran and corncobs, meal of groundnut shells and Lentil bran (seed husk of *Lens esculentus*) are examined for xylanase production. Presence of important co-secreted enzymes viz., β-xylosidase (β-1,4-D-xyloside xylohydrolase, EC 3.2.1.37) and α-L-arabinosidase (α-L-arabinofuranosidase arabinfuranohydrolase, EC 3.2.1.55), that play a crucial role in total xylan hydrolysis was also studied. Properties of xylanase, important for its successful application, such as thermostability, specific activity, pH and temperature optima were also investigated.

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MATERIALS AND METHODS

Microorganism: *Thermomyces lanuginosus* NK-2 was isolated from a compost sample and was grown on YpSs agar (Cooney and Emerson, 1964) composed of (g L^{-1}): yeast extract: 4.0; soluble starch: 15.0; $MgSO_4.7H_2O$: 1.0; K_2HPO_4: 1.0; agar: 20.0 at 45°C . It was maintained on the slants of same media at 4°C. This study was conducted at Department of Applied Microbiology and Biotechnology, Dr. Hari Singh Gour Vishwavidyalaya, Sagar (MP) India.

Pretreatment of lignocelluloses: Different natural, locally available lignocelluloses namely, Wheat Bran (WB), Corn Cobs (CC), Lentil Bran (LB) and Groundnut Shells (GS) were selected for this study. Among these, wheat bran and lentil bran were thoroughly washed in running tap water and oven dried while corncobs were chopped, dried and ground in a hammer mill. Empty groundnut shells were washed to remove any soil, dried and ground. The ground materials were separated by sieves and fractions of approximately identical particle size (~2 mm) were used for xylanase production.

Enzyme production: Erlenmeyer flasks (250 mL) containing different lignocelluloses (1 g) and 50 mL Czapek's mineral salt solution were autoclaved at 15 psi for 20 min. These were inoculated with mycelial discs (6 mm) of 4-5 days old cultures of *T. lanuginosus* NK-2 and incubated under stationary condition at 45°C for 7 days. The contents were filtered through preweighed Whatman filter paper No.1 and culture filtrate was examined for xylanase activity.

The fungus was tested for its ability to produce xylanase at different incubation temperatures and pH. For this, it was cultured on Czapek's salt solution containing wheat bran (2% w/v) at different temperatures and pH (initial pH adjusted with 0.1 N HCl or NaOH). The effect of carbon supplementation to wheat bran medium on the production of xylanase by *T. lanuginosus* NK-2 was studied by adding different carbohydrates (1% w/v). Effect of nitrogen source on xylanase yield was studied by replacing sodium nitrate by different N-sources. After appropriate amendments, flasks were inoculated with mycelial discs (6 mm) of 4-5 days old cultures of the fungus and incubated at 45°C for a period of seven days. Afterwards, the contents of the flask were filtered through a preweighed Whatman filter paper No. 1 and the mycelial mat was washed, dried and weighed for biomass. The filtrate was centrifuged at 8000 g to remove any particulate impurity and was used as enzymes source.

Enzyme assays: The xylanase (β-1,4-D-xylan xylanohydrolase, EC 3.2.1.8) assay was carried out in 0.2M acetate buffer (pH 5.0) for 5 min. The substrate was prepared by dissolving 1% xylan (oatspelt xylan, Sigma) in acetate buffer. The reaction mixture contained 0.5 mL of substrate solution and 0.5 mL suitably diluted enzyme sample. After incubation for 5 min at 50°C the reaction was stopped by adding 3 mL of DNS reagent and boiling in a waterbath for 15 min (Miller, 1959). The samples were allowed to cool and their absorbance was read at 550 nm. Carboxymethylcellulase (Endo-1, 4-β-glucanase, EC 3.2.1.4) activity was determined by using 1% (w/v) carboxymethylcellulose as substrate by the DNS method. One unit of enzyme activity was defined as the amount of enzyme that liberated 1 μmol of reducing sugar in one minute. β-xylosidase (β-1,4-D-xyloside xylohydrolase, EC 3.2.1.37) and α-L-arabinosidase (α-L-arabinofuranosidase arabinfuranohydrolase, EC 3.2.1.55) were estimated by measuring the liberation of p-nitrophenol (at 410 nm) from p-nitrophenyl-β-D-xylopyranoside and p-nitrophenyl-α-L-arabinofuranoside, respectively at 50°C in 30 min reaction time. One unit of β-xylosidase and α-arabinosidase was defined as amount of enzyme that liberated 1 μmol of p-nitrophenol per minute.

Partial purification and properties of xylanase: Precipitation of total proteins of crude culture filtrate was done by salting out proteins with ammonium sulphate. The protein precipitate thus obtained was resuspended in distilled water and was used in finding pH and temperature optima and specific activity of the xylanase. The protein content was estimated according to Lowry *et al.* (1951). The effect of pH on xylanase activity was determined by incubating 0.1 mL of protein precipitate in 0.4 mL of appropriate buffers (0.1 M citrate-phosphate buffer: pH 4 and 5; 0.1M phosphate buffer: pH 6, 7 and 8; 0.1 M Tris-HCl buffer: pH 9; 0.1 M glycine-NaOH buffer: pH 10 and 11). To this, 0.5 mL of xylan (1% w/v in distilled water) was added and the reaction mixture was incubated at 50°C for 5 min. The effect of temperature was determined by incubating 0.5 mL of suitably diluted enzyme and 0.5 mL of xylan (1% w/v in 0.2M sodium acetate buffer, pH 5) for 5 min at different temperatures. Thermal stability of NK-2 xylanase at high temperatures was studied by incubating partially purified xylanase at 50, 60, 70 and 80°C for one hour in waterbath. Samples were withdrawn at regular time intervals and xylanase activity in terms of liberation of xylose from xylan was estimated by DNS method. All the experiments and assays were conducted in triplicate and the average values±SD. are reported as results.

RESULTS AND DISCUSSION

Xylanase production on lignocelluloses: *T. lanuginosus* NK-2 could utilize and grow on all four test lignocelluloses to produce varied levels of xylanase (Fig. 1). Maximum secretion of xylanase was noticed on wheat bran (1418 IU mL^{-1}), followed by corncobs (1004 IU mL^{-1}). Significant xylanase titres were noticed on relatively less reported substrates, lentil bran (941 IU mL^{-1}) and groundnut shells (355 IU mL^{-1}). We have reported use of these lignocelluloses in xylanase production by thermotolerant *Emericella nidulans* where they were found to support xylanase yield in similar order, WB being the best followed by CC, LB and GS (Kango *et al*., 2003). As the use of pure xylan as substrate is not cost-effective, cheap and abundant lignocellulosic residues with prominent xylan content make suitable alternatives. In the present study, wheat bran supported maximum xylanase yield. Nevertheless, corncobs, lentil bran and meal of groundnut shells could also be used successfully as per local availability and concerns. A perusal of literature shows that several strains of *T. lanuginosus* have been tested for xylanase production under different culture conditions on different lignocelluloses. Alam *et al.* (1994) reported *T. lanuginosus* isolate from self-heated juite compost to produce 1899.6 IU g^{-1} of xylanase in seven days incubation period in solid state cultures on wheat bran at 55°C. Gomes *et al.* (1993) have employed xylan and barley husk as substrates for xylanase production from *T. lanuginosus* and obtained 9168 and 9502 nkat mL^{-1} yields, respectively, in liquid fermentation at 55°C in six days. Hoq and Deckwer (1995) have compared xylanolytic abilities of two different strains of the fungus namely, *T. lanuginosus* RT9 and *T. lanuginosus* MH4 on different media and noticed enormous differences in their xylanase producing ability. Chadha *et al.* (1999) have compared xylanase producing abilities of different *T. lanuginosus* strains from repositories and a soil isolate BS1. They found all these strains to produce varied levels of xylanase on corncobs (1.35% w/v) containing medium while BS1, showed maximum activity equal to 1018 IU mL. In the present study our isolate NK-2 produced 1418 IU mL on wheat bran in liquid stationary condition at 45°C in 7 days. The difference in xylanase production on different lignocelluloses can be related to the composition of complex substrates, especially with their xylan content.

Effect of culture conditions and supplements on xylanase production: The present study proceeds by examining effect of pH and temperature on the growth and xylanase production on wheat bran medium. Results indicated the ability of fungus to grow and produce xylanase over a wide range of pH (4.0-9.0) and temperature (28-65°C). It produced maximum biomass (0.200 g) and xylanase (1443.7 IU mL) at 55°C while among different initial pH values, 7.0-8.0 supported better xylanase production.

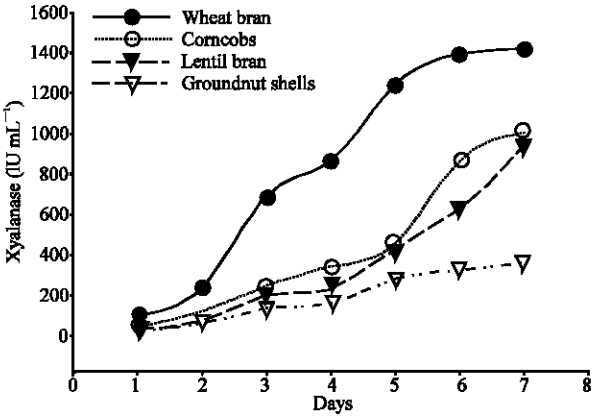

Fig. 1: Xylanase production by *Thermomyces lanuginosus* NK-2 on lignocellulosic substrates after seven days of incubation at 45°C in stationary conditions

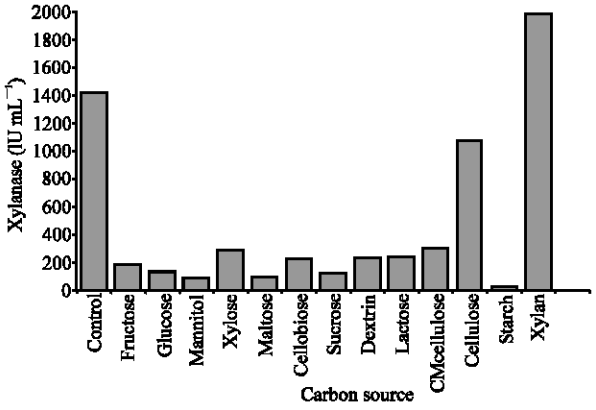

Fig. 2: Effect of carbon supplementation on xylanase production by *T. lanuginosus* NK-2

Effect of C-supplementation to WB containing medium was studied by incorporating different carbohydrates (1% w/v). The effect of C-supplements was noticeably very significant as the test organism showed very high biomass production but drastic fall in xylanase titres excepting in case of cellulose and xylan (Fig. 2). Wheat bran containing medium was found to support best xylanase production. The results showed repression of xylanase synthesis by low molecular weight sugars and only xylan (oatspelts) enhanced xylanase production by 39%. High titres of xylanase were witnessed in unsupplemented wheat bran medium indicating that any

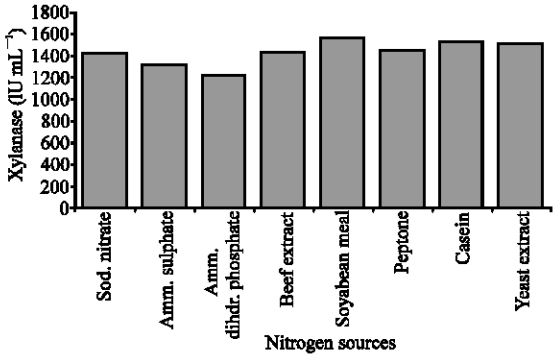

Fig. 3: Effect of Nitrogen sources on xylanase production by *T. lanuginosus* NK-2

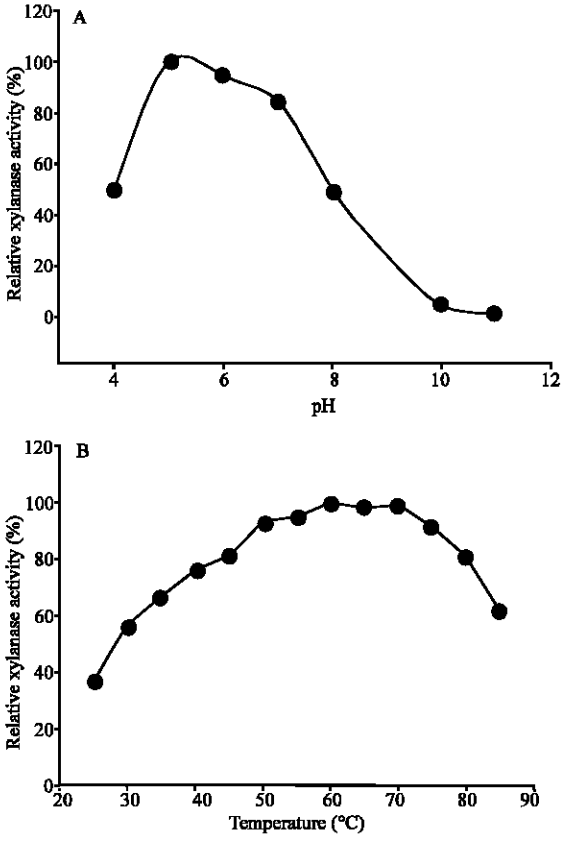

Fig. 4: Effect of pH (A) and temperature (B) on *T. lanuginosus* NK-2 xylanase activity

inducer is not required. Organic N-sources viz. soyabean meal, casein, yeast extract and peptone were found to support better xylanase yields (Fig. 3).

Partial purification and properties of NK-2 xylanase: Protein precipitate obtained by salting out with 80% ammonium sulphate saturation showed 1107 IU mg^{-1}

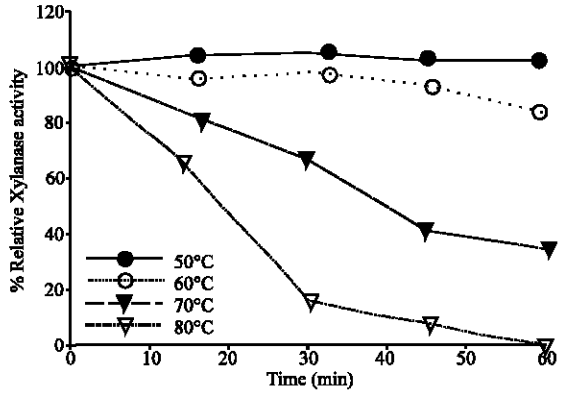

Fig. 5: Thermostability of *T. lanuginosus* NK-2 xylanase at high temperatures

xylanase, indicating 91% of xylanase recovery. Activity of partially purified enzyme over pH and temperature profiles is shown in Fig. 4A and B. The enzyme was active over a broad range of pH (4.0-9.0) and temperature (25-80°C) and had temperature and pH optima at 60°C and 5.0. Among other enzymes tested, CMCase and α-arabinosidase were not detected while 0.007 IU mg^{-1} of β-xylosidase activity was noticed. This is in accord with the reports on *T. lanuginosus* strains producing high amount of cellulase-free xyalanase but very low levels of other hemicellulases (Singh *et al*., 2003). Thermal stability of *T. lanuginosus* NK-2 xylanase at different temperatures as studied by incubating partially purified enzyme at high temperatures (50-80°C) for a period of one hour showed its excellent thermal stability. The enzyme remained fully active after one hour of incubation at 50°C and retained 83.4% activity after one hour at 60°C while the half life of enzyme at 70°C was 40 min (Fig. 5). Gomes *et al*. (1993) have reported of a *T. lanuginosus* xylanase showing optimal activity at pH 5.1 and 80°C while Alam *et al*. (1994) have described a *T. lanuginosus* strain from juite compost producing xylanase optimally active at pH 6.0 and 70°C and more importantly, the xylanase activity was fully retained upto 80°C. Xylanases from *T. lanuginosus* DSM 5828 and *T. lanuginosus* SSBP have been reported to have pH optima at 6.5 and 7.0 and showed maximum activity at temperatures 60-70 and 70-75°C, correspondingly (Cesar and Mrsa, 1996; Lin *et al*., 1999). It is important to note that all these xylanase preparations were cellulase-free and although there are significant differences in the properties, the fungus certainly seems to be a natural potent xylanase producer. Xylanase synthesis was repressed by all carbohydrates except xylan. It was interesting to note that the fungus could utilize LB and CC for its growth and production of

thermostable xylanase and these cheap substrates can be explored further by optimization of media components.

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