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Full Length Research Paper

Production, purification and characterization of celullase-free xylanase from *Aspergillus terreus* UL 4209

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***Aspergillus terreus*, UL 4209 strain, isolated from the soil in South Africa was used to produce an extracellular cellulase-free xylanase in shake flask cultures containing oat spelt and/or birchwood xylans. Maximum xylanase activity (35 U/ml) was observed after 96 h at 35ºC and pH 6 in 1% oat spelt xylan. The xylanase was purified to homogeneity by gel filtration on Sephacryl S-200. This enzyme was found to be a single subunit protein of 22 kDa showing optimal activity at 35ºC and pH 6. The enzyme retained 95% activity at 35 - 40ºC after 4 h incubation at pH 6 and at 50ºC the half-life was 5.8 h. The apparent K_m and V_{max} values were 3.57 mg/ml and 55.5 µmol/min per mg protein, respectively. MALDI-TOF and LC mass spectroscopy gave 8 peptide ions whose sequence alignments showed that the xylanase produced by this strain has homology with those of other *Aspergillus* strains such as *A. terreus* and *A. versicolor*. These observations showed that our strain produced a low molecular weight, acidophilic, and thermostable xylanase that may be considered for processes operated at moderate temperatures and pH such as preparation of baked cereal food, clarification of fruit juices and saccharification of agro-residues.**

Key words: *Aspergillus terreus,* cellulase-free xylanase, purification, MALDI-TOF.

INTRODUCTION

Xylan is the second most abundant biopolymer after cellulose and the major hemicellulosic polysaccharide found in the plant cell wall (Timell, 1967). It has a complex structure consisting of β-1,4-linked xylose residues in the backbone to which short side chains of O-acetyl, α-L-arabinofuranosyl, D-α-glucuronic and phenolic acid residues are attached (Coughlan and Hazlewood, 1993). This biopolymer constitutes one third of all renewable organic carbon sources on earth (Poorna and Prema, 2007). Considerable amount of xylan is found in solid agricultural and agro-industrial residues, as well as in effluents released during wood processing, which, due to frequent inappropriate discard, cause great damage to the ecosystem (Biely, 1985; Prade, 1995). Hydrolysis of xylan is an important step towards the proper utilization of lignocellulosic material in nature (Poorna and Prema, 2007). Chemical hydrolysis of lignocelluloses results in harzardous byproducts, forcing the use of microbial enzymes which are specific in action for xylan hydrolysis, and are an environmentally friendly option (Biely, 1985). Due to structural heterogeneity of xylan, complete degradation of this biopolymer requires synergistic action of different xylanolytic enzymes such as endo-xylanase, β-xylosidase, α-glucuronidase, α-arabinofuranosidase and esterase (Poorna and Prema, 2007). Among these, the most important one is endo-1,4-β-xylanase (1,4-β-D-xylan xylohydrolase, EC 3.2.1.8), which is also known as xylanase and it initiates the degradation of xylan into xylose and xylooligosaccharides of different sizes (Collin

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et al., 2005).

There are different types of xylanases varying in substrate specificities, primary sequences, folds and physicochemical properties (Wong et al., 1988; Collins et al., 2004). These are produced by a number of bacteria and fungi (Kulkarni et al., 1999; Subramaniyan and Prema, 2002). Filamentous fungi have been reported to be interesting and good producers of xylanases from industrial point of view due to extracellular release of the enzymes, higher yield compared to yeast and bacteria and also the production of several auxiliary enzymes that are necessary for debranching of substituted xylans (Haltrich et al., 1996). However, fungal xylanases are generally associated with concurrent production of cellulases (Steiner et al., 1987). Traditionally, the application of xylanases in conjunction with cellulocytic enzymes has been mainly considered for the bioconversion of lignocellulosic materials, especially residues and wastes produced by agriculture and forestry to produce higher-value products such as ethanol fuel and other chemicals (Biely, 1985; Mandels, 1985). Other potential applications of crude xylanase preparations containing cellulases, β-glucanases, or pectinases include bread making, fruit juice extraction, beverage preparation, increasing digestibility of animal feed, converting lignocellulosic substances to feedstock and fibre separation (Beg et al., 2001; Subramaniyan and Prema, 2002), paper and pulp industries (Bajpai, 1997; Kenealy and Jeffries, 2003). However, in the paper and pulp industry, cellulase-free xylanases are required to avoid adverse effects of damaging the pulp fibres (Haltrich et al., 1996). Moreover, specific xylanases can be used in the prebleaching of craft pulps in order to reduce the amount of chlorine required to achieve target brightness (Viikari et al., 1994) and consequently reduce the chloroorganics released in the effluent (Christakopoulos et al., 1996). Cellulase-free xylanases or xylanases containing negligible cellulase activity could be obtained by using suitable separation methods or using genetically engineered organisms to produce exclusively xylanase or by applying screening methods and selection of appropriate growth conditions (Balakrishnan et al., 1992).

Characterization of xylanolytic enzymes is important for their biotechnological applications. The cost of the enzyme is one of the main factors determining the economics of a process and this can be partially achieved by optimizing fermentation media (Shah and Madamwar, 2005). Several industrial processes can be carried out using whole cells as sources of enzymes, but efficiency can be improved by using isolated and purified enzymes. Criteria for selection of a particular method of isolation and purification depend on the end use. A high state of purity is generally not required in food processing, detergent as well as paper and pulp industries, but it may be necessary to exclude certain contaminating enzymes (Price and Stevens, 1999).

Recently, interest in microbial xylanases has markedly increased due to their potential in boiotechnological applications and attempts are being made to isolate new strains (Lee-Chiang et al., 2006; Schmeisser et al., 2007). In a screening programme, we have recently isolated from the soil in South Africa (SA), a strain of *Aspergillus terreus,* UL 4209, showing xylanase producing ability. In the present investigation, we report on the production of cellulase-free xylanase using this strain and some of the biochemical properties of the purified enzyme.

MATERIALS AND METHODS

Fungal strain and maintenance

Aspergillus terreus-UL 4209 was isolated from soil sample collected from George (SA) by enrichment culture technique followed by culture purification on basal media containing xylan. The purified culture was maintained on 5% malt extract agar (pH 6.5). The isolate was identified by the SA Agricultural Research Council (Plant Protection Institute), Biosystem Division.

Spore production and culture conditions

Fungal spores were propagated on 5% malt extract agar plate and after seven days of incubation at 35°C the spores were harvested by addition of 10 ml of 1% (v/v) Tween 80 solution (Merck). The spores were gently brushed using an inoculating loop and counted using a hemocytometer. The experimental cultures were carried out in duplicate in 250 ml Erlenmeyer flasks containing 100 ml of medium which comprised 0.3% yeast extract, 0.3% malt extract, 0.3% peptone (YMP) recommended by Saha (2002) and supplemented with 1% oat spelt xylan (Sigma) at pH 6. Spores ($1x10^5$ spores/ml) were used to inoculate the flasks, and incubated at 35ºC in an orbital shaking incubator (Labcon) at 200 rpm for 204 h. On harvesting, the mycelia was filtered out using Whatman No.1 filter paper and the culture filtrate was centrifuged at 8000 x g (J2-21 centrifuge, Beckman) for 15 min at 4°C and the clear supernatant was assayed for xylanase and cellulase activities.

Xylanase assay

Endoxylanase activity was measured according to the method of Saha (2002) using 0.5 ml of 1% (w/v) solution of oat spelt xylan incubated with 0.5 ml of an appropriately diluted enzyme in 50 mM acetate buffer (pH 6) for 30 min at 50ºC The released reducing sugars were assayed using the DNS method (Miller, 1959). One unit of xylanase activity was defined as the amount of the enzyme that liberated 1 µmol of xylose equivalents per minute under the assay conditions.

Cellulase assay

Cellulase (CMCase/endocellulase) activity was assayed by adding 0.5 ml of appropriately diluted enzyme to 0.5 ml of 1% (w/v) carboxymethyl cellulose (CMC) (Sigma) in 50 mM sodium acetate buffer (pH 6) and incubating at 50ºC for 30 min (Saha, 2002). The amount of reducing sugars released during the reaction was measured using the DNS method (Miller, 1959) and D-glucose solution was used as the standard. One unit of cellulase activity was defined as the amount of enzyme that liberated 1 µmol of glucose equivalents under the assay conditions.

Effect of temperature on xylanase production

The optimal temperature for xylanase production by *A. terreus* in shake flask cultures containing 100 ml YMP medium supplemented with 1% oat spelt xylan was determined at various temperatures (25 - 50ºC) in an orbital shaking incubator at 200 rpm for 168 h. Clear supernatant solutions obtained after centrifugation were assayed for xylanase activity.

Effect of different carbon sources on xylanase production

To investigate the effect of various carbon sources on xylanase production, *A. terreus* UL 4209 was cultivated for 240 h at 35°C in the YMP medium containing either 1% oat spelt xylan (Sigma), or 1% birchwood xylan (Fluka BioChemika) or a 1% mixture of both as the sole carbon source. Xylanase activity was assayed by the DNS method at various time intervals.

Effect of initial pH on xylanase production

The optimal pH for xylanase production was determined by adjusting the initial YMP culture medium supplemented with oat spelt xylan (1% v/w) as carbon source. The culture media were adjusted with 50 mM acetate buffer (pH 3 and 4), 50 mM Tris-base buffer (pH 6) and 50 mM sodium phosphate buffer for pH 8-10) and incubated at 35°C in an orbital shaking incubator at 200 rpm. The enzyme activity was assayed at 50ºC and pH 6 by the DNS assay method.

Xylanase purification

The crude culture filtrate was concentrated 15 times using Amicon Ultra-15 centrifugal device with a molecular cut-off point of 10 kDa. Five milliliters of the concentrated enzyme was loaded onto a 60 x 1.5 cm Sephacryl gel filtration column (Sephacryl S-200 HR, Sigma) that had been pre-equilibrated with 50 mM acetate buffer (pH 5). Elution was carried out at a flow rate of 0.70 ml/min with protein elution being followed by monitoring absorbance at 280 nm using a Bio-Rad Econo UV monitor. Fractions of 5 ml were collected and assayed for xylanase activity.

The amount of protein in the crude fractions and active fractions from gel filtration was estimated by the bicinchoninic acid (BCA) protein assay (Smith et al., 1985), using bovine serum albumin (BSA) as a standard. Enzyme purity for the collected fractions was determined on 15% SDS-PAGE according to Laemmli, (1970).

Optimal temperature and pH for the pure xylanase

To investigate the optimum temperature for the purified xylanase activity, 0.5 ml of the enzyme solution was incubated with 0.5 ml of 1% oat-spelt xylan substrate at different temperatures in the range of 20 – 50°C, for 30 min at pH 6. For optimum pH determination, 10 mM buffer solutions ranging from pH 3 - 10 were used at the optimal temperature (35°C). Sodium acetate buffer was used for pH 3 - 5, Tris-base buffer for pH 6 and sodium phosphate buffer for pH 8 - 10. The enzyme activity was measured as described above.

Thermal stability

To determine the xylanase thermal stability, the purified enzyme preparations were pre-incubated, in the absence of the xylan substrate, in 10 mM acetate buffer pH 6 at 30, 40, 50 and 70°C. Samples of 0.5 ml were taken at regular intervals for residual xylanase activity assay as described above.

Kinetic parameters

The effect of oat spelt xylan concentration on xylanase activity was evaluated under optimal assay conditions. Diluted enzyme solution (0.5 ml) was incubated with 0.5 ml of various concentrations (0 - 10 mg/ml) of soluble oat spelt xylan in 50 mM sodium acetate buffer pH 6 at 50°C for 10 min. Xylanase activity was assayed as described above. The kinetic parameters (Michaelis-Menten constant, K_m and maximal reaction velocity, V_{max}) were estimated by linear regression from double-reciprocal plots according to Lineweaver and Burk (1934)

In-gel tryptic digestion of the xylanase

Gel bands were excised from the gels and incubated in 300 µl of 200 mM NH_4HCO_3 (ABC) in 50% acetonitrile (ACN) at 32°C for 30 min. This incubation was repeated twice to remove SDS. The protein bands were then reduced by incubation in 300 µl of 20 mM dithiothreitol (DTT), 200 mM ABC, 50% ACN at 32°C for 1 h. After the reduction, the bands were washed in 300 µl of 200 mM ABC, 50% ACN three times to remove the DTT.

The cysteines were alkylated in fresh 100 µl 50 mM iodoacetamide (IAA), 200 mM ABC, 50% ACN at room temperature in the dark for 20 min. To remove the IAA, the bands were washed three times with 500 µl of 20mM ABC in 50% CAN. The treated gel bands were cut into 2 x 1 mm pieces, spun in the Microfuge at 13,000 rpm for 2 min and covered with 100% ACN until they turned white. The ACN was decanted and discarded and the gel pieces were allowed to dry. The gel pieces were swelled in 29.5 µl of 50 mM ABC containing 0.5 µl trypsin (0.2 µg), (Promega) at 4°C. The tops of the tubes were sealed with Nescofilm and incubated at 32°C for 16 h.

Mass spectrometry analysis

MALDI-TOF MS

An aliquot of 0.5 µl tryptic digest was spotted onto a MALDI target plate with 0.5 µl a-cyano-4-hydoxycinnamic acid (CHCA) matrix. The sample was then analysed in a Voyager DE-STR Matrix Assisted Laser Desorption Mass Ionisation-Time of Flight Mass Spectrometer (MALDI-TOF MS, Applied Biosystems). The peptide mass fingerprint data collected was searched against a non-redundant database using either MS-Fit (Protein Prospector) (Clauser et al., 1999) or Mascot (Matrix Science Ltd.) search engines.

LC-MS

Tryptic peptides from a digest were separated on an Ultimate LC system (Dionex) prior to nanospray mass spectrometric analysis on a LCQdeca mass spectrometer (ThermoScientific). Peptides were separated on a PepMapC_{18} (3 µm, 100 A; 75 µm x 15 cm) column (LC Packings) at a flow rate of 200 ml/min. The column was equilibrated with solvent A (aqueous, 0.5% formic acid) and eluted with a linear gradient from 0 to 70% solvent B (90% acetonitrile containing 0.45% formic acid) over 60 min. MS data was collected in a data dependent manor using Excalibur software (Thermo-Scientific). The ms/ms data files were then searched against a non-redundant database using TurboSequest software (ThermoScientific) or Mascot (Matrix Science Ltd.) search engine.

RESULTS

Time course of xylanase production and temperature effect

Xylanase production by *Aspergillus terreus*-UL4209 was

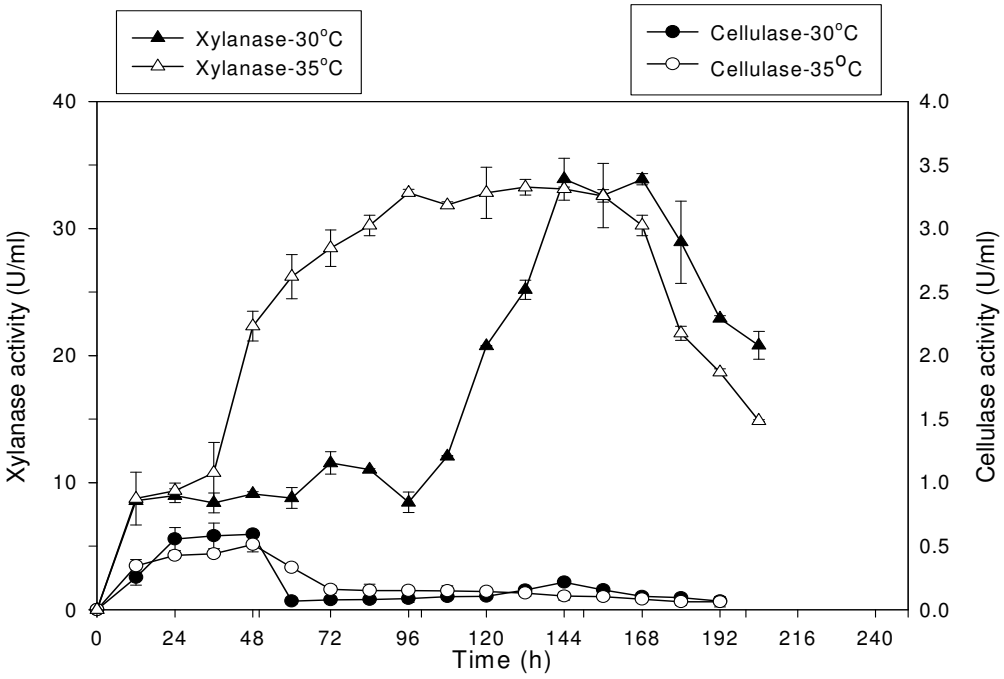

Figure 1. Effect of cultivation temperature on xylanase production by *A. terreus*-UL 4209. The culture media used was YMP supplemented with 1% oat spelt xylan at pH 6.0 in shake flasks. The enzyme activity was assayed at 50°C and pH 6.0 by DNS assay method. The results were the means of triplicate experiments ± SD.

studied at various temperatures (25 - 50ºC) for 240 h in the YMP medium supplemented with 1% (w/v) oat spelt xylan in shake flask cultures at an initial pH of 6 and shaking speed of 200 rpm. The xylanase production profile is shown in Figure 1. Secretion of xylanase into the medium started only after 36 h at all the temperatures used. Maximum xylanase activity (35 U/ml) was observed after 96 h at 35ºC and remained at that level until 156 h when the activity started to decrease. At 30ºC the enzyme activity only increased after 108 h and reached the maximum (35.3 U/ml) after 144 h. After 84 h, maximum xylanase activity (25 U/ml) was observed at 25ºC and it remained at that level until 168 h. The results showed that the optimum temperature for xylanase production was 35ºC and that higher temperatures were not suitable for production. During the production of xylanase at 30 and 35ºC, negligible cellulase activities (< 0.06 U/ml) were detected in the culture filtrates (Figure 2)

Effect of initial production pH

The effect of initial pH (3 - 10) on xylanase production by the fungal isolate was investigated in the YMP medium supplemented with 1% (w/v) oat spelt xylan under the conditions described in Materials and Methods. Xylanase production started at 48 h when the initial pH was 6 and reached the maximum activity (28.6 U/ml) after 96 h and remained at that level until after 144 h when the activity decreased steadily (Figure 2). At pH 8, the enzyme activity only started increasing after 60 h and peaked at 108 h (31.2 U/ml), but the activity decreased rapidly thereafter. Lowest enzyme activities were observed at acidic (pH 3) and alkaline (pH 10) pHs. Moderate activity (18.0 U/ml) was observed at pH 4. The results suggested that an initial pH of 6 was suitable for xylanase production by the isolate.

Xylanase production on various substrates

Of the two substrates tested, oat spelt xylan (1% w/v) was found to be more effective (45.6 U/ml after 120 h) than birch wood xylan (38.2 U/ml after 96 h), although the latter could also be used (Figure 3). A combination of oat spelt and birch wood xylans rather showed a decrease in the enzyme activity (30.2 U/ml after 72 h). Interestingly, xylanase activity peaked earlier but decreased more rapidly in the birch wood medium than in the oat spelt medium where the enzyme activity remained stable from 120 to 216 h.

Purification of the xylanase from the culture supernatant

The xylanase was initially concentrated 15 times using

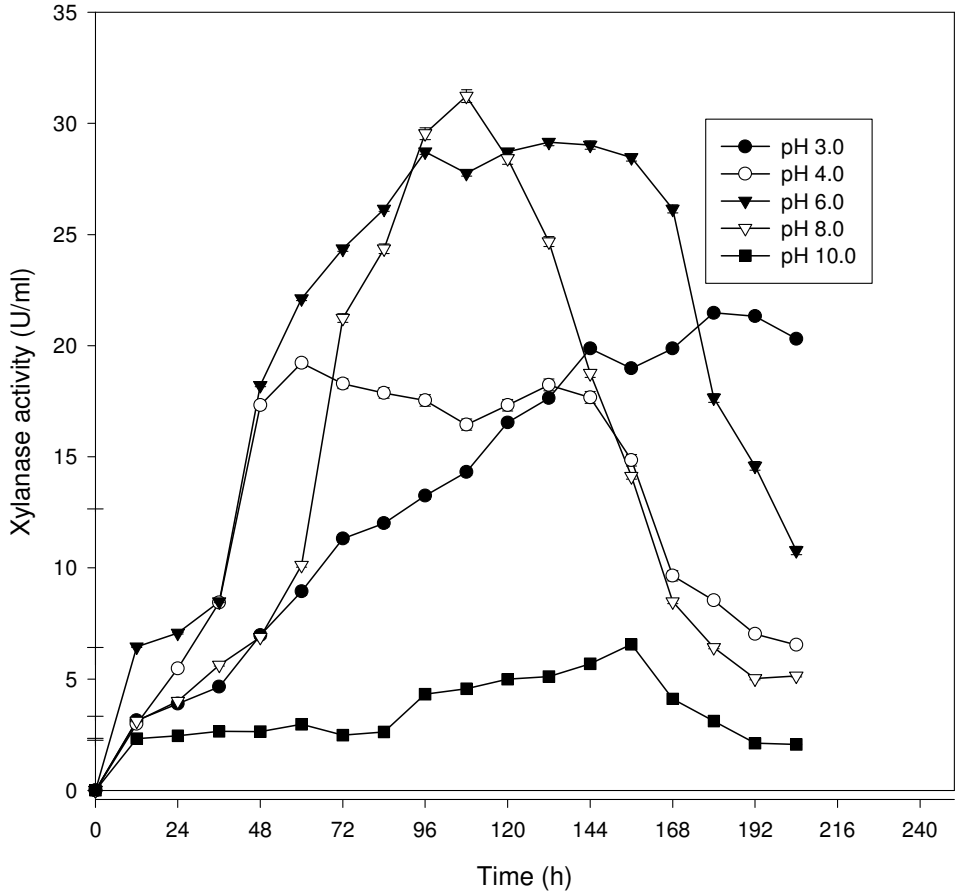

Figure 2. Influence of initial pH on production of xylanase by *A. terreus*–UL 4209 in shake flask cultures. The culture medium was YMP supplemented with 1% oat spelt xylan incubated at 35℃ with shaking at 200 rpm. The enzyme activity was assayed at 50℃ and pH 6.0 by DNS assay method.

membrane ultrafiltration. This resulted in 56% recovery of enzyme with a specific activity of 31 U/mg and a purification fold of 1.2. Sephacryl S-200 gel filtration was performed on the concentrated enzyme. The gel filtration step gave a yield of 0.6% and a specific activity of 12 U/mg. As observed in the gel filtration elution profile, part of the xylanase activity was eluted in an early peak, peak 1, and another part in a larger and more prominent peak, peak 2 (Figure 4). In between the two peaks was a broad elution of xylanase activity that did not present a distinct peak.

Peak 1 (fractions 10 to 13) and Peak 2 (fractions 36 - 49) fractions were pooled and concentrated by ultra-filtration. SDS-PAGE analysis of the concentrated peaks 1 and 2 showed that Peak 2 fractions consisted of a single band of 22 kDa (Figure 5) while peak 1 fractions concentrated to the same level as peak 2 did not show the 22 kDa protein (Figure 5). The native molecular weight of the xylanase in peak 2 as deduced from the elution of the most active fractions in peak 2 on a pre-calibrated column was estimated to be 27 kDa which is

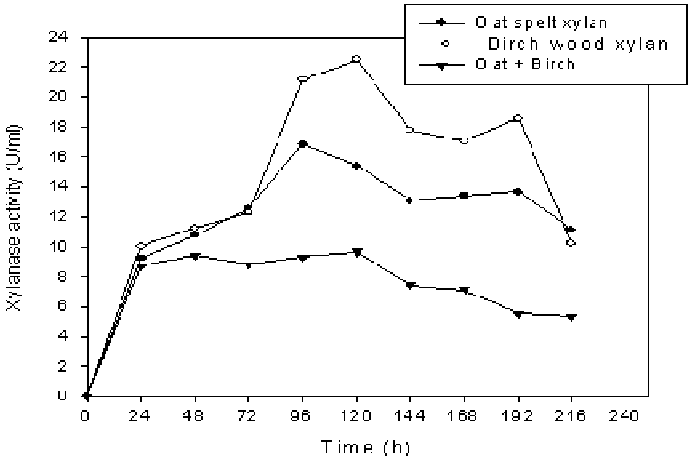

Figure 3. Effect of xylan substrates on xylanase production by *A. terreus*-4209 in shake flask cultures using YMP media supplemented by 1% oat spelt xylan or 1% birch wood xylan or a mixture of the two (0.5% each). The cultures were incubated at 35℃ and pH 6.0 with shaking at 200 rpm. The enzyme activity was determined by DNS method.

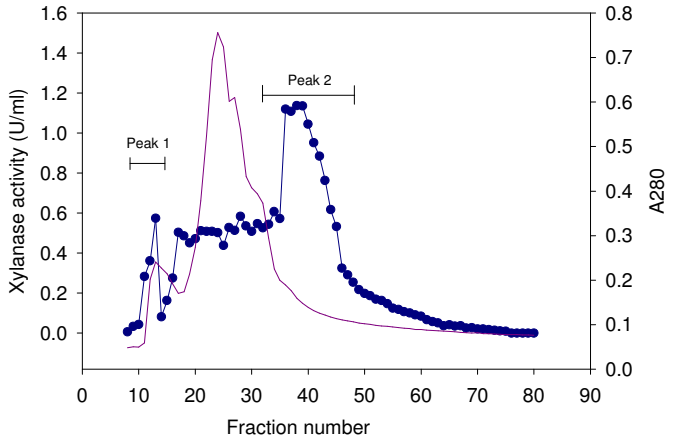

Figure 4. Sepharcryl S-200 gel filtration of the *A. terreus* xylanase. The concentrated culture supernatant (5 ml) was loaded onto a Sepharcryl S-200 column (1.5 x 60 cm) and eluted with 50 mM Acetate buffer pH 5 at a flow rate of 0.7 ml/min. Fractions of 5 ml were collected. Protein content (—) and xylanase activity (–●–).

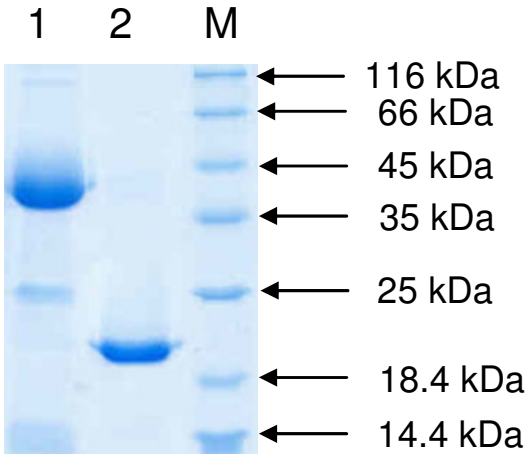

Figure 5. SDS-PAGE analyses of xylanase fractions. Lane 1: Peak 1; lane 2: Peak 2 and lane M: Molecular weight marker.

relatively close to the SDS-PAGE molecular weight.

Effect of pH on enzyme activity

The activity of the purified xylanase was markedly affected by pH. Relative activities and stability were determined at pH values ranging from 4 to 8. The enzyme was most active at pH 6, but the activity decreased as the pH was increased (Figure 6). Significant decrease in activity was observed under extreme acidic (pH 4) and basic (pH 8) conditions. After 3 h the enzyme activities decreased by 20 and 56% at pH 4 and pH 8, respectively. There was negligible change in activity at pH 6 after 3 h (Figure 7) showing that the optimum activity of the enzyme occurred at pH 5 - 6.

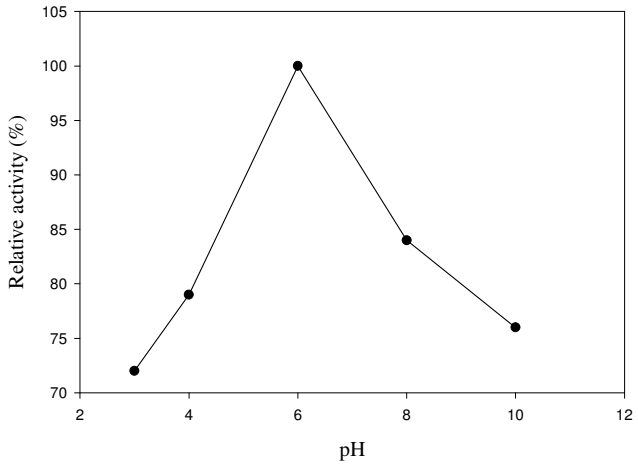

Figure 6. Influence of pH on the activity of purified xylanase produced by *A. terreus*-4209. The activity of the enzyme was determined at different pH values (pH 3 - 10) incubated for 30 min at 50^{0}C and assayed by the DNS method. The buffers used were: 50 mM sodium acetate (pH 3.0 - 5.0), 50 nM tris-base (pH 6.0) and 50 mM sodium phosphate (pH 8.0 - 10).

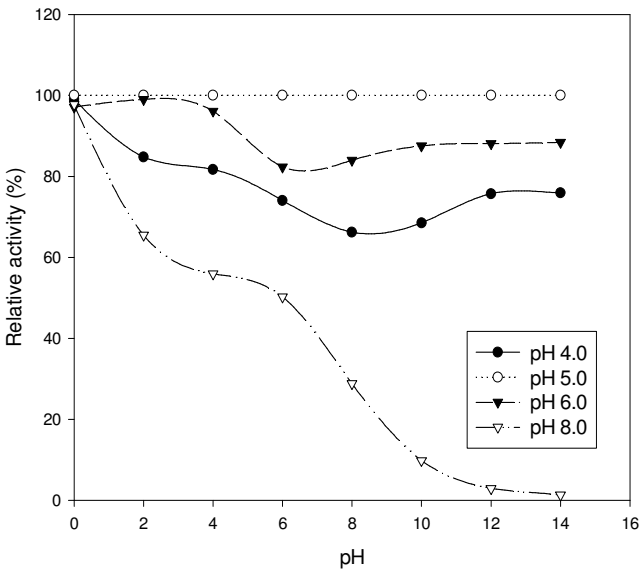

Figure 7. Effect of pH on purified xylanase stability. pH stability was performed by incubating diluted enzyme solution at 50^{0}C for 30 min. at different pHs (4.0 - 8.0). After the incubation period, the enzyme activity was assayed by DNS method.

Effect of temperature on xylanase activity and thermostability

Initial reaction rates for the purified xylanase were determined at temperatures between 25 and 50ºC for 30 min at pH 5. The optimum temperature for the enzyme activity was 35ºC and the activity decreased rapidly after 40ºC (Figure 8). Drastic reduction in enzyme activity (to

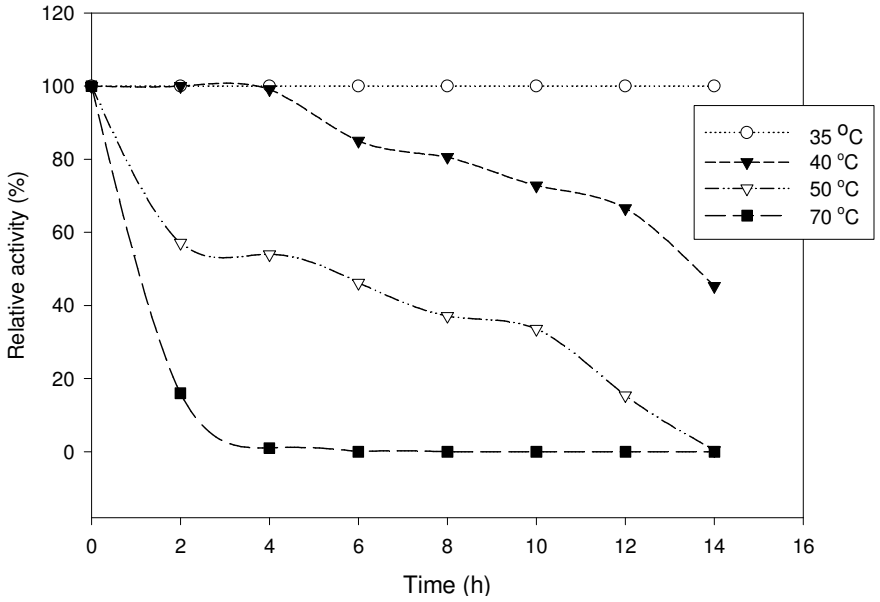

Figure 8. Thermostability of purified xylanase of *A.terreus*-4209. Thermal stability was performed by pre-incubating the enzyme solution in the absence of substrate up to 14 h in a temperature range of 35 - 70^{0}C. Residual enzyme activity was determined at 50^{0}C and pH 6.0 by DNS method and expressed as percentage of control.

17% residual activity) occurred at 25 - 30ºC and 50ºC. Thermal stability studies were carried out by pre-incubating the enzymes up to14 h in a temperature range of 35 - 70ºC. The xylanase was sensitive to 70ºC and complete loss of activity occurred after 2 h (Figure 8). The enzyme retained 100% activity for 3 h at both 35 and 40ºC and no losses of activity were observed up to 14 h during 35ºC incubation. At 50ºC the half-life of the enzyme was 5.8 h.

Kinetic parameters

The rate dependence of enzymic reaction on oat spelt xylan concentration at pH 6.0 and 50ºC followed by Michaelis-Menten kinetics and the double reciprocal plots showed apparent K_m and V_{max} values of 3.57 mg ml^{-1} and 55.5 µmol mg^{-1} protein min^{-1}, respectively.

Mass spectroscopy of the *A terreus* xylanase

Tryptic digests of the coommassie blue stained 22 kDa xylanase bands were analysed using MALDI-TOF and LC-MS mass spectroscopy. The analyses gave 8 peptides. A MASCOT (Matrix Science Ltd) database search on these peptides produced matches against a number of xylanases. Amino acid sequence alignments using Clustal-W (Higgins et al., 1996) showed that the xylanase produced by this strain of *A. terreus* compares well with a xylanase cloned from cDNA of another strain of *A. terreus* BCC129 (Chantasingh et al., 2006) and a xylanase from *A. versicolor* (Figure 9). The peptides matched cover 22% of the *A. terreus* xylanase sequence cloned by Chantasingh et al. (2006). However, the xylanases cloned and expressed in yeast by Chantasingh et al. (2006) was a 33 kDa protein which is larger than the 22 kDa xylanase isolated in this study.

DISCUSSION

The fungal strain used in this study was identified as *Aspergillus terreus* strain and it was found to have the potential to produce xylanase (1,4-β-D-xylan xylohydrolase, EC 3.2.1.8). This is the first report on production, purification and characterization of the enzyme produced by this strain. In a shake flask culture, the medium supplemented with oat spelt xylan was found to be more suitable than that containing birch-wood xylan for cellulose-free xylanase production. Interestingly, a combination of both xylans in the medium showed the lowest productivity. Xylanases are known to be inducible enzymes (Cai et al., 2003) and generally, efficient production of xylanolytic enzymes is known to be dependent upon the choice of an appropriate inducing substrate and the medium composition (Kulkarni et al., 1999). Generally, xylanases are induced in most microorganisms during growth on substrates containing xylan (Purkarthofer and Steiner, (1955). Hence oat spelt and birch wood xylans were capable of playing a key role in the regulation of xylanase biosynthesis. During produc-

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A. terreus BCC129     MVYIKSIASGVLLASTAASAYSVIGRRQNATGLNSAFVAHGKKYWGTCGDQGTLSESANV
A. versicolor         MVYIKTLASGLLLASTTASAYSVLGRRQNATGLNSAYVAHGKKYWGTCGDQGTLSESANV
A. terreus 4209       ----------------------------QNATGLNSAFVAHGKK-----------SIASGV
                                                   *********:******           * ::.*

A. terreus BCC129     DVIKANFGQITPENSMKWDATEPSQGSFSFDGSDYLVNFAQENNLLIRGHTLVWHSQLPS
A. versicolor         DVIKANFGQITPENSMKWDATEPTQGSFSFDGSDYLVNFAQENNLLVRGHTLVWYSQLPS
A. terreus 4209       LLASTAASAYSVIGRKWLDAGVPIDGIGSQSG---------------------WNPGSAR
                       : .:  .  :  .    **  * :*  * .*                      * .  .

A. terreus BCC129     WVQSITDKDTLTDVLKNHITTVMTQYKGKIYAWDVVNEVLNEDGTLRSDVFYDVLGEDYI
A. versicolor         WVQGITDRDTLIGVMKNHITTVMTQYKGQIYAWDVVNEALAEDGSLRDDAFSNVIGEDYI
A. terreus 4209       HLGLPTPASTSRGTVNSDGGTYNIYTATRYNAPSIIGTATFT--QYWSSVFYNVLGEDFV
                      :   *  .*  ..::..  *       :  * .::. .        ...* :*:***::

A. terreus BCC129     RIAFETAREVDPDAKLYINDYNLDDANYSKTQGMVSLVKKWLDAGVPIDGIGSQSHLGLP
A. versicolor         QIAFETAREVDPDAKLYINDYNLDDANYAKTQGMVNLVSNLLAAGVPIDGIGSQSHLGST
A. terreus 4209       RIAFETAR-------LYINDYNLDNANYAKVR----------------------------
                      :*******       *********:***:*.:

A. terreus BCC129     TPASTSRLVSVSPPGE-----------------------------------------------
A. versicolor         WPSSGVEAALASLASTGVSEVAITELDIAGAAPEDYVNVANACLNVETCVGITSWGVSDK
A. terreus 4209       ------------------------------------------------------------


A. terreus BCC129     --SPTSESPLLFDSSFQPKEAFTAVLNAL
A. versicolor         DSWRASDSPLLFDSNYQPKEAYTAVMNAL
A. terreus 4209       -----------------------------
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Figure 9. Clustal W alignment of peptides obtained from tryptic digests of *A. terreus* 4209 xylanase against full sequences other *Aspergillus* xylanases; *A. terreus* and *A. versicolor*.

tion, xylanase activity increased only after 36 h at 25 - 35ºC in the oat spelt xylan-supplemented medium (Figure 3). Probably, the lag phase was required for the induction of certain constitutive hydrolases (present at low levels) that are required to produce effective inducers.

Possible events that take place at molecular level during xylanase synthesis have been reviewed by Haltrich et al. (1966). The most prominent catabolite derived from xylan found to provoke xylanase synthesis in yeasts and fungi is xylobiose together with other xylooligosaccharides (Royer and Nakas, 1990; Hrmova et al., 1991). Clevage of xylobiose to xylose can result in catabolite repression of xylanase synthesis, but xylose can also be a potent inducer of xylanase formation, depending on the microbial strain used (Maheshwari and Kamalam, 1985; Ghosh and Nanda, 1994; Pukarthofer and Steiner, 1995). In the present study, xylanase activity in the birchwood xylan medium peaked earlier but decreased more rapidly than in the oat spelt xylan medium. Probably, the composition and the source of the xylans and accessibility of the substrate to the fungal strain influenced the production profiles. Since the birchwood xylan (Fluka, BioChemika) contained ≥ 95% xylose whereas the oat spelt xylan (Sigma) contained 10% arabinose and 15% glucose, there could be selective xylanase induction/repression in the different media used. In a pre-screening study using *Aspergillus sp.* (2M 1 strain) for xylanase production, Angelo et al. (1997) reported varying titers of xylanase activity when different xylans were used to induce the enzyme.

When xylanase production was examined at different temperatures, maximum enzyme activity was noticed at 35ºC but at higher temperatures (40 - 50ºC) the enzyme production decreased considerably. The results suggest that the *Aspergillus terreus* stain is a mesophilic fungus. A number of reports have indicated that the optimal temperatures for production of most fungal xylanases ranged between 30 and 50ºC (Haltrich et al., 1996; Subramaniyan and Prema, 2002). Most *Aspergillus* species are regarded as mesophilic fungi. For example, the optimal temperature for the production of xylanase by *A. terreus* and *A. niger* was reported as 35ºC (Gawande and Kamat, 1999), 30ºC for *A. ochraceus* and *A. versicolor* (Biswas et al., 1990). Although the physiological changes induced by high temperatures during enzyme production is not very clear, it has been suggested that at high temperatures, microorganisms may synthesize reduced number of proteins that are probably essential for growth and other physiological processes including enzyme production (Gawade and Kamat, 1999). Another important factor that significantly affected the production of xylanase was the initial pH of the medium. Xylanase production by this isolate was observed in the pH range of 3.0 - 10.0. Maximum xylanase production

occurred at pH 6.0 after 84 h and production remained stable until 144 h (Figure 2). Although marginal increase in production was observed at pH 8, the activity of the enzyme decreased rapidly after 120 h. These results indicated that the optimal pH for xylanase production by the fungal isolate was 6.0 and it is acidophilic in nature. With rare exceptions, fungi are reported to produce xylanases at initial pH lower than 7.0 (Subramaniyan and Prema, 2002; Shah and Madamwar, 2005). Other reports have shown that initial pH range of 4 - 7 is optimal for xylanase production by *Aspergillus* species (Fernandez-Espinar et al., 1992; Ghosh et al., 1993; Raj and Chandra, 1995; Carmona et al., 1997).

The xylanase activity in the gel filtration was distributed across most of the elution profile. This is probably due to the expression of a wide spectrum of the xylanases by *A. terreus* as observed with *A. niger* strains (Berrin et al., 2000). A combination of this broad elution of xylanase and loss in specific activity, possibly due to removal of some stabilizing elements in the culture supernatant on purification of the enzyme, may be largely accountable for the low yields of activity in the selected distinct peak, peak 2. Despite the low yields, the isolation procedure provided pure xylanase that allowed charcaterisation of the protein and preliminary studies on the amino acid sequence. The *A. terreus* xylanase is a single subunit protein of 22 kDa. The xylanase isolated is, therefore, similar in size to xylanases from other fungi such as *Paecilomyces themophila* (Li et al., 2006) and *Penicillium citrinum* (Tanaka et al., 2005). This group of xylanases that is characterized by a small molecular weight and lack of cellulase activity has been classified under glycoside hydrolase (GH) family 11 of xylanases, a group viewed favourably in the pulp and paper industry (Henrissat, 1991; Henrissat and Bairoch, 1993; Henrissat and Davies 1997). Absence of cellulase activity is attractive in the paper and pulp industry as the cellulose required for paper manufacture is not degraded during the enzymatic bleaching (Haki and Rakshit, 2003). The small size of the enzyme is also advantageous as it facilitates easy penetration of the woody structures in the application of the xylanase (Haki and Rakshit, 2003).

SDS-PAGE of xylanase peak 1 revealed that the 22 kDa protein was absent from peak 1 (Figure 5). Thus the xylanase activity in peak 1 can not be attributed to the xylanase found in peak 2. Chantasingh et al. (2006) cloned and expressed a xylanase from the *A. terreus* strain BCC129. The xylanase expressed in *Pichia pastoris* was a 33 kDa monomeric enzyme, and therefore is a member of the family 10 xylanases. Hence, this enzyme is different from the one in this study.

Peptide sequence alignment showed areas of sequence homology between the 33 kDa (Family 10) (Chantasingh et al., 2006) and our 22 kDa (Family 11) xylanase. However, there are peptides that clearly show differences between the two enzymes, implying that while the two may be related to some extent in their sequences, there are some distinguishing regions on these proteins.

Thermal stability is a desirable property of xylanases when considering their industrial application. The optimal temperature for the purified enzyme was found to be 35ºC but it retained over 95% activity at 40ºC after 30 min incubation. Thermostability studies showed that the original activity was retained at 35 - 40ºC for 4 h at pH 6 but the activity was completely lost after 4 h at 70^{v}C. It has been widely reported that many cellulase-free xylanases produced by mesophilic microorganisms lack high thermostability (Fernandez-Espinar, 1992; Cuotto et al., 1998; Subramaniyan and Prema, 2002). These results indicate that for industrial application of the xylanase from our isolate, the suitable temperature range is 35 - 40ºC. These observations suggest that the cellulase-free xylanase produced by the isolate may not be thermostable at temperatures commonly used in the bleaching of pulp, but may be used in processes operated at moderate temperatures and pH which may include preparation of baked cereal food products saccharification of agro-residues, aiding extraction and clarification of fruit juices.

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