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Full Length Research Paper

Purification and characterization of a xylanase from the endophytic fungus *Alternaria alternata* isolated from the Thai medicinal plant, *Croton oblongifolius* Roxb.

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Xylanases are a class of enzymes that degrade β-1, 4-xylan, a linear polysaccharide found as hemicellulose in plant cell walls, in xylose. They are one of the most important enzyme groups used in industry and agriculture. Fifty-four endophytic fungi were isolated and examined for xylanase production. Xylanase activity was found in thirty of the isolates in primary screening by growing on solid xylan agar plates. After secondary screening for xylanase activity in xylan liquid culture, the isolate that yielded the highest xylanase-production (PTRa9) was selected for further evaluation. Optimal xylanase production was achieved after 4 days of culture with 2% (w/v) rice bran and 0.1% (w/v) ammonium sulfate as the carbon and nitrogen source respectively. This xylanase was enriched 60.8-fold to apparent homogeneity by sequential ammonium sulphate precipitation, Diethylaminoethyl (DEAE)-cellulose ion exchange and Superdex 75 gel filtration chromatography. The resultant 54.8 kDa protein had a specific activity of 161.1 U/mg protein, an optimal temperature of 45°C, with >90% activity from -20 to 45 ^{o}C, a broad pH range of 3.0 to 11.0 (optimal at pH 5.0), and was sensitive to most divalent cations but especially by Hg^{2+}, Cu^{2+} and EDTA. From the kinetic analysis, it had a K_m of 0.421 mg/ml and a V_{max} of 0.826 U/mg protein.

Key words: Xylanase, endophytic fungi, Thai medicinal plant.

INTRODUCTION

Enzymes are distinct biological polymers that catalyze specific chemical reactions, converting substrates into particular products. They are specific in function and speed up reactions by providing alternative pathways with lower activation energy without being consumed. Although enzymes are fundamental elements for cellular and extracellular biochemical processes, they are commercially utilized in a number of food processing industries (Haq et al., 2006). The manipulation of biotechnological techniques has played an important role in recent advancement, for example, the baking industry.

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Agricultural countries, such as Thailand, produce agro-industrial wastes and by-products in abundance, such as wheat bran, sugar cane bagasse, cassava waste, corn cobs, rice bran and so on, and these form some of the prominent waste materials from the allied food and energy industries. These waste materials, if not handled properly, are sources of environmental pollution as well as, if correctly handled, a potentially valuable add on value to the agroindustries. Thus, producers are increasingly interested to utilize these otherwise waste resources to improve the economic viability of agriculture as well as safeguard the environment. One such increasing drive is to utilize such neglected materials as carbon or nitrogen sources for the bioproduction in the production of enzymes that can then be further employed and further processed (Mohammadi et al., 2006; Okafor et al., 2007).

Xylan is the second most abundant biopolymer in the biosphere 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). A considerable amount of xylan is found in solid agricultural and agro-industrial residues, as well as in effluents released during wood processing, but asides representing a significant source of wasted products their inappropriate disposal causes significant damage to the ecosystem (Biely, 1985; Prade, 1995). Hydrolysis of xylan is an important step towards the degradation of lignocellulosic material in nature for non-toxic recycling, or for further metabolism, such as biofermentation, to utilize the trapped carbon and nitrogen sources (Poorna and Prema, 2007). However, the chemical hydrolysis of lignocelluloses results in hazardous byproducts, forcing the use of microbial enzymes that are specific in action for xylan hydrolysis, and so are environmentally friendly options (Biely, 1985). Due to the structural heterogeneity of xylan, complete degradation of this biopolymer requires the 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 which initiates the degradation of xylan into xylose and xylooligosaccharides of different sizes (Collins et al., 2005).

There are different types of xylanases that differ in their substrate specificities, primary sequences, folds and physicochemical properties (Wong et al., 1988; Collins et al., 2005). 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 good producers of xylanases of interest from an industrial point of view, due to the extracellular release of the enzymes, their higher yield compared to that produced by yeast and bacteria and also that they produce several auxiliary enzymes that are necessary for debranching of substituted xylans (Haltrich et al., 1996). However, fungal production of xylanases is generally associated with the 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 lingo-cellulosic 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 the digestibility of animal feed, converting lignocellulosic substances to feedstock and fiber separation (Beg et al., 2001; Subramaniyan and Prema, 2002), paper and pulp industries (Bajpai, 1997). However, in the paper and pulp industry, cellulase-free xylanases are required to avoid the adverse effects of damaging the pulp fibers (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 those xylanases containing negligible cellulose activity can be obtained by (1) using suitable separation methods, (2) using genetically engineered organisms to produce exclusively xylanase or (3) 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 that determine the economics of a process and this can be partially achieved by optimizing the fermentation media (Shah and Madamwar, 2005). Several industrial processes can be carried out using whole cells as sources of enzymes, but the efficiency can be improved by using isolated and purified enzymes. The criteria used to select a particular method for the isolation and purification depends on the intended end use. A high state of purity is generally not required in the food processing, detergent and 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 biotechnological applications and attempts are being made to isolate new strains (Lee-Chiang et al., 2006; Schmeisser et al., 2007).

Endophyte typically describes an organism living within a plant, although the actual meanings vary depending on

researchers and have not been unified yet. Although the existence of endophytes has been known for a long time (Lewis, 1924), many of the known endophytic fungi have only been isolated and studied in the last two decades (Findlay et al., 1995; Hata and Futai, 1995; Hata and Futai, 1996; Koga et al., 1997; Hata et al., 1998). It is apparent that endophytes will produce novel metabolites some of which should benefit the host plant, but their potential use to humans is less known. Certainly, some endophytes have been reported to produce some substances harmful to predators of the host plant (Bacon et al., 1977; Clay, 1989), whilst *Acremonium* sp. isolated from *Taxus baccata* was found to produce leucinostatin A, which was active against breast cancer (Strobel et al., 1997). Other endophytes are also known to be producers of bioactive compounds (Findlay et al., 1995, 1997; Ju et al., 1998; Lu et al., 2000; Rodrigues et al., 2000). However, very few microorganisms living in plants, for example, the ericoid mycorrhizal fungus *Hymenoscyphus ericae* producing β-1, 4-endoxylanase (Burke and Cairney, 1997), have been reported to be xylanase producers even though one of the major components of the plant cell wall is xylan. In this study, we focus on the inside part of plants as a new source for isolating microorganisms producing xylanase and describe the purification and characterization of a xylanase from one such endophytic fungal isolate.

MATERIAL AND METHODS

Chemical materials

Agricultural wastes residues were purchased from a local market in Bangkok, Thailand. Birch wood xylan, dinitrosalisylic acid (DNS) and xylose were purchased from Sigma-Aldrich (USA). The reagents used in polyacrylamide gel electrophoresis (PAGE) were obtained from Plusone Pharmacia Biotech (Sweden), except the low molecular weight calibration kit, used as standard molecular weight marker proteins, which was purchased from Amersham Pharmacia Biotech (UK). All other biochemical reagents and general chemicals used in the investigation were of analytical grade.

Isolation of endophytic fungi

Endophytic fungi were isolated using a modification of Petrini's method (Petrini, 1992). The leaves of *Croton oblongifolius* were cleaned with tap water, dried in a laminar air flow, cut into small pieces (5 x 5 mm) and then surface sterilized by immersing the cut pieces sequentially into 95% (v/v) ethanol for 1 min, 12% (w/v) sodium hypochlorite for 5 min and then 95% (v/v) ethanol for 30 s. Finally, they were washed in sterilized water twice, dried with sterile tissue paper and placed on the surface of potato dextrose agar (PDA) plates. Plates were then incubated at room temperature and examined for signs of fungal germination every day. Fungal endophytes germinating from the leaf pieces were transferred to fresh PDA medium plates by hyphal tip transfer, and incubated for 7 to 14 days at room temperature. Their potential purity was determined by colony morphology. Fungal isolates with a different morphology were collected for further study. This sample set was supplemented with 28 other isolates of endophytic fungi (22 isolates from the leaves of mangrove and six isolates from palm leaves), obtained from the culture collection of the Microbiology Department, Faculty of Science, Chulalongkorn University, Thailand.

Screening of endophytic fungi for extracellular xylanase production

All endophytic fungal isolates were cultured on PDA plates for 7 days. A single agar disc (0.5 mm in diameter) was punched from the leading edge of the growing colony and inoculated onto a selective xylan-agar plate containing 1% (w/v) birchwood xylan. After 5 days, the plates were flooded with 2% (w/v) aqueous Congo red and left for 15 min. The stain was then washed from the agar surface with distilled water and the plates were then flooded with 1 M NaCl to destain for 15 min. The NaCl solution was then removed. Xylan degradation around the colonies (as xylanase activity) appeared as a yellow-opaque area against the red color of the undegraded xylan. In order to select the best xylanase producer, strains with apparent xylanase activity on the plates were then secondary screened by culturing in 100 ml of basal medium [peptone 1% (w/v), KH_2PO_4 0.15% (w/v), $NaNO_3$ 0.2% (w/v), NaCl 0.05% (w/v), $MgSO_4$ 0.05% (w/v), $CaCl_2$ 0.025% (w/v), $FeSO_4$ 0.0001% (w/v), $ZnSO_4$ 0.0001% (w/v), $CuSO_4$ 0.0001% (w/v) and birchwood xylan 1% (w/v)] at pH 7.0. Each flask was inoculated with three 0.5 cm diameter agar plugs and kept at 30°C in a rotary shaker at 150 rpm. Then, the cultures were filtered through filter paper and the filtrate was screened for the presence of xylanase activity in the culture medium as detailed in the next section.

Assay for xylanase activity

Xylanase 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 the appropriately diluted culture supernatant or enzyme enrichment fraction in 20 mM acetate buffer (pH 5.0) for 30 min at 50°C 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 mol of xylose equivalent per minute under the assay conditions. Values are given as the mean ± 1 standard error (SE), derived from triplicate samples.

Identification of the endophytic fungi isolate

The endophytic fungal strain which showed the highest level of xylanase production was then identified with species using morphological and molecular systematic approaches. Morphological identification used both macroscopic and microscopic characters, whilst the molecular identification was based upon the DNA sequence similarity of the internal transcribed spacer (ITS) regions of the ribosomal ribonucleic acid (rDNA), comparing this isolate to those in the National Center for Biotechnology Information (NCBI) GenBank database. Genomic DNA was prepared from fresh mycelial cultures of the selected endophytic fungal isolate and extracted with cetyltrimethylammonium bromide (CTAB), as described by Zhou et al. (1999). Polymerase chain reaction (PCR) amplification of the internal transcribed spacer (ITS) was performed in a total volume of 35 μl which was comprised of approximately 100 ng genomic DNA, 1 × PCR master Mix (Fermentas, Califonia, USA), and 100 nM of ITS1F primer and 500 nM ITS4 primer. The amplification was performed in a thermocycler with a PCR profile of 94°C for 5 min, followed by 38 cycles of 94°C for 1 min, 51°C for 1 min and 72°C for 1 min, plus a final extension of 72°C for 5 min. The PCR reactions were purified using the NucleoSpin® (Macherey-Nagel Inc., Easton, USA) and were direct sequenced on both the leading and lagging strands (using the ITSF1 and ITS4

primers, respectively) commercially by Macrogen (Seoul, Korea). The complete consensus sequence was then used to BLASTn search the NCBI GenBank database using the default settings, with the top 100 highest sequence similarity hits being recorded and compared. Species annotation of the deposited ITS sequences in the GenBank database were taken on trust and used to convert the molecular operational taxonomic unit (MOTU) designation of the fungal isolate to a likely species designation where the % sequence similarity was high enough (>97%).

Xylanase productio n

For xylanase production, the selected endophytic fungal isolate was cultivated in a modified basal medium, as described previously and the effect of the carbon and then the nitrogen sources and concentrations on the extracellular xylanase (activity) production level were detected by a sequential univariate approach. With respect to the optimal carbon source for extracellular xylanase production, various types of different agricultural residues were tested to determine their effect on xylanase production over a 20 day culture period, replacing the 1% (w/v) birchwood xylan in the basal medium with similar concentrations of one of chaff, bagasse, rice bran, rice straw and sawdust. After selecting the best carbon source, and then concentration (from 0.5, 1 and 2% (w/v)), the peptone was replaced by one of soybean powder, yeast extract, corn steep liquor and urea as the organic nitrogen source or the sodium nitrate was replaced by one of ammonium sulfate, ammonium persulfate, ammonium hydrogen phosphate and ammonium chloride as the inorganic nitrogen source, each at one of three different concentrations (0.1, 0.2 and 0.5% (w/v)). Note that since this was performed as a univariate and not a multivariate analysis, then any potential interaction between these components is not ascertained. All experiments were done in triplicate flasks, with the results reported as the mean ± 1 SE.

Protein content determination

Protein contents were determined by the Bradford assay (Bradford, 1976), using 5, 10, 15 and 20 μg/ml of bovine serum albumin (BSA) as the standard to construct the calibration curve. For each serial two-fold dilution of the sample in deionized water, 50 μl aliquots were transferred into each of three wells of a microtiter plate and 50 μl of Bradford's reagent [100 ml contains: 10 mg coomassie brilliant blue G-250 and 10 ml of 85% (v/v) phosphoric acid, dissolved in 95% (v/v) ethanol] was added to each well. The plate was shaken (Biosan, OS-10, Latvia) for 5 min and then left for 10 min before reading the absorbance at 595 nm using an enzyme linked immunosorbent assay (ELISA) plate reader (Biotek Synergy HT, Biotek instrument, USA). The obtained OD was converted to the protein concentration using the linear equation computed from the standard curve. During the column chromatographic separations, the elution peak profiles of proteins were determined by measuring the absorbance at 280 nm.

Purification of xylanase

All the procedures were performed at 4°C, unless otherwise stated.

$(NH_4)_2SO_4$ Precipitation

To 5 liters of culture supernatant, $(NH_4)_2SO_4$ was slowly added with stirring to a final 80% saturation and then left to stand overnight at 4°C. The precipitate was collected by centrifugation at 10,000 × g for 30 min (Beckman Coulter, USA), and dissolved in distilled water, dialyzed (3,500 MWCO) against three changes of 5 L distilled water at 4°C and then concentrated by lyophilization (Labconco, USA) . This fraction is referred to hereafter as the "ammonium sulfate cut fraction".

Diethylaminoethyl (DEAE)-cellulose ion exchange chromatography

DEAE-cellulose ion exchange chromatography was performed with a 1.6 × 15 cm column using an automatic liquid chromatography system (AKTA prime, Amersham Pharmacia Biotech, Sweden). The column was equilibrated with five column-volumes of 50 mM Tris-HCl (pH 7.0). Thereafter, 5 ml samples (400 mg protein) of the ammonium sulfate cut fraction were injected into the column and eluted with the same buffer at a flow rate of 1.0 ml/min, collecting 10-ml fractions before a linear 0-1.0 M NaCl gradient in the same buffer was applied over the next 55 fractions. The eluted fractions were monitored for protein content with a UV detector at 280 nm and for xylanase activity as described previously. The fractions containing xylanase activity from the column were pooled, dialyzed against three changes of 5 L of distilled water and concentrated, and is referred to as the "post-DEAE-cellulose xylanase fraction".

Superdex-75 gel filtration chromatography

The post-DEAE-cellulose xylanase fraction was then further enriched by preparative Superdex-75 gel filtration column (1.6 × 60 cm) chromatography. The column was equilibrated with two column -volumes of 100 mM NaCl / 50 mM Tris-HCl (pH 7.0), and then 2 ml of the post- DEAE-cellulose xylanase fraction solution (50 mg protein) was injected and eluted in the same buffer at a flow rate of 0.5 ml/mi n, collecting 5 ml fractions. Fractions were monitored for protein with a UV detector at 280 nm and for xylanase activity as described previously. The xylanase active fractions were pooled , dialyzed against three changes of 5 L of distilled water and concentrated, and this is referred to as the "enriched xylanase fraction".

Determination of enzyme purity by native-PAGE and xylanase activity staining (zymography)

The enzyme from each step of purification was analyzed by its native protein pattern following native PAGE resolution and its purity according to the method of Bollag et al. (1996). Native PAGE was performed with 10 and 5% (w/v) acrylamide separating and stacking gels respectively, with 100 mM Tris-glycine (pH 8.3) as the electrode buffer. Electrophoresis was run at a constant current of 20 mA per slab at room temperature in a Mini-Gel Electrophoresis unit (Hoefer model miniVE, Pharmacia Biotech, UK). After electrophoresis, proteins in the gel were visualized by coomassie blue R-250 (sigma) staining and activity staining.

For protein staining, gels were stained by immersion in 0.1% (w/v) coomassie blue R-250 in 10% (v/v) acetic acid / 45% (v/v) methanol for 45 min. Destaining was performed by immersing the gel in 10% (v/v) acetic acid / 45% (v/v) methanol, with several changes of this destaining solution until the background was clear.

For xylanase activity staining (zymography), after native-PAGE resolution the gel was directly immersed in 1% (w/v) birchwood xylan / 50 mM Tris-HCl (pH 7.0) at room temperature for 30 min. The gel was then transferred to a 0.1% (w/v) solution of Congo red (sigma) and incubated at 25°C with constant shaking for 10 min. The gel was destained by washing with 1 M sodium chloride. The activity band was clearly visible as yellowish clearances against a deep red background by the end of 10 min of destaining

(Karnchanatat et al., 2008).

Molecular weight determination by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Discontinuous reducing 0.1% (w/v) SDS-PAGE was performed according to the procedure of Laemmli (1970) using 15 and 5% (w/v) acrylamide resolving and stacking gels respectively. Samples were treated with reducing (2-mercaptoethanol containing) sample buffer and boiled for 5 min prior to application to the gel. Electrophoresis was run at a constant current of 20 mA per slab at room temperature in a Mini-Gel Electrophoresis unit. High and low molecular weight standards were coresolved on each gel and used to determine the subunit molecular weight of the enriched lipase enzyme. After electrophoresis, proteins in the gel were visualized by staining with coomassie blue R-250 as described previously.

Effect of temperature on the xylanase activity and thermostability

The effect of temperature on the xylanase activity was determined by incubating the enriched xylanase fraction in 20 mM sodium acetate buffer pH 5.0 at various temperatures (-20 to 90°C at 10°C intervals) for 30 min assaying the xylanase activity. The thermostability of the xylanase was investigated by preincubating the enriched xylanase fraction at various temperatures (-30 to 60°C at 10°C intervals) in the same buffer for the indicated fixed time intervals (10 to 120 min), cooling to 4°C and then assaying the residual xylanase activity as described previously.

pH-dependence of the xylanase activity

Incubating the enriched xylanase fraction in buffers of broadly similar salinity levels, but varying in pH from 2 to 14, was used to assess the pretreatment pH stability and the pH optima of the xylanase. The buffers used were (all 20 mM) glycine-HCl (pH 2.0 to 4.0), sodium acetate (pH 4.0 to 6.0), potassium phosphate (pH 6.0 to 8.0), Tris-HCl (pH 8.0 to 10.0) and glycine-NaOH (pH 10.0 to 12.0). The enriched xylanase fraction was mixed in each of the different pH-buffer compositions, plus the control (20 mM sodium acetate buffer pH 5.0). For pH stability, the above xylanase-buffer mixtures were left for 30 min at room temperature and then adjusted back to 20 mM sodium acetate buffer pH 5.0 and assayed for xylanase activity as described previously. The control incubation was set at 100% activity and the activity of the samples from the different pH buffers were expressed as the % activity relative to that of the control (set at 100%).

For evaluation of the pH optima of the enriched xylanase, the different pH buffer-enzyme mixtures were adjusted in substrate concentration, as per section 2.4 for xylanase activity assay, and performed over 30 minutes. The activity of the enzyme in each pH was then related to that of the control, set to 100%.

Effect of metal ions on the xylanase activity

The effect of preculture with different divalent metal cation salts (mostly chloride anions but also two sulfate anions) and the chelating agent ethylenediamine tetraacetic acid (EDTA), on the xylanase activity of the enriched xylanase fraction was evaluated. The enriched xylanase fraction was incubated for 30 min with one of Ca^{2+}, Fe^{2+}, Hg^{2+}, Mg^{2+}, Mn^{2+} (all as chlorides), Cu^{2+} or Zn^{2+} (as sulfates) or EDTA, at one of three concentrations (1, 5 and 10 mM) with continuous shaking. The residual xylanase activity was then evaluated, and from this, the relative xylanase activit y (%) was calculated taking t he residual xylanase activity found in the control samples (without the addition of metal salts or EDTA) as 100%.

Determination of kinetic parameters

The Michaelis constant (K_m) and maximum velocity (V_{max}) values of the enriched xylanase fraction were determined by measuring the rate of birchwood xylan hydrolysis under standard assay conditions. The reaction mixture was 20 mM sodium acetate buffer pH 5.0 with the birchwood xylan substrate at concentrations ranging from 1.0 to 20 mg. The values for K_m and V_{max} were then determined from the Lineweaver-Burk plot.

RESULTS AND DISCUSSION

Isolation and screening of xylanase producing from endophytic fungi

Fifty-four endophytic fungal cultures were isolated from Thai medicinal plants (Table 1), and were subjected to rapid screening for extracellular xylanase production using birch wood xylan agar plates. Although the basal medium containing birch wood xylan used for the screening of xylanolytic fungi has previously been reported to be suitable for such by several workers (Bhalla and Joshi, 1993; Kvesitadze et al., 1999; Abdel-Sater and El–Said, 2001), it is important to note the potential fastidious nature of many endosymbionts, and thus the unknown proportion of these endophytic fungi that are actually clonally cultureable and what preselection this has imposed. The species list given here (Table 1) then should not be viewed as representing the complete or even representative constituents in any ecological sense, but rather is just a list of endophytic fungi and their detectable xylanase secretion. Even though a clearing zone was obtained after 5 days of incubation the clarity of the clearing zone was signifi-cantly increased when the medium was stained with 0.1% (w/v) Congo red, a widespread method for the screening of hemicellulase activity (Teather and Wood, 1982; Pajni et al., 1989), followed by washing with 1 M NaCl as reported Bhalerao et al. (1990) and Techapun et al. (2001).

From the 54 endophyte isolates initially screened, 30 were found to be positive for xylanase activity. Amongst these 30 active isolates, one isolate (PTRa9) produced a much larger clearing zone was observed than the others (Table 1). Nevertheless, to ensure that the best xylanase producer was selected, all 30 of these positive isolates were evaluated by secondary screening for extracellular xylanase production in liquid culture in basal medium and subjected to quantitative analyses of the xylanase activity level in the culture media. Again, isolate PTRa9, obtained from the host plant *C. oblongifolius* (Plao yai), was found to produce the highest level of extracellular xylanase (Table 1). In this study, this isolate was selected , identified to species level and further evaluated for the factors influencing the xylanase production level and the

Table 1. Endophytic isolates, host plant and xylanase production levels in primary and secondary screening.

Endophyte isolate	Host plant	Primary screening: Xylanase activity (halo diameter in cm)	Secondary screening: Xylanase activity (U/ml)
A238R	*Croton oblongifolius*	1.50±0.05	1.52±0.03
A522R	„	2.10±0.07	1.02±0.04
CCP 1	„	2.00±0.00	0.89±0.05
PTM 5	„	1.50±0.14	0.95±0.06
PTM 7	„	0.90±0.08	0.70±0.13
PTM 8	„	0.50±0.03	0.33±0.04
PTM 9	„	1.30±0.14	1.46±0.05
PTM 11	„	2.10±0.11	0.97±0.05
PTRa 2	„	0.90±0.05	0.04±0.00
PTRa 7	„	1.40±0.05	0.92±0.08
PTRa 8	„	0.50±0.03	0.78±0.03
PTRa 9	„	2.60±0.05	2.59±0.06
PTRa 10	„	2.10±0.08	2.28±0.03
PTRa 11	„	1.00±0.11	0.08±0.00
PTVa 6	„	1.10±0.17	1.08±0.10
PTVa 7	„	1.10±0.17	1.05±0.29
PTVa 10	„	1.10±0.03	0.46±0.02
PTMa 2	„	1.70±0.08	0.69±0.04
PTMa 4	„	0.80±0.10	1.46±0.01
PTMa 5	„	0.80±0.03	0.60±0.11
PTMa 7	„	0.40±0.03	0.96±0.16
PTMa 9	„	1.20±0.14	1.13±0.14
PTMa 12	„	0.80±0.05	0.15±0.02
PTMa 13	„	1.30±0.03	1.39±0.03
PTMa 14	„	0.50±0.05	1.45±0.03
RAP 45	„	1.90±0.11	1.05±0.13
Collectotrichum sp.	*Lumnitzera littorea*	0.90±0.05	0.59±0.03
Pestalotiopsis sp.	*Rhizophora apiculata*	1.10±0.05	0.70±0.04
Phomopsis sp.	*Thespesia populnea*	0.90±0.05	0.75±0.01
Xylaria sp.	*Thespesia populnea*	0.50±0.03	0.80±0.04

enzyme activity and kinetics. The other isolates of course remain of interest and will be selectively screened in the future.

Identification of endophytic fungi

The isolate PTRa9, which showed the highest xylanase activity production, was identified to a likely species level based on morphological and molecular systematics. With respect to morphological identification, the isolate showed a green-black colony on PDA medium. The isolate was identified by the help of molecular sys-tematics using the DNA sequence of the ITS1-5.8S rDNA-ITS2 rDNA region. The BLASTn search of the obtained sequence revealed several highly similar (>97% identity) ITS sequences but these were all from *Alternaria alternata* isolates, with the highest sequence identity being to *A. alternata* isolate VC38 (GQ916545.1) at 99% sequence identity. Isolate PTRa9 is therefore most likely to be an isolate of *A. alternata* and very closely related to isolate VC38 (http://www.ddbj.nig.ac.jp).

Molecular techniques have been successfully used for identifying endophytic fungi and several recent studies have shown that genetic methods exhibit high sensitivity and specificity (Promputtha et al., 2005; Sette et al., 2006; Tedersoo et al., 2006; Morakotkarn et al., 2007). Indeed, m of the known endophytic fungi have been detected and identified by comparison of their 18S rDNA or internal transcribed spacer (ITS1-5.8S rDNA-ITS2) sequences (Harney et al., 1997; Guo et al., 2000; Lacap et al., 2003). These have the advantage over random amplified polymorphic DNA (RAPD), which has been used for detecting genetic diversity of endophytic fungi from medicinal plants and for pre-selection of these isolates for bioactive screening program (Tejesvi et al., 2007), in that

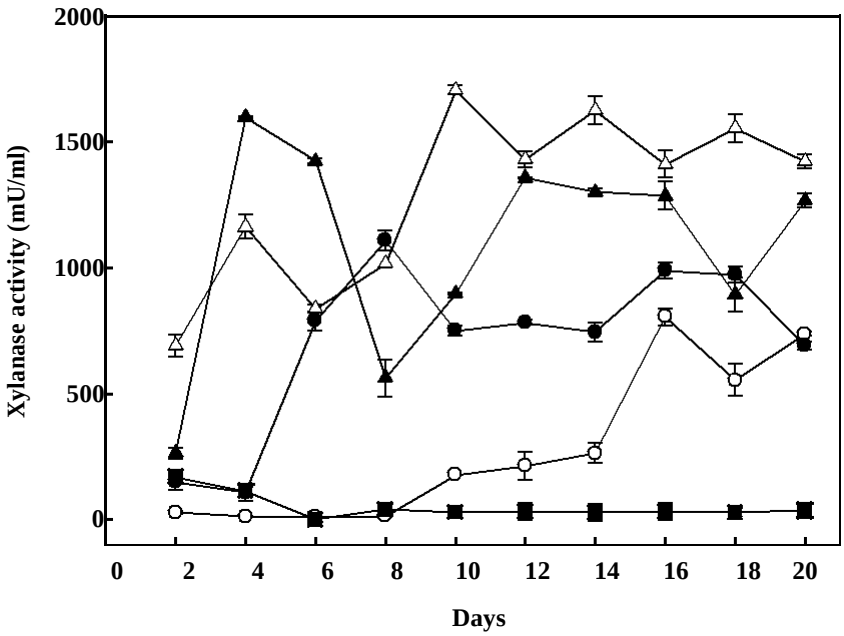

Figure 1. Time course of xylanase production by endophytic fungus *A. alternata* PTRa9 grown on different 1% (w/v) carbon sources as; chaff (●), bagasse (○), rice bran (▼), rice straw (Δ) and sawdust (■). Data are shown as the mean ± 1 SEM and are derived from three repeats.

any contaminating host or other minor endophytic DNA does not compound the pattern but is clearly recognized. As discussed previously, some or many endophytic fungi may be lost during the isolation processes, but molecular techniques can be used to alleviate the requirement of cultivation and so measure the diversity of endophytes in the natural environment (Guo et al., 2000).

Production of xylanase

Effect of the carbon source on xylanase production

The results for the ability of the different carbon sources to promote the development of fungal mycelium and stimulate the secretion of the xylanolytic enzyme are shown in Figure 1. Sawdust was essentially ineffective, whilst bagasse and chaff supported only a low (<1 U/ml) level of xylanase production after 16 and 8 days of culture respectively. In contrast, rice bran and rice straw yielded 1.59 and 1.67 U/ml after 4 and 10 days of culture respectively. Thus, with respect to the yield and culture time, xylanase production by rice bran as the carbon source seemed more optimal and was selected.

With varying the rice bran concentration, the highest level of total xylanase activity (1.95 U/ml) was produced with 2% (w/v) of rice bran after 4 (and again at 20) days growth (Figure 2). Although, xylanase yield (1.83 U/ml) was found almost as high as 0.5% (w/v) rice bran, this was not attained until after 16 to 18 days of culture, and so a carbon source of 2% (w/v) rice bran was selected. Note, that when the concentration of each carbon source was increased above 2.0% (w/v), the enzyme activity level in the culture medium decreased (data not shown), presumably because the higher levels of substrates reduced the culture agitation resulting in a reduced accessibility of the fungus to the substrates, and hence, a lower observed enzymatic activity. That rice bran produced the maximum enzyme activities may be due to the fact that it is fairly diversified in its composition and contains most minerals. These findings are also in accord with the findings of Coelho and Carmona (2003), who reported that wheat bran as good substrate for xylanase production, as well as other researchers (Chen et al., 1999; Ferriera et al., 1999; Park et al., 2002; Haq et al., 2002). However, since wheat bran was not included in this study (it is not grown or produced to any significant extent in Thailand in contrast to rice), *A. alternata* isolate PTRa9.

Effect of the nitrogen source on xylanase production

The results for the effect of the nitrogen source on the production of xylanase by the selected endophytic fungi isolate (PTRa9) are shown in Figure 3. Among the nitrogen sources tested, $(NH_4)_2SO_4$ at 0.1% (w/v) was found to support the highest level of xylanase production (1.95 U/ml) in basal medium at 4 days, slightly higher than that for NH_4NO_3. The other three ammonium salts

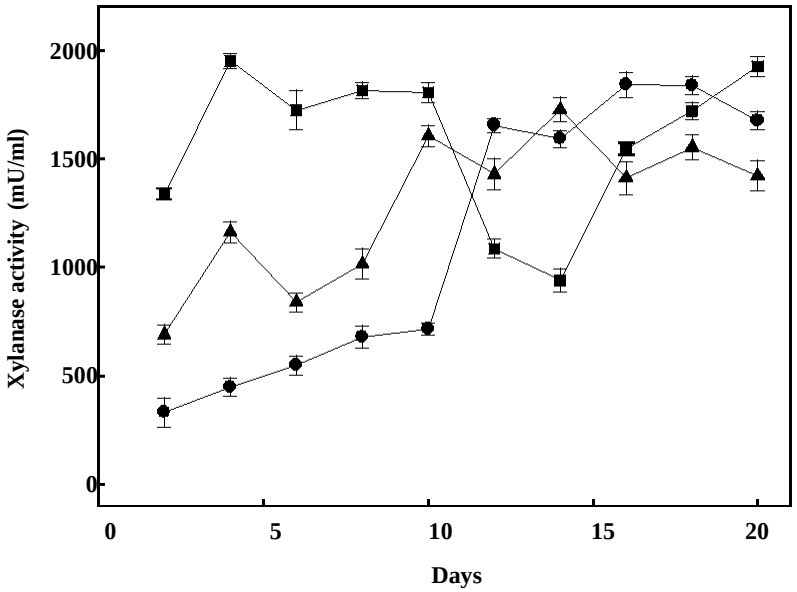

Figure 2. Effect of the rice bran concentration on xylanase activity. *A. alternata* PTRa9 was grown in minimal media supplemented with 0.5% (●), 1.0% (o) and 2.0% (▼) (w/v) rice bran. Data are shown as the mean ±1 SEM and are derived from three repeats.

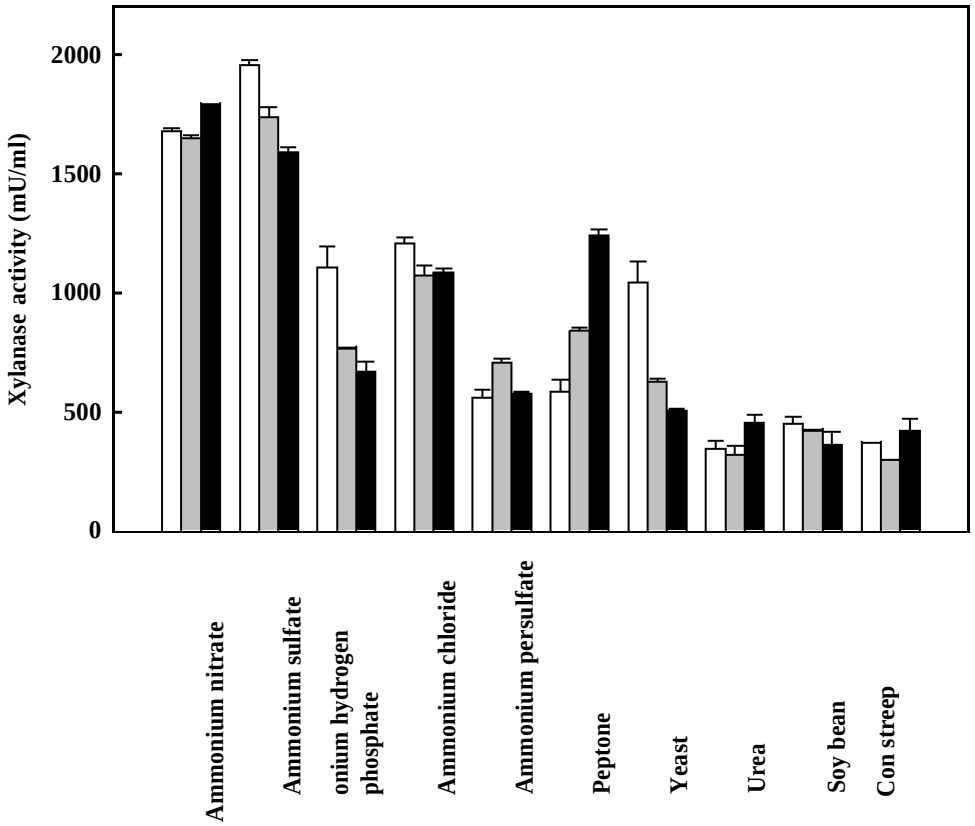

Figure 3. Effect of the nitrogen source and concentration on xylanase activity. *A. alternata* PTRa9 was grown in minimal media supplemented with the indicated nitrogen sources at 0.1% (white), 0.2% (grey) and 0.5% (black) (w/v). Data are shown as the mean ± 1 SEM and are derived from three repeats.

Table 2. Enrichment summary for the xylanase from *A. alternata* isolate PTRa9.

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Culture filtrate	765.22	2027.90	2.72	100.00	1.00
80% $(NH_4)_2SO_4$ cut	86.27	402.13	4.74	19.81	1.81
DEAE-cellulose	11.14	118.31	10.73	5.84	4.02
Sephadex-75	0.21	26.49	161.10	1.32	60.82

as inorganic nitrogen sources were less effective, and all the organic nitrogen sources, except for yeast and peptone were more than three-fold less effective than $(NH_4)_2SO_4$. The nitrogen source used in the production medium is one of the major factors affecting enzyme production and level. With respect to inorganic nitrogen sources, in accord with this study, $(NH_4)_2SO_4$ has been previously reported to yield better results (Xu et al., 2005), whilst NH_4NO_3 was optimal for *Schizophyllum commune* (Haltrich et al., 1993). However in contrast, $NaNO_3$ and $(NH_4)_2HPO_4$ were reported to be optimal for *Trichoderma harzianum* (Abdel-Sater and El-Said, 2001) and *Thermomyces lanuginosus* RT9 (Hoq et al., 1994) respectively. In summary, it can be concluded that production of extracellular xylanase activity by *A. alternata* PTRa9 is maximal with 2% (w/v) rice bran and 0.1% (w/v) $(NH_4)_2SO_4$ as the carbon and nitrogen source, respectively.

Purification of xylanase

The extracellular xylanase from the culture media of *A. alternata* PTRa9 was enriched to apparent homogeneity by sequential ammonium sulfate precipitation, DEAE-cellulose ion exchange and Superdex-75 gel filtration column chromatography. After fractionation of the culture filtrate with 80% saturation ammonium sulfate approximately 19.8% of the xylanase activity was recovered for a 1.8-fold enrichment (Table 2).

With DEAE-cellulose ion-exchange chromatography, the xylanase activity eluted as a sharp peak in the unbound fraction (fractions 3 to 5) allowing separation from a significant amount of the bound proteins including the peak in fractions 40 to 75 (Figure 4A). Thus, the elution pattern showed a single xylanase activity peak which was harvested and pooled. Compared to the ammonium sulfate cut fraction, the post-DEAE-cellulose xylanase fraction showed a 87% reduction in the total protein content for only a loss of 70% xylanase activity (Table 2).

Next, t he post-DEAE-cellulose xylanase fraction was further fractionated using Superdex-75 gel filtration column chromatography, where a sharp peak was eluted free of most of the other xylanase activity negative proteins (Figure 4B). Compared to the post-DEAE-cellulose xylanase fraction, although the enriched xylanase fraction showed a 98.2% reduction in the total protein content this was achieved at the cost of a 77.6% loss of xylanase activity (Table 1). In Overall, a 60.8-fold enrichment for a 2.21% yield was obtained after the three enrichmnt stages, compared to the crude culture filtrate (Table 2). The enriched xylanase fraction, with a specific activity of 161.1 U/mg of protein (Table 2), that was enrinched to or near to apparent homogeneity, was used for all further enzyme characterization. That the xylanase activity in the Superdex-75 gel filtration was distributed across most of the elution profile is probably due to the expression of a wide spectrum of xylanases by filamentous fungi, as observed with *Aspergillus 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. Despite the low yields, the isolation procedure provided pure xylanase that allowed characterization of the protein and preliminary studies on the amino acid sequence. In addition, the findings of the present study regarding the enrichment of xylanase are in accord with the results of Carmona et al. (1998), including the specific activity in the crude xylanase extract (33.79 U/mg), compared to that of the xylanase preparation from *A. alternata* PTRa9 reported here at 161.1 U/mg.

Determination of enzyme purity and protein pattern on native-polyacrylamide gel electrophoresis (PAGE)

The xylanase containing fractions from each step of the enrichment process was analyzed for purity and protein pattern by native-PAGE with protein and enzyme activity staining (Figure 5A). Whilst the post-DEAE-cellulose xylanase fraction still showed multiple components, the enriched xylanase fraction showed a single protein band on native-PAGE. On the same gel, a rapid visualization of the enzyme activity with 1% (w/v) birch wood xylan and subsequent staining with Congo red, revealed a single enzyme activity band at the same migration distance as the protein band, indicating that the single protein band seen in the enriched xylanase preparation contained the

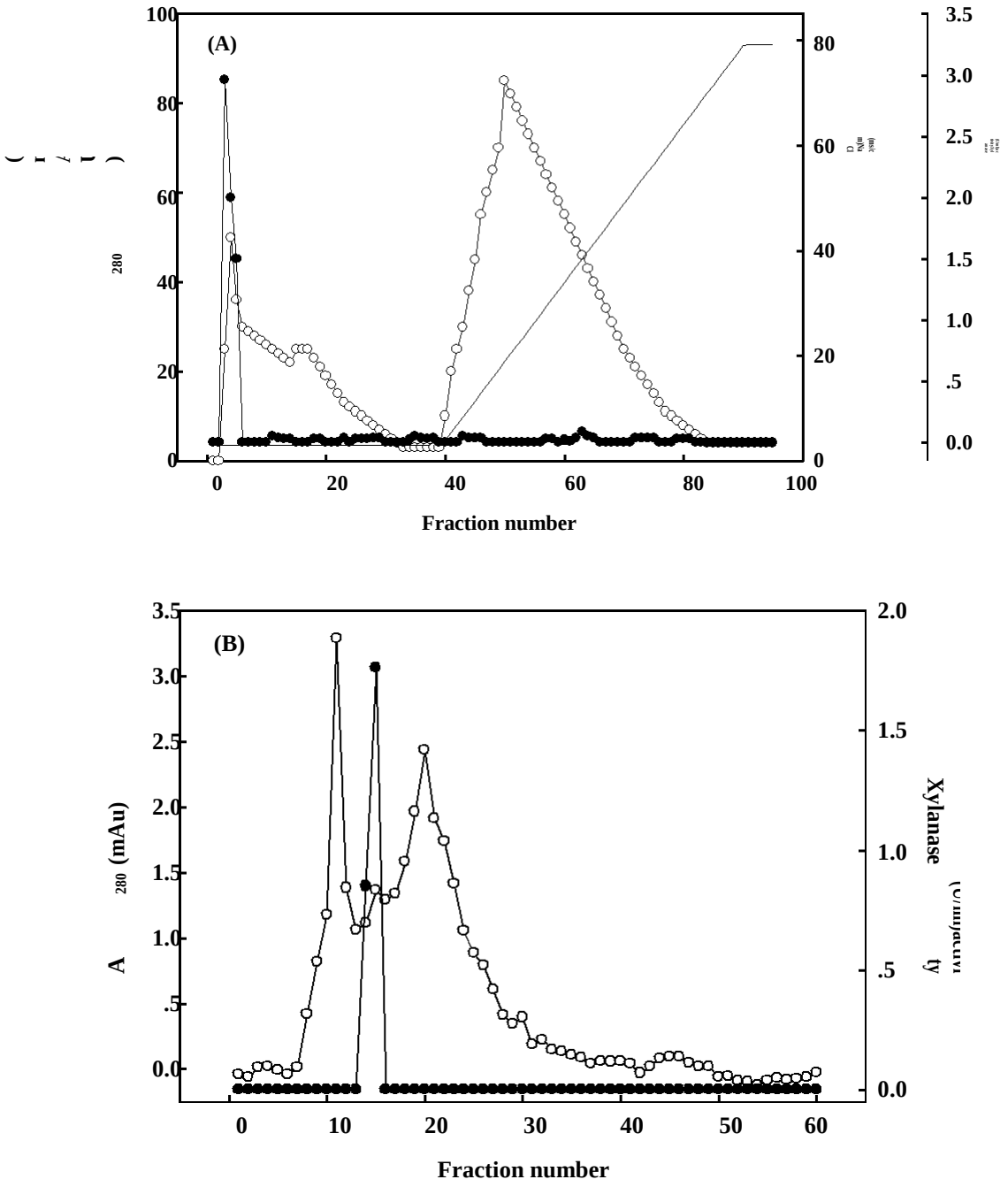

Figure 4. Profile of the enrichment of the *A. alternata* isolate PTRa9 extracellular xylanase extract by (A) DEAE-cellulose ion-exchange chromatography of the ammonium sulfate cut fraction (400 mg protein) eluted in 50 mM Tris-HCl (pH 7.0) with a 0 to 1 M NaCl linear gradient, and (B) Superdex-75 gel chromatography of the post-DEAE-cellulose xylanase fraction (50 mg) eluted in 100 mM NaCl / 50 mM Tris-HCl (pH 7.0). In both panels A and B, (○) absorbance at 280 nm and (●) xylanase activity.

entire enzyme.

Molecular weight determination

Discontinuous reducing SDS-PAGE resolution of the enriched xylanase fraction revealed a single strong band with an apparent molecular weight of 54.8 kDa after Coomassie blue R250 staining (Figure 5B), although up to four very faint smaller bands between 14 and 45 kDa were seen. This supports the enrichment to near homogeneity and suggests that the enriched xylanase

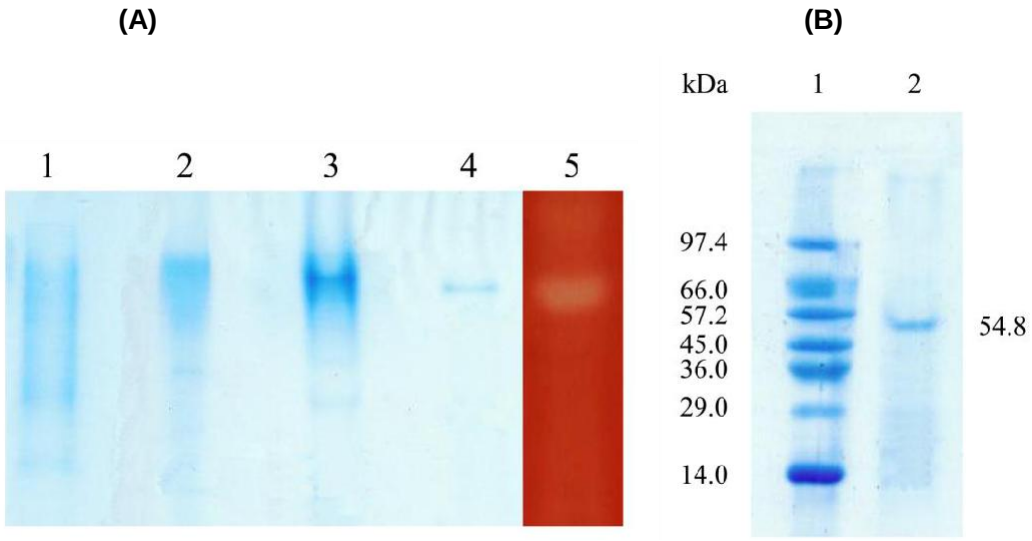

Figure 5. (A) Coomassie blue stained native-PAGE analysis of the *A. alternata* isolate PTRa9 xylanase fractions from each step of the enrichment procedure stained for protein by coomassie blue (Lanes 1 to 4) or for xylanase enzyme activity (Lane 5). Lane 1, crude enzyme (20 μg of protein); Lane 2, ammonium sulfate cut fraction (20 μg of protein); Lane 3, post-DEAE-cellulose xylanase fraction (15 μg of protein); Lanes 4 and 5, enriched xylanase fraction (10 μg of protein). (B) Reducing SDS-PAGE analysis, after coomassie blue staining of the enriched xylanase fraction from *A. alternata* isolate PTRa9: Lane 1, Low molecular weight protein markers (10 mg / lane); Lane 2, enriched xylanase fraction (5 μg of protein).

could be a monomeric protein, or at least if a multimeric one that dissociates into subunits under these enrichment conditions, that this 54.8 kDa subunit has xylanase activity alone.

Effect of temperature on the xylanase activity and thermostability

Figure 6A depicts the effect of different temperatures on the relative activity of the enriched xylanase from *A. alternata* PTRa9. The xylanase activity was relatively unchanged from -20 to 30°C and then slightly increased with an increase in the temperature up to 40°C, its maximum activity before declining sharply at 50°C with no activity at 60°C or higher. This temperature-optima at 40°C is similar to that reported for xylanase obtained from *Aspergillus ficuum* AF-98 or *Trichoderma reesei* at 45°C (Fengxia et al., 2008; Tenkanen et al., 1992). However, slightly higher optimal temperatures have been reported for other xylanases, such as 55°C for the purified xylanase from the fungus *Aspergillus nidulans* KK-99 (Kavita et al., 2002) and *Aspergillus versicolor* (Carmona et al., 1998), and 75°C for the xylanase from *Thermomyces lanuginosus* (Damaso et al., 2000).

The thermal stability of the xylanase from *A. alternata* PTRa9 was determined by maintaining the enzyme at various temperatures ranging from 30 to 60°C for 120 min in 20 mM sodium acetate buffer pH 5.0. The enzyme retained more than 90% of its original activity between 50 to 60°C, whilst at 70°C and above the enzyme retained less than 50% of its original activity (data not shown). The thermostability of the enzyme was also determined by incubating the enzyme for one hour at 30, 40 and 60°C at pH 5.0. The enzyme showed essentially full activity after 120 min at 30 or 40°C with interestingly, a slightly higher activity; after all, pre-incubation time points at 40°C than at 30°C (Figure 6B). At 50°C, the activity decreased after 20 min of pre-incubation but was still at >45% activity after 120 min. In contrast, at 60°C, the enzyme rapidly lost activity falling to 50% and no activity after <5 and 120 min, respectively.

Effect of pH on xylanase activity and stability

The optimum pH for xylanase activity was 5.0 when assayed at room temperature for 60 min, giving a relative xylanase activity of 112.8%, but the activity level was maintained at over 90% across the broad pH range of 3.0 to 11.0 for 60 min, with less than 20 to 40% residual activity at pH 2.0 and 12.0 respectively (Figure 7). The considerable stability at acid-alkaline pH values of this xylanase from *A. alternata* isolate PTRa9 makes it potentially effective for use in industry. These results are in accord with that reported by Kavita et al. (2002) that maximum activity of the xylanase from *Aspergillus nidulans* KK-99 was in the pH range of 4.0 to 9.5.

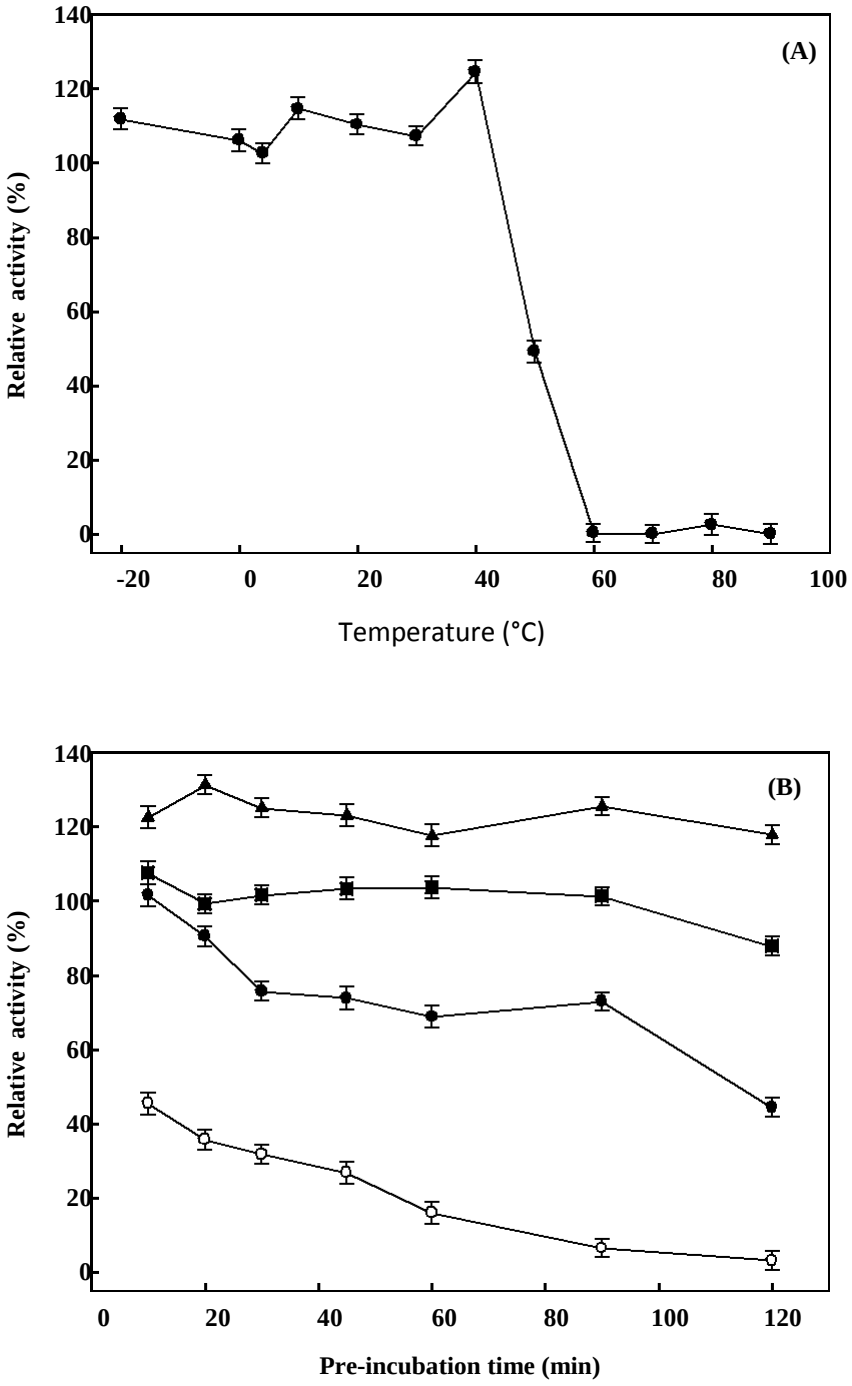

Figure 6. (A) The optimal reaction (enzyme) temperature and (B) thermostability of the enriched xylanase fraction from *A. alternata* isolate PTRa9, assayed in 50 mM Tris-HCl (pH 7.0) at (■) 30°C, (▲) 40°C, (●) 50°C and (○) 60°C. For both panels A and B the data are shown as the mean ± 1 SEM and are derived from three repeats.

However, the xylanase from *Rhizopus stolonifer* showed a maximal activity at the more neutral pH range of 6.0 to 7.0 (Goulart et al., 2005), whilst that from *Trichoderma koningii* showed an optimal activity at pH 5.5 and 60°C (Huang, 1991).

Effect of metals and reagents

Xylanase activity was strongly inhibited by Hg^{2+} in a dose-dependent manner (Table 3). EDTA and Cu^{2+} were also found to be inhibitory causing up to 57 and 80%

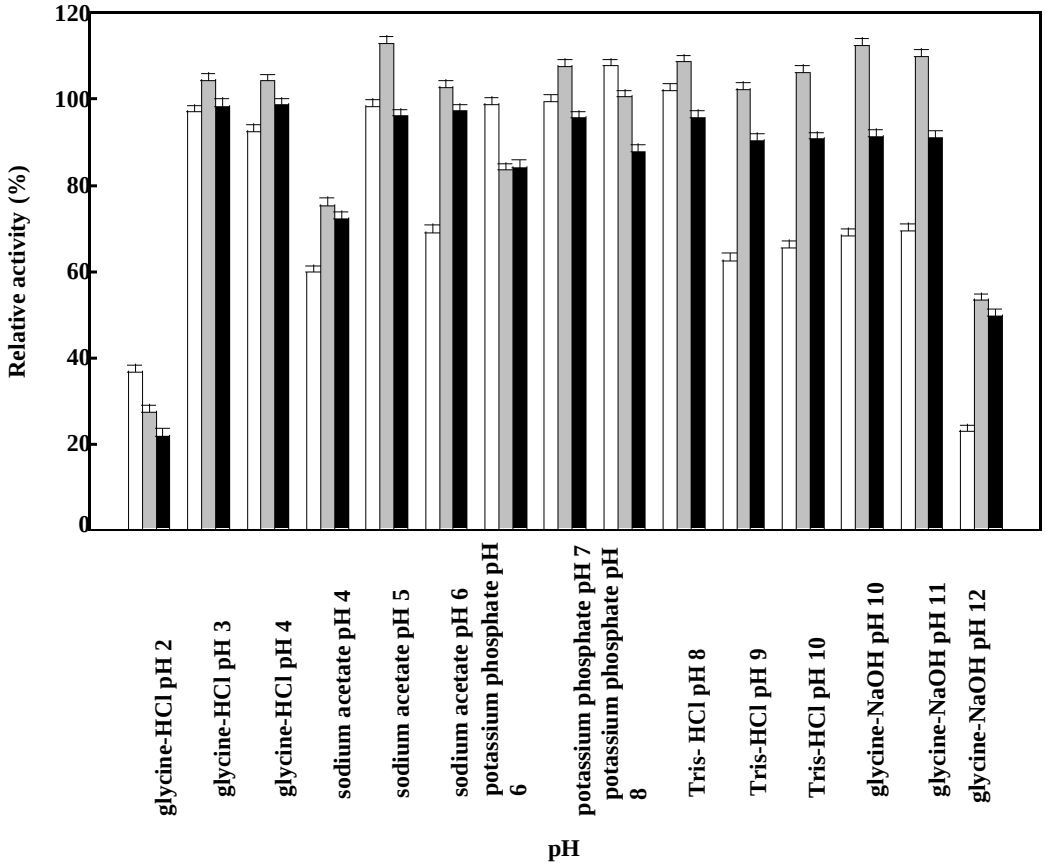

Figure 7. Effect of pH on the activity of the enriched xylanase fraction from *A. alternata* isolate PTRa9. The effect of pH on xylanase activity was evaluated in (all 20 mM) glycine-HCl buffer for pH 2.0 to 4.0, sodium acetate buffer for pH 4.0 to 6.0, potassium phosphate buffer for pH 6.0 to 8.0, Tris-HCl buffer for pH 8.0 to 10.0 and glycine-NaOH buffer for pH 10.0 to 12.0 at various time for (white) 30, (grey) 60 and (dark) for 90 min. The data are shown as the mean ± 1 SEM and are derived from three repeats.

inhibition of the enzyme activity at 10 mM (Table 3). Some other metal ions like Mg^{2+}, Mn^{2+}, Fe^{2+} and Zn^{2+} decreased the enzyme activity in a dose-dependent manner but to a much lower extent, whilst Ca^{2+} caused a low level of inhibition at 1 mM but this was negated at higher ion concentrations to no inhibition at 10 mM. The inhibition of the enzyme activity by Hg^{2+} ions may be due to its interaction with sulphydril groups, suggesting that there is an important cysteine residue in or close to the active site of the enzyme. Hg^{2+} has previously been reported to completely inhibit the activity of xylanase from several different sources (Bataillon et al., 2000; Khandeparker and Bhosle, 2000; Khanna and Gauri, 1993; Qureshy, 2002).

Determination of kinetic parameters

The Lineweaver-Burke plot of the xylanase activity at 30 min and 50°C in 20 mM sodium acetate buffer pH 5.0 with various concentrations of xylan as the substrate is shown in Figure 8. The relationship was linear (R^2 = 0.997), giving estimates of the K_m and V_{max} values of the enzyme as 2.37 mg/ml and 2.14 U/min/mg protein respectively. In spite of the narrow high specific activity of this enzyme towards natural xylan, the K_m of this enzyme is similar to that reported for xylanases from other sources (Camacho and Aguilar, 2003; Araki et al., 1998; Gupta et al., 2000).

Conclusion

Xylanase is one of the most important enzymes used in industry and agriculture and it is produced by many microorganisms, such as bacteria, yeast and fungi. According to the data presented here, the endophytic fungus “PTRa9” is likely to be *A. alternata* and closely related to isolate VC38 with which it shares a 99% sequence identity in the ITS1-5.8S rDNA-ITS2 region. *A. alternata* isolate PTRa9 produces the highest extra-cellular xylanase levels, sufficient for enrichment and

Table 3. The effect of divalent cation salts and the chelating agent EDTA on the xylanase activity of the enriched xylanase fraction from *A. alternata* isolate PTRa9.

Reagent	Relative xylanase activity (%)[a]		
	1 mM	5 mM	10 mM
Control[b]	100.0 ± 0.00	100.0 ± 0.00	100.0 ± 0.00
$MgCl_2$	89.8 ± 0.42	75.5 ± 0.80	64.7 ± 0.15
$MnCl_2$	79.4 ± 0.14	78.7 ± 0.11	73.7 ± 0.53
$CuSO_4$	65.7 ± 0.46	22.8 ± 0.40	19.5 ± 0.14
$CaCl_2$	86.4 ± 0.31	90.7 ± 0.02	100.2 ± 0.02
$ZnSO_4$	94.7 ± 0.04	84.6 ± 0.05	57.9 ± 0.13
$FeCl_2$	66.7 ± 0.25	55.7 ± 0.76	55.0 ± 0.61
$HgCl_2$	50.3 ± 0.06	21.0 ± 0.94	6.78 ± 0.02
EDTA	57.0 ± 0.15	46.6 ± 0.05	43.3 ± 0.09

[a]The relative activity was determined by measuring the xylanase activity at 30 min at 50°C in 20 mM sodium acetate buffer pH 5.0 after pre-incubation at 30°C for 30 min with the indicated reagents and concentrations; [b]using the activity seen in the absence of such reagents in 20 mM sodium acetate buffer pH 5.0 alone as 100%. Results are shown as the average ± 1 SEM from a representative assay performed in triplicate. Means within a column or across a row that are followed by a different lower case letter are significantly different.

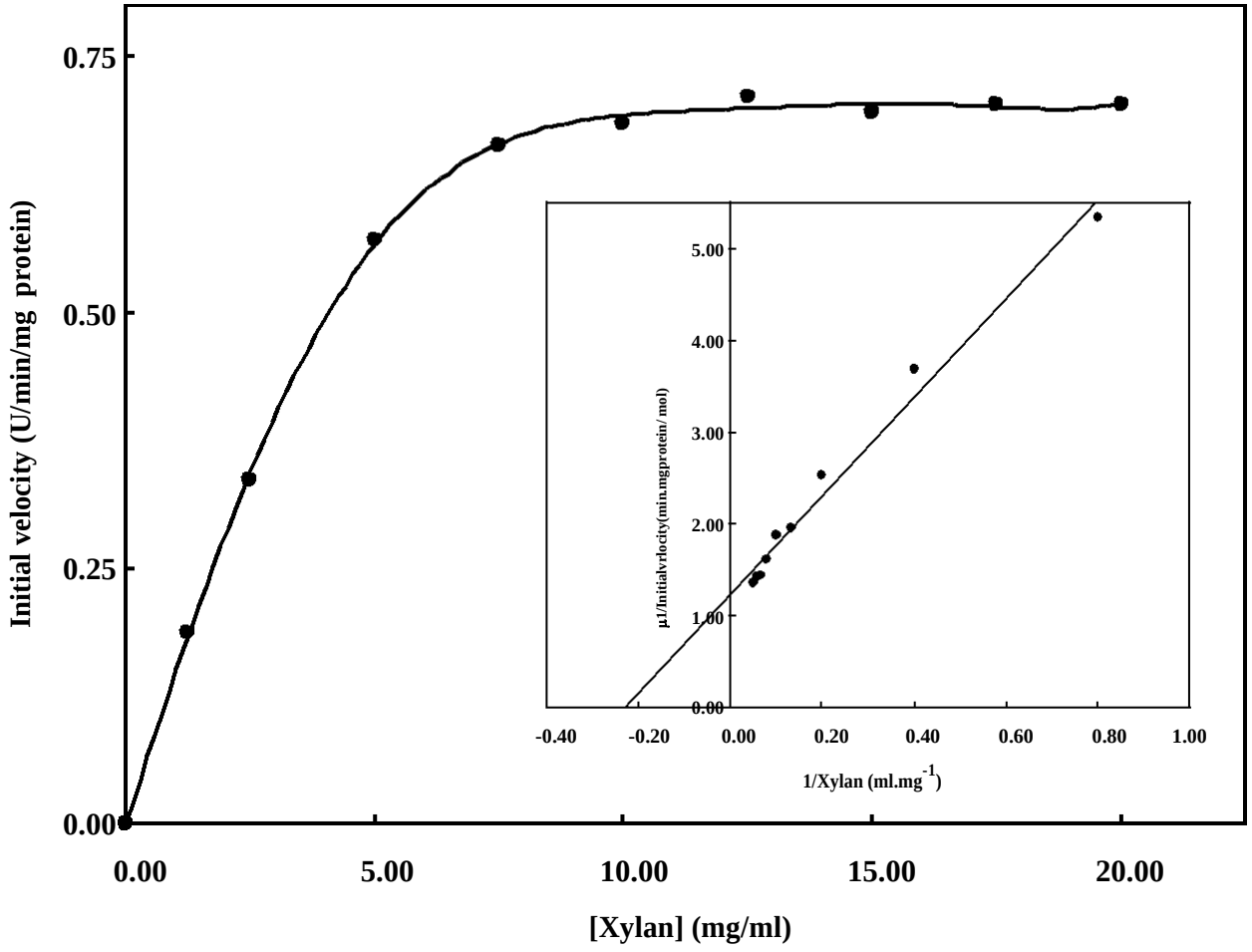

Figure 8. Lineweaver-Burk plot of the enriched xylanase fraction from *A. alternata* isolate PTRa9. The xylanase fraction was incubated with different concentrations of birch wood xylan (1.25 to 20 mg) as substrate. Data are shown as the mean ± 1 SD, and are derived from three repeats, with the best fit linear regression and 95% CI lines shown.

and use, when grown in basal medium for four days with 2% (w/v) rice bran and 0.1% (w/v) $(NH_4)_2SO_4$ as the carbon and nitrogen source respectively. This xylanase was enriched (60.1-fold) to apparent homogeneity by sequential ammonium sulphate saturation, DEAE-cellulose ion exchange and Superdex 75 gel filtration chromatography, but with only a 1.3% final yield. The xylanase appeared to be monomeric with a molecular weight of 54.8 kDa, and showed a broadly similar substrate affinity to other xylanases with a K_m of 2.37 mg/ml. The enzyme was active over a broad pH range of 3.0 to 11.0, and was thermostable up to 40°C. The enzyme was inhibited to some extent by all tested divalent metal cations, but especially by Hg^{2+} and Cu^{2+}.

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