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Purification and Characterization of a Low Molecular Weight Endo-xylanase from Mushroom *Termitomyces clypeatus*

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Abstract A low molecular weight endo-xylanase (EC 3.2.1.8) was purified from an edible mushroom *Termitomyces clypeatus* grown in submerged medium with oat spelt xylan. Xylanase was purified to apparent homogeneity by ammonium sulfate fractionation and gel filtration chromatography. Its molecular weight was determined by gel filtration chromatography and sodium dodecyl sulfate-polyacrylamide gel electrophoresis to be 12 kDa. The enzyme was found to be most active at 50°C and pH 5.0, being most stable at pH 6.5. The K_m for oat spelt xylan was determined to be 10.4 mg/ml. The specificities of the enzyme was observed to be highly specific towards oat spelt xylan and was inhibited by mercuric chloride ($HgCl_2$), N-bromosuccinimide, and trans-1,2-diaminocyclohexane-*N*′,*N*′,*N*′,*N*′-tetraacetic acid strongly. The inhibitory action of N-bromosuccinimide on enzyme confirmed the presence of one tryptophan residue in its substrate-binding site. Amino acid analysis for xylanase showed the presence of high amount of hydrophobic serine, glycine, threonine, and alanine residues. The N-terminal sequencing study for the previously purified and characterized 56 kDa xylanolytic amyloglucosidase reveal the presence of 33.30% identity with glucoamylase chain A from *Aspergillus awamori*. The N-terminal sequence analysis of the present 12 kDa enzyme showed highest similarity (72.22% identity) towards xylanase from *Neurospora crassa*.

Keywords Endo-xylanase · *Termitomyces clypeatus* · N-terminal sequencing

Introduction

Xylan, the most abundant of the hemicelluloses in terrestrial plant cell walls, has a linear β-1,4-linked xylopyranosidic back bone with side chain glucose, arabinose, glucuronic acids, and arabinoglucuronic acid residues on its D-xylose backbone [1]. Larch wood xylan also contains similar backbone of β-1,4-linked D-xylopyranosyl residues with every fifth or sixth xylose residue substituted at C_2 with 4-O-methyl-D-glucuronic acid and at C_3 with arabinofuranosyl

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units, where none of the xylosyl residue have more than one branch [1, 2]. The presence of glucose in xylan has already been reported [3–5]. Commercially available oat spelt xylan upon enzymatic hydrolysis liberates glucose molecule in varied amount, which is also known to contain glucose as per the manufacturer's technical specification (Sigma Chemicals Co., USA). Based on hydrophobic cluster analysis and amino acid sequence homologies, endo-xylanase has been classified into two families, mainly family 10 and 11 [6, 7]. Xylanase production by many micro-organisms is observed to secrete extracellularly with inducible substrates. It is also produced in significant amounts by bacteria and filamentous fungi to degrade xylan as a major source of energy [1, 8]. In recent years, microbial enzymes depolymerizing these polysaccharides got remarkable application in the market values mainly in food industry as animal feed [9] and application in paper and pulp industries [10, 11], baking industry [12], and degumming of plant fibers like flax, hemp, jute, etc. [13].

It was reported that the mushroom *Termitomyces clypeatus* produces one xylanase when the carbon source was dextrin [14]. The enzyme was found to liberate glucose from xylan which was later identified as a 56 kDa xylanolytic amyloglucosidase [14, 15].

The present paper reports purification and characterization of a low molecular weight endo-xylanase from *T. clypeatus*.

Materials and Methods

Materials

Xylan from oat spelt, dinitrosalicylic (DNS) acid, 2-mercaptoethanol, Bradford reagent, polyvinylidene difluoride (PVDF) membrane, *N*-cyclohexyl-3-aminopropanesulfonic acid (CAPS) buffer, and Ponceau S solution were all purchased from Sigma, USA. N-bromosuccinimide (NBS), neuraminidase inhibitor (NAI), fluoro-2,4-dinitrobenzene (FDNB), 3′,5′-dithiobis (2′-nitrobenzoic acid) (DTNB), *p*-chloro mercuric benzoic acid (PCMB), trans-1,2-diaminocyclohexane-*N*′,*N*′,*N*′,*N*′-tetraacetic acid (CDTA), 2-hydroxy-5-nitrobenzyl bromide (HNBB), guanidine-HCl, and carbodimide were products of Aldrich, USA. Polyacrylamide gel electrophoresis reagents were obtained from BIORAD, USA. Amino acid standards and reagents for N-terminal sequencing were obtained from Applied Biosystems, USA. Molecular weight markers were procured from Pharmacia, Sweden. All the other chemicals and medium components used were of analytical grade or better.

Methods

Organism and Culture Condition

The basidiomycetes, mushroom, was used for enzyme production. Maintenance and growth of the mycelial culture of *T. clypeatus* were carried out by the ways described previously [16]. The synthetic medium (0.5 l) contained 1% (*w*/*v*) oat spelt xylan with minerals percentage (*w*/*v*), i.e., $ZnSO_4 \cdot 7\ H_2O$, 0.03; $MgSO_4 \cdot 7\ H_2O$, 0.05; H_3BO_3, 0.05; $MnCl_2 \cdot 4\ H_2O$, 0.0036; $NaMoO_4 \cdot 4\ H_2O$, 0.0032; $CaCl_2 \cdot 2\ H_2O$, 0.037; and sodium monobasic ($NaH_2PO_4 \cdot H_2O$), 0.15; yeast extract, 0.2 as a complex nutrient supply and nitrogen source; and ammonium sulfate $(NH_4)_2SO_4$, 2.0; maintained at pH 5.0. Mycelial growth of *T. clypeatus* was made in complex medium (glucose, 1.0%; malt extract, 1.0%; KH_2PO_4, 0.015%; and potato extract, 10%) and grown for 3 days. Enzyme production was initiated in synthetic medium (stated above) by 10% (*v*/*v*) inoculums and allowed to grow for 5 days at 30°C [16].

Fermentative Production of Xylanase

For the production of endo-xylanase enzyme, three different concentrations of oat spelt xylan (0.5%, 1.0%, and 2.0%) and yeast extract (1.0%, 0.5%, and 0.2%) were used as medium composition as given in Table 1.

Purification of Enzyme

Ammonium Sulfate Fractionation

The fermented broth was filtered through Whatman No.1A and used as a crude enzyme solution. The entire process of enzyme purification was carried out at 10±5°C, unless specifically mentioned. Initially, culture filtrate was brought to 30% saturation with addition of solid ammonium sulfate. The culture filtrate (0–30% saturation) was kept overnight and then centrifuged at 100,000×g for 1 h. The pellet containing very low activity was discarded, and the supernatant was again subjected to 30–50% saturation with solid ammonium sulfate followed by overnight precipitation and ultra centrifugation. The subsequent 50–70% and 70–90% ammonium sulfate precipitation was carried out as described above accordingly. Maximum xylanase activity was precipitated in 50–90% and used for further processing in the next step. The pellet obtained in each fraction was then resuspended in minimum volume of 0.05 M sodium phosphate buffer, pH 6.5 (buffer A), and assayed for xylanase activity and its protein content. The fractions with higher enzyme activity were selected and taken for the next steps of purification by high-performance gel permeation liquid chromatography (HPGPLC).

HPGPLC on BioSuite 125

The enzyme solution obtained from the previous step, precipitated in 50–90% ammonium sulfate, dissolved in 1.2 ml buffer A, and injected 0.15 ml into high-performance liquid chromatography (HPLC) column (BioSuite 125, 7.5×300 mm, 13.25 ml, Waters, USA), was eluted with buffer A containing 0.2 M NaCl. The HPGPLC consisted of Waters HPLC module, 2489 UV/Vis detector, 515 pump, and Millennium software for data analysis. The flow rate and total run time was 0.5 ml/min and 30 min, respectively. Protein peaks were identified as

Table 1 Xylanase activity as a function of substrate concentration.

Substrate with nutrient supply and minerals		Xylanase production (U/ml) fourth day	Xylanase production (U/ml) fifth day	Xylanase production (U/ml) sixth day
Xylan (0.5%)	a	3.588±0.87	2.883±0.06	2.997±0.19
	b	8.383±0.13	8.427±0.09	8.327±0.25
	c	18.052±0.49	17.343±0.21	19.09±0.13
Xylan (1%)	a	3.951±0.21	4.350±0.22	10.210±0.21
	b	9.012±0.46	18.746±0.21	18.435±0.13
	c	27.278±0.23	33.952±0.06	34.646±0.23
Xylan (2%)	a	3.705±0.35	5.818±0.40	8.995±0.21
	b	10.225±0.33	18.762±0.32	20.199±0.22
	c	26.097±0.40	32.778±0.40	30.643±0.24

Production distribution of xylanase with different concentrations of oat spelt xylan containing yeast extracts, (*a*) 1.0%, (*b*) 0.5%, and (*c*) 0.2%, respectively. Results represented are two sets of triplicate. ±Standard error is less than 5%.

absorbance at 280 nm, xylanase activities of peaks were measured, and active peaks were collected. The two active peaks were further analyzed by rechromatography on same column.

Enzyme Assay

Xylanase activity was determined by DNS acid reagent (Bailey et al. [17]) as modified by Sengupta et al. [18]. Reaction mixture (2 ml) containing enzyme solution and 1% (*w*/*v*) oat spelt xylan in 0.1 M sodium acetate buffer, pH 5.0, was incubated at 50°C for 10 min. The reducing sugar released was determined by DNS reagent. Activity was expressed in terms of micromoles of xylose equivalent liberated per minute under the assay condition.

Starch hydrolyzing activity of the enzyme was determined by Somogyi–Nelson method [19, 20]. Briefly, the reaction mixture (0.4 ml) contained enzyme solution and 0.5% (*w*/*v*) starch or related substrate in 0.1 M sodium acetate buffer, pH 5.0, incubated at 50°C for 10 min. Liberation of free-reducing sugar was estimated by Nelson's reagent considered as glucose equivalent measured at 500 nm, A_{500}.

Enzymatic formation of glucose was estimated according to the method of glucose oxidase and peroxidase at absorbance of 505 nm, A_{505} [21]. The incubation mixture (0.08 ml) containing enzyme solution and 0.05% respective substrate (trehalose/maltose) was incubated at 50°C for 10 min, and liberation of free glucose was measured.

Desalting on PD 10 Column

The enzyme solution was desalted whenever necessary by passing through PD 10 column following methodology provided by supplier (GE Healthcare, USA).

Determination of Protein Concentration

Amount of protein concentration was measured by the method of Lowry et al. [22] with bovine serum albumin (Sigma) as the standard and during HPGPLC elution as absorbance at 280 nm.

Polyacrylamide Gel Electrophoresis

Native PAGE

Purified protein sample from BioSuite 125 (step 4) was subjected to native gel electrophoresis. Native polyacrylamide gel electrophoresis was carried out at pH 8.5 on a 7.5% resolving gel preparation [23] using Mighty Small electrophoresis unit, Hoefer, USA. The movement of the proteins entirely depends on the net average negative charge exhibited in the presence of glycine (Tris-glycine buffer, pH 8.5). The single protein band was visualized on staining with Coomassie Brilliant Blue R-250.

SDS-PAGE

Denaturizing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of BioSuite 125 purified protein sample (Step 4) was carried out at pH 8.5 on an 18% resolving gel (8×8×1.5 mm slab) preparation using same Mighty Small electrophoresis unit, Hoefer, USA using discontinuous Laemmli buffer system as per GE Healthcare technical bulletin, code RPN 755 [24]. Denatured protein bands were resolved by a constant

supply of current (20 mA/slab) according to the technical bulletin provided by the manufacturer (Hoefer, USA). 2-mercaptoethanol (5%) and ethylenediaminetetraacetic acid (EDTA; 1 mM) was added to the sample buffer as a solubilizing agent. Low molecular weight markers obtained from GE Healthcare (code RPN 755), USA, were loaded and run in parallel lanes, and gels were stained with Coomassie Brilliant Blue R250 (Pierce, USA) by overnight shaking at 37°C.

Molar Mass Estimation by HPGPLC

The eluted active peaks (step 4) from the same BioSuite 125 column were further injected into the same column for molecular weight determination. The molecular weight of xylanase was derived from a plot of log (standard molar mass) versus K_{av} values of standard gel filtration marker proteins (Ribonuclease A, 13.7 kDa; chymotrypsinogen A, 25 kDa; ovalbumin, 43 kDa; and albumin, 67 kDa) according to the supplied technical bulletin by the manufacturer (GE Healthcare, USA). Standard proteins and xylanase were run in a BioSuite 125 HPLC column separately as described in "HPGPLC on BioSuite 125".

Effect of Different Chemical Modifiers

A wide variety of chemical modulators was used for the study of xylanase modification. The purified enzyme solution (7.155×10^{-2} µg in 25 µl) was incubated with different chemical inhibitors (25 µl), concentration (listed in Table 4) at 30±1°C for 15 min. The enzymatic reaction was initiated by the addition of the above-stated enzyme and inhibitor mixture (50 µl) to pre-equilibrated reaction mixture (1.95 ml) containing oat spelt xylan 1% (*w*/*v*) in 0.1 M sodium acetate buffer, pH 5.0, kept and maintained at 50°C. The reaction process was allowed to continue for 10 min at 50°C and terminated by subsequent addition of 3 ml of DNS acid solution followed by boiling for 10 min. The residual xylanase activity was determined as per the reducing sugar released by DNS acid reagent [18].

Titration of Xylanase with NBS

Modification of enzyme by NBS was carried out by incubation of xylanase 50 µl (1.8166×10^{-3} µM) in 0.1 M acetate buffer, pH 5.0, with varying concentration (0.6, 0.8, 1.0, and 1.2 µM) of NBS for different time periods (10, 20, 30, and 40 min). The termination of chemical modification as well as initiation of enzyme reaction was made by addition of 1% oat spelt xylan solution, and determination of residual xylanase activity was measured. The pseudo-first order rate constant (k) were obtained from the slopes of the best-fit plots of logarithm of the residual xylanase activity against time of reaction. The number of tryptophan residues modified for 100% inactivation of enzyme activity was calculated from the slopes of the plots of logarithm of pseudo-first order rate constant (k) against logarithm of concentrations of the inhibitor [15, 25].

Amino Acid Analysis

Amino acid analysis was performed in a PICO TAG system according to the operation manual (Waters, USA). Amino acid present in unknown sample was determined quantitatively by comparing the peak areas of amino acids present in standard H. The numbers of residues were determined on the basis of molecular weight of 12,000 Da as determined for xylanolytic amyloglucosidase [15].

Tryptophan content was determined spectrophotometrically according to Edelhoch [26]. Enzyme solution (protein content 0.2 mg/ml) was prepared in 0.02 M sodium phosphate buffer, pH 6.5, containing 6 M guanidine hydrochloride; and molar extinction coefficients at 280 (EM_{280}) and 288 nm (EM_{288}) were determined [15].

N-terminal Sequencing

The active fractions retention time (RT) 13.58 min (xylanolytic amyloglucosidase) and RT 17.77 min (endo-xylanase) purified through BioSuite 125 column was collected, desalted by passing through PD10 disposable column (GE Healthcare, USA), and reduced its volume by lyophilization separately. Later, 20 μg of enzyme protein was loaded for SDS-PAGE analysis [24]. The protein resolved in the gel was then transferred to a PVDF membrane in the presence of 10 mM CAPS buffer, pH 11.0, with constant current of 400 mA for 5–6 h at 8–10°C in Mighty Small Transphore unit (Hoefer, USA) as per the manufacturer's instruction. The membrane was removed from the trans-blotting sandwich, and transferred protein bands on PVDF were stained and identified with Ponceau S solution. The identified protein bands of interest were excised for N-terminal sequencing studies. The desired bands were analyzed by N-terminal sequencing using Model 491 Procise protein/peptide sequencer from Applied Biosystems, USA.

Results and Discussion

Production of Xylanase

The optimum xylanase production was achieved in the presence of oat spelt xylan (1%) as an inducible carbon source for 5 days where enzyme activity was found to be 34 U/ml (Table 1). The production was comparable with those of other strains as 0.58 U/ml by *Aspergillus niger* [27], 22.2 U/ml by *Aspergillus carneus* [28], and 35 U/ml by *Aspergillus terreus* [29], utilizing oat spelt xylan as a carbon source. However, 16.55 U/ml of xylanase was obtained by *Penicillium citrinum* in wheat bran [30] and 56.0 U/ml by *Sporotrichum thermophile* in corn cob medium [31]. Mycelial culture of the mushroom *Pleurotus ostreatus* also produces xylanase at 24.98 U/ml in medium containing both corn cob and wheat bran [32].

It has been presented in Table 1 that low enzyme production was obtained when 0.5% (*w*/*v*) xylan was supplied, irrespective of addition of various concentration of yeast extract. The maximum xylanase production has been observed on the fifth day with 1% (*w*/*v*) xylan containing 0.2% (*w*/*v*) yeast extract. Previously, it has been found that in absence of yeast extract, xylanase production on fourth, fifth, and sixth day was 14.04, 23.02, and 20.22 U/ml, respectively (data not shown); and the cell biomass produced in supplement with yeast extract was approximately doubled, when compared with in absence of the same (data not shown). The effect of nitrogen sources on mushroom enzymatic activity was variable, depending up on the supplied nutrient altogether.

Purification of Xylanase

Ammonium Sulfate Fractionation

The crude enzyme, (culture filtrate) with 30.27 U/ml of xylanase activity, was subjected for ammonium sulfate fractionation (Table 2). During the process of ammonium sulfate

Table 2 Purification of xylanase activity.

Steps	Total volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Recovery (%)	Fold purification
Culture filtrate (CF)	710	89.60	21,495.03	239.90	100	1
Ammonium sulfate (50–90%)	6.66	13.57	14,983.36	1,104.23	69.70	4.60
BioSuite125; RT 17.94 min, RT 16.5–20 min (1.75 ml×8)	14	7.33	9,045.75	1,234.07	42.08	5.14
BioSuite125; RT 17.77 min, RT 17–19 min (1.0 ml×8)	8	1.10	7,608.42	6,916.74	35.39	28.83

Purification and assay protocol was described in "Materials and Methods".

fractionation, it has been found to yield enzyme activity of 69 U/ml, 2.43% of the total enzyme activity in 0–30% saturation. With increasing ammonium sulfate concentration to 50% saturation, only 1.58% of the total activity was precipitated with 141 U/ml. The amount of protein precipitated was estimated to be 1.03 (0–30%) and 0.32 mg/ml (30–50%), respectively. The purification was further proceeded with 50–70% ammonium sulfate fractionation, where maximum of 10.07 mg of protein was salted out with 2,560 U/ml xylanase activities showing 3.67-fold purification with 41.33% recovery. Similarly, in 70–90% fractionation, the protein precipitated was 3.49 mg, where the xylanase activity was 1,909 U/ml. The protein recovered during 70–90% fraction was 28.37% with 7.28-fold purification.

However, considering the specific activity of xylanase, it may be considered that 50–90% ammonium sulfate fractionation results best in terms of percentage recovery, and it is represented in Table 2 where the total precipitated protein was found to be 13.57 mg with 4.6-fold purification, and the total enzyme activity was found to be 14,983.36 U, which is about 69.70% of the total enzyme recovery. The protein precipitated during 50–70% fractionation was three times more than the protein obtained at 70–90% fractionation, where the fold purification was found to increase from 3.67% to 7.28%, respectively. The maximum specific activities obtained during purification were 881.64 and 1,746.51 U/mg at 50–70% and 70–90% fractionation, respectively. So, in the future, purification step 50–90% ammonium sulfate fractionation has been made (Table 2, step 2).

HPGPLC on BioSuite 125

During HPGPLC of step 2 (Table 2) enzyme on BioSuite 125 column, xylanase activities were found in two fractions eluted at RT 13.95 and 17.94 min, respectively (step 3; Fig. 1a). The RT 13.95 min peak was further rechromatographed and eluted at RT 13.58 min, showing both xylanolytic and amylolytic activity which was identified earlier as xylanolytic amyloglucosidase [14, 15]. The RT 17.94 min enzyme gave 646.12 U/ml of xylanase activity with 7.33 mg of total protein, where the fold purification and recovery obtained were 5.14 and 42.08, respectively. The enzyme peak of RT 17.94 min on rechromatography was eluted at RT 17.77 min (step 4). The step 4 enzyme has xylanase activity of 951.05 U/ml and seen to achieve a total of 28.83-fold purification with 35.39% recovery (Table 2, Fig. 1b). Although, the protein recovery in step 4 enzyme solutions was found to be very low (1.10 mg), the purification was significantly high with further 5.5-folds.

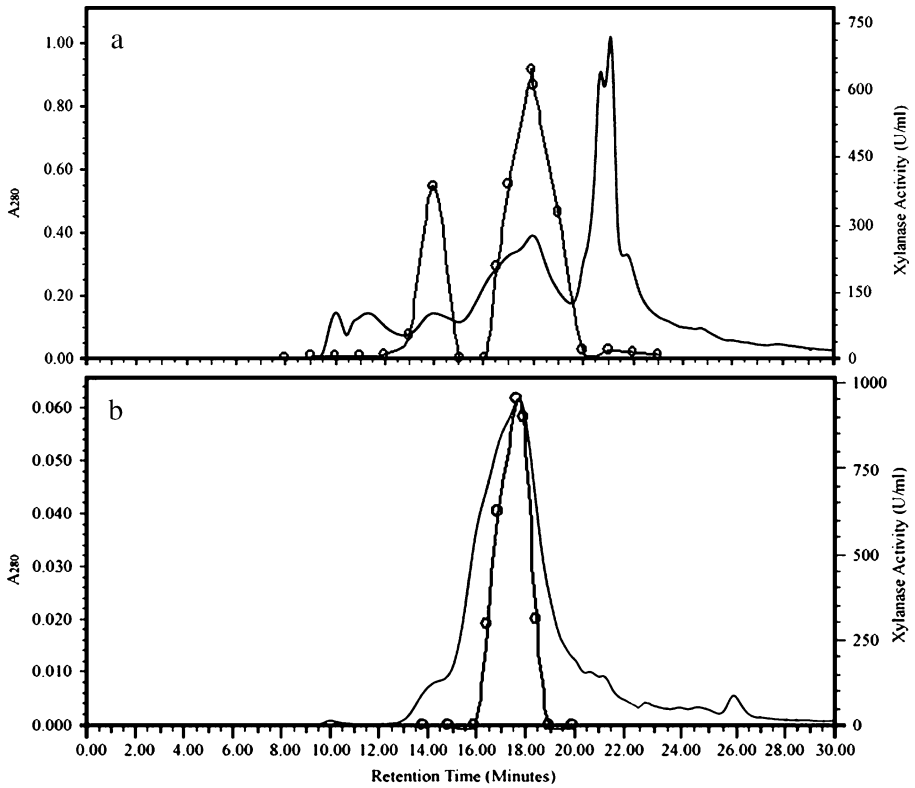

Fig. 1 Elution profile of xylanase activity during high-performance gel permeation liquid chromatography. (**a**) Enzyme solution, *step 2*, was eluted from BioSuite 125 column, and activity were measured according to xylanase assay protocol stated in "Materials and Methods" section, and (**b**) *step 3* enzymes was rechromatographed on the same column, and xylanase activity was measured. Eluted protein as absorbance measured at 280 nm (—) and xylanase activity as U/ml (–○–) are shown in secondary Y axis

Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis was carried out with step 4 purified protein samples. Initially, native gel (7.5%) electrophoresis was run in presence of Tris-glycine buffer pH 8.5, and homogeneity was checked by resolving to a prominent single band (data not shown). The purified enzyme sample when run on SDS-PAGE (18%), also resolved into a single band. The band was relatively diffused as usually obtained for low molecular weight protein shown in the technical bulletin supplied by GE Healthcare (code RPN 755). It was calibrated with corresponding low molecular weight marker by estimating the relative mobility (R_f) value; the M_r was approximated to be 12 kDa (Fig. 2).

The low molecular weight xylanase purified from various strains reported were *Trichoderma viride* (22 kDa), with specific activity 152.6 U/mg and 12.5-fold purification [33]; *Trichoderma koningii* xylanase, with 6,089.2 U/mg protein and 11.1-fold purification [34]; and *Aspergillus fumigatus* Fresenius xylanase (19 and 8.5 kDa), with 1.32-fold purification having specific activity of 7.10 U/mg protein [35]. Recently, the xylanase from *Streptomyces cyaneus* was purified to 2.25-fold with specific activity 893.56 U/mg proteins [36]. A low molecular weight xylanase (13 kDa) from *Aspergillus fischeri* was purified to 29-fold with specific activity of 1,455 U/mg protein [37].

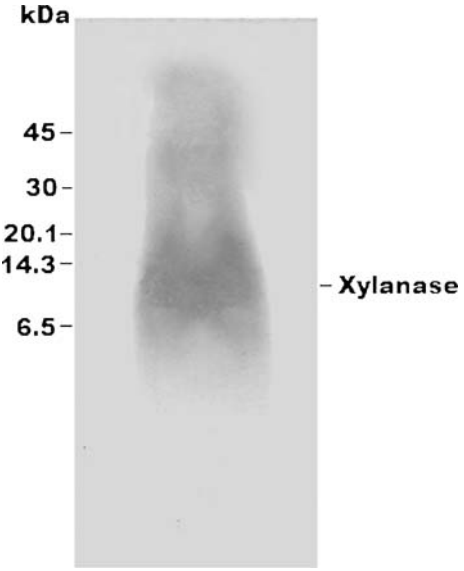

Fig. 2 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of xylanase preparation. The polyacrylamide gel electrophoresis of *step 4* enzyme was carried out on 18% gel preparation described in "Materials and Methods" section. In this figure, protein band marked as xylanase, compared with standard markers. Aprotinin, 6.5 kDa; lysozyme, 14.3 kDa; trypsin inhibitor; 20.1 kDa, carbonic anhydrase, 30 kDa; and ovalbumin, 45 kDa; Amersham Bioscience, UK, product code RPN 755. Molar mass was estimated by SDS-PAGE from a plot of R_f (relative migration values) versus log (molar mass) of standard low molecular weight markers

Biochemical Characterization of Xylanase

K_m and V_{max} of Xylanase

The purified enzyme showed a K_m of 10.4 mg/ml with oat spelt xylan (2–28 mg/ml) and V_{max} value 6.91×10^3 μmol min^{-1}/mg of protein when calculated from Lineweaver–Burk plot (data not shown).

Similar K_m and V_{max} for low molecular weight xylanase had already been reported by other research groups with K_m of 10 mg/ml for a protein of molecular weight 20.4 kDa from thermotolerant *Streptomyces* sp. using larch wood xylan [38] and K_m of 11.1 mg/ml for a protein of molecular weight 20.5 kDa monomeric xylanase from *S. cyaneus* using birch wood xylan as a substrate [36].

Determination of Molecular Mass

The molecular mass of the protein was resolved to be 12 kDa using low-range rainbow molecular weight markers and approximately 12 kDa by gel filtration chromatography, when eluted and calibrated against standard Gel filtration markers (GE Healthcare) through BioSuite 125 column. Molecular weight of endo-xylanase was determined by gel filtration chromatography, eluted at RT 17.77 min (step 4 enzyme) during HPLC (Fig. 1b) and found to be 12 kDa from calibration curve with standard proteins (data not shown). Later, it was verified from SDS-PAGE and found to be 12 kDa (Fig. 2). Low molecular weight xylanase were reported for *A. fumigatus* Fresenius, 8.5 and 19 kDa [35]; *A. niger*, 13 kDa [27]; and

A. fischeri, 13 kDa [37], and 17 kDa was found in *Acrophilophora nainiana*, a hot water fungal isolates from Brazil [39].

Activity of Purified Xylanase towards Different Substrates

The enzyme was found to hydrolyze various substrates as enlisted in Table 3. The maximum level of hydrolysis was observed for oat spelt xylan (0.5%, *w*/*v*). The least hydrolysis seen by the enzyme was with guar gum. The enzyme had showed almost no activity towards CM-cellulose, α-glucan, trehalose, maltose, and *O*-nitrophenyl-β-D-xyloside. Substrate specificity of the enzyme appeared to undergo hydrolysis of starch (3.17%) compared to oat spelt xylan (100%; Table 3). Beside oat spelt xylan, other substrates like arabinoxylan and arabinogalactan have been hydrolyzed by the present enzyme with 17.30% and 4.18%, respectively, whereas the enzyme showed least specificity towards amylopectin (1.67%) and guar gum (0.33%). Previously, the 56 kDa xylanolytic amyloglucosidase had been observed to liberate maximum glucose from starch, where the enzyme was less specific towards oat spelt xylan (36.98%) [14]. Thus, the present 12 kDa enzyme may be considered as an endo-xylanase which could cleave the main backbone of xylan consisting 1,4-β-xylopyranoside residue.

Effect of pH and Temperature on Activity and Stability of Xylanase

The purified enzyme of *T. clypeatus* was found most stable at pH 6.5 with 94% residual xylanase activity when kept at room temperature (30±1°C) in different buffer (pH 3.0 to pH 10.0) systems. The pH optima of xylanase was studied in the presence of 1% oat spelt xylan with different buffer (pH 3.0 to pH 10.0) systems, and maximum percentage activity of xylanase was obtained at pH 5.0. On the course of temperature optima determination, the maximum percentage of residual xylanase activity was measured at 50°C (data not shown).

Studies on the physiochemical properties of endo-xylanase reveals that the enzyme has a pH optimum of 5.0, stability of 6.5, and temperature optimum at 50°C where the pure

Table 3 Activity of purified xylanase towards different substrates.

Substrate	Concentration (% *w*/*v*)	Percentage enzyme activity (U/ml)
Xylan (oat spelt)	0.5	100.00
Arabino xylan	0.5	17.301
Arabino galactan	0.5	4.187
Starch	0.5	3.173
Guar gum	0.5	0.333
Amylopectin	0.5	1.676
1-*O*-methyl-β-D-glucopyranoside	0.5	2.178
1-*O*-methyl-β-D-xylopyranoside	0.5	1.608
1-*O*-methyl-β-L-arabinopyranoside	0.5	1.608
p-nitrophenyl-β-D-glucoside (PNPG)[a]	0.12	1.999

The enzyme gave maximum activity with oat spelt xylan when 29.90 μg of protein was used for enzyme assay, incubated for 10 min according to methods described in "Material and Methods", which is considered as 100%. Activities with other substrates are represented as relative percentage.

Substrate like CM-cellulose, α-glucan, trehalose, maltose, and *O*-nitrophenyl-β-D-xyloside[a] did not show any enzymatic activity.

[a] Liberated nitro phenols have been measured spectrophotometrically at absorbance OD 400, A_{400}.

enzyme was stable up to 55°C. The crude enzyme on different buffer system (pH 3.0–10) when placed at room temperature was found most stable at pH 7.0, similar to other enzymes [39] except for some thermophilic fungal xylanases [40].

Effect of Chemical Modification on Xylanase Activity

The effect of different chemical modifying agents on pure enzyme was studied (Table 4). The enzyme was inhibited strongly by Hg^{2+}, Ag^{2+}, and Fe^{2+}; whereas $CuSO_4$, NaN_3, $MgSO_4$, and iodoacetic acid did not show any inhibitory effect.

Among the chemical modifying agents, Hg^{2+}, Ag^{2+}, and Fe^{3+} were found the most potent inhibitors which are similar to the enzyme reported for *T. koningii* [34] and *A. terreus* [41], while $CuSO_4$, NaN_3, $MgSO_4$, and iodoacetic acid were found with no inhibitory activity much similar for the enzyme reported by *Penicililum citrinum* [30] and *Trichoderma harzinum* [42]. The purified enzyme when treated with NAI (5 mM) and PCMB (1 mM) showed 15% of the residual activity each, suggesting the presence of hydrophobic tyrosine and cysteine residues, respectively, which were usually required for maintenance of its normal active structure. The presence of aromatic tyrosine residue was again confirmed by FDNB (20 mM), as it alters net stability of enzyme by charge

Table 4 Effect of inhibitors on xylanase.

Inhibitors	Concentration (mM)	Residual xylanase activity (%)
CDTA	0.05	81.56
	0.50	45.56
NBS	0.05	85.10
	0.50	2.90
NAI	05.0	15.5
	50.0	3.0
$HgCl_2$	01.0	60.28
	02.0	29.06
$AgNO_3$	0.20	62.20
	01.0	23.50
EDTA	02.0	76.30
	20.0	52.76
$FeSO_4$	0.20	70.7
	02.0	40.5
Carbodimide	10.0	63.5
HNBB	10.0	40.6
EGTA	20.0	57.0
PCMB	01.0	15.0
	10.0	2.0
FDNB	02.0	37.3
	20.0	15.5

Effect of listed inhibitors on xylanase activity was tested at indicated concentration. Briefly, 7.155×10^{-2} μg of enzyme protein were incubated in presence of respective inhibitors at 30°C for 15 min, and enzymatic reaction were started with the addition of oat spelt xylan taken in assay buffer in a final of volume 2.0 ml and assayed according to assay method given in "Materials and Methods". $CuSO_4$, NaN_3, $MgSO_4$, and Iodoacetic acids caused no inhibition. Data expressed are the average of best duplicate values.

displacement (nucleophiles) leading to the loosening of its enzymatic behavior (substrate specificity). The chemical agents like carbodimide (10 mM), HNBB (10 mM), and ethylene glycol tetraacetic acid (EGTA; 20 mM) did not show any inhibitory effects with residual xylanase activity of 63.5%, 40.6%, and 57.0%, respectively. The irreversibly highest modification occurred with the least residual xylanase activity was 2.9% with 0.5 mM NBS. The inactivation of this enzyme by NBS (0.05 mM) resulted 85.10% activity and NBS (0.5 mM) resulted 2.9% activity, respectively.

Titration with NBS

The presence of tryptophan residue at the active binding site and its role in substrate binding was determined by titration of purified enzyme with NBS (Fig. 3). The concentration-dependent treatment of NBS with the enzyme retained only 15.28% of residual xylanase activity at NBS concentration of 1.2 μM for 40 min. The number of tryptophan residue present at the active site of the enzyme was determined by the logarithm of percentage residual activity as a function of time at various concentrations of NBS, when plotted, exhibited a pseudo-first-order kinetics with respect to time at any given concentration of inhibitor (Fig. 3). The pseudo-first-order rate constants (k) were derived from the slope of the plots of logarithm of the residual xylanase activity against the time of reaction. The order of the reaction was calculated from the slopes of the plots of log of pseudo-first-order rate constant, k against log of inhibitor concentrations (Fig. 3, inset) [15]. The slope revealed the loss of enzyme activity resulted from alteration of only one tryptophan residue per molecule of enzyme during the reaction. A similar work had been reported for xylanase from *Streptomyces* sp. [38], *Chainia*

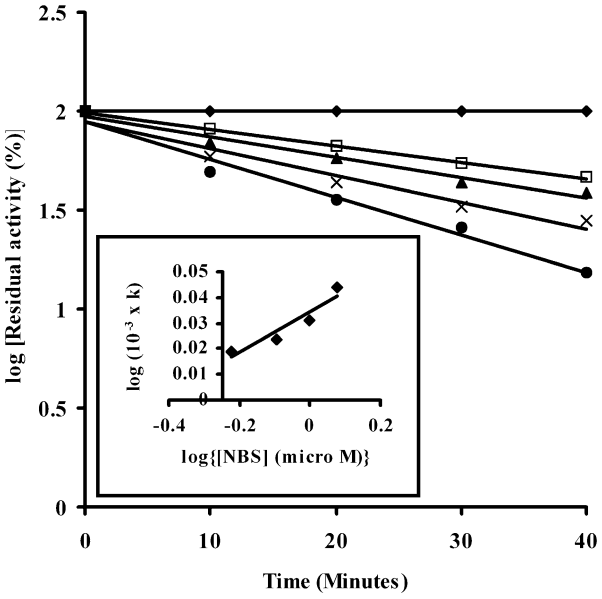

Fig. 3 Kinetics of xylanase inactivation by N-bromosuccinimide (NBS). The enzyme (0.45 U) was used, and xylanase activity were determined, as per assay methods stated in "Materials and Methods" section, in the presence of NBS (*diamonds* 0 μM, *square* 0.6 μM, *triangle* 0.8 μM, *x symbol* 1.0 μM, and *circle* 1.2 μM). The *inset* shows the apparent order of reaction with respect to inhibitor concentration. The logarithmic pseudo-first order rate constants (k) calculated for each was plotted against the logarithm of inhibitor concentration. Data presented are average of three sets of experiment values

sp., and alkalophilic/thermophilic (AT) *Bacillus* [43], where two, one, and three residues of tryptophan were seen to be essential for xylanase activity.

Amino Acid Analysis

Amino acid analysis of *T. clypeatus* endo-xylanase showed that the enzyme is rich in amino acid, in descending order, serine, glycine, threonine, alanine, histidine, valine, asparagine/aspartic acid, glutamine/glutamic acid, etc. The lesser amount of residues present was lysine, phenylalanine, and tyrosine, where almost negligibly present was cysteine residue (Table 5). The enzyme is rich in serine, glycine, threonine, alanine, and least in phenylalanine and sulfur containing cysteine residues, which are in contrast to xylanase reported from *Trichoderma harzianum* which contained cystine (8.85%) and phenylalanine (10.65%; Table 5) [44]. So, the present enzyme is rich in less hydrophobic amino acids and least in very hydrophobic amino acids. A similar amino acid composition rich in asparagine, glycine, and glutamic acid was reported for *Acrophialophora nainiana*, *Aureobasidium pullulans*, *T. viride*, and *Bacillus amyloliquefacens* [45].

N-terminal Sequencing

The N-terminal sequencing analysis was carried out for both xylanolytic amyloglucosidase and endo-xylanase. The N-terminal sequencing of 56 kDa xylanolytic amyloglucosidase was not reported earlier [14, 15]. The purified xylanolytic amyloglucosidase have the N-terminal amino

Table 5 Amino acid composition of *Termitomyces clypeatus* xylanase.

Residues	Percentage content
Asparagine (N)/aspertic Acid (D)	06.29
Glutamin (Q)/glutamic acid (E)	06.00
Serine (S)	16.41
Glycine (G)	10.50
Histidine (H)	07.22
Arginine (R)	04.51
Threonine (T)	09.16
Alanine (A)	09.08
Proline (P)	02.80
[a]Tyrosine (Y)	01.95
Valine (V)	06.53
Metionine (M)	03.56
Cystine (C)	0.079
Isoleucine (I)	03.53
Leucine (L)	05.66
Phenylalanine (F)	01.02
Lysine (K)	02.14
[a]Tryptophane (W)	04.00

Amino acid composition was determined by PICO. TAG system as described in "Materials and Methods" section [15].

[a] Tyrosine (Y) and tryptophane (W) content was estimated spectrophotometrically [26].

Table 6 Comparison of N-terminal amino acid sequence of 56 kDa xylanolytic amyloglucosidase and 12 kDa endo-xylanase with known protein database sequences by LALIGN/PLALIGN.

Organism/Acc ID	Molecular weight	Sequences																					Identity
56 kDa xylanolytic amyloglucosidase																							
Query sequences	56,000 Da	D	P	G	M	V	Y	G	F	D	K	I	A	P	S	x	G	L	T	W	T		
Neosartorya fischeri [A1CVL1]	58,323 Da	E	*P*	*G*	*M*	*V*	*Y*	*G*	*F*	E	N	*I*	E	*P*	*S*	A	K	*L*	M	*W*	*T*		68.42% identity in 19 amino acid overlap
Aspergillus nidulans [Q5ATS0]	50,827 Da	G	Q	*G*	*M*	*V*	*Y*	*G*	*F*	E	D	*I*	E	*P*	*S*	A	*G*	*L*	*T*	*W*	A		68.42% identity in 19 amino acid overlap
Magnaporthe grisea [A4RGJ5]	50,636 Da	G	Q	*G*	*M*	*V*	H	S	*F*	*D*	A	*I*	I	*P*	*S*	A	N	L	*T*	*W*	*T*		63.15% identity in 19 amino acid overlap
Candidatus Kuenenia stuttgartiensis	–	*D*	A	*G*	*M*	*V*	*Y*	*G*	*F*	K	V	D	E	Y	D	K	–	–	–	–	–		50.00% identity in 14 amino acid overlap
Aspergillus terreus [XP_001213869]	53,229 Da	*D*	*P*	*G*	*M*	*V*	*Y*	*G*	*F*	*D*	S	P	C	D	R	A	E	T	L	S	Q		47.36% identity in 19 amino acid overlap
Alpha proteobacterium [ZP_01446650]	59,784 Da	S	E	N	A	L	D	K	I	S	*K*	*I*	L	*P*	*S*	V	*G*	*L*	S	*W*	*T*		42.10% identity in 19 amino acid overlap
Methanococcoid burtonii [YP_566984]	45,139 Da	I	A	E	L	G	L	E	*F*	*D*	M	*I*	*A*	*P*	*S*	H	*G*	V	I	*W*	R		42.10% identity in 19 amino acid overlap
Bacillus cereus [YP_084058]	47,563 Da	A	I	*G*	*M*	S	*Y*	*G*	*F*	N	N	*I*	*A*	G	G	Q	L	I	*T*	D	P		42.10% identity in 19 amino acid overlap
Drosophila virilis [GJ 17124]	103,618 Da	T	N	*G*	*M*	L	*Y*	*G*	*F*	*D*	*K*	T	L	C	L	K	R	F	E	L	K		36.84% identity in 19 amino acid overlap
Acinetobacter sp. [YP_045844]	39,439 Da	K	I	K	A	Q	*Y*	*G*	*F*	*D*	*K*	S	*A*	*P*	E	R	F	W	I	M	L		36.84% identity in 19 amino acid overlap
Yarrowia lipolytica [XP_505415]	101,759 Da	Q	N	*G*	L	*V*	*Y*	*G*	Y	*D*	*K*	*I*	P	K	G	R	L	D	L	R	C		36.84% identity in 19 amino acid overlap
Aspergillus awamori [1GAH_A]	–	*D*	S	*G*	I	*V*	V	A	S	P	S	T	D	*P*	D	Y	F	Y	*T*	*W*	*T*		33.30% identity in 19 amino acid overlap

Frankia species [YP_001506322]	41,261 Da	K	G	A	S	T	W	Y	*F*	*D*	*K*	*I*	*A*	*P*	*S*	M	T	E	A	G	V	31.57% identity in 19 amino acid overlap
Tetrahymena thermophila	79,066 Da	K	M	V	Q	F	*Y*	*G*	*F*	*D*	*K*	*I*	W	N	R	L	N	N	I	T	E	31.57% identity in 19 amino acid overlap
Penicillium funiculosum [1TE1_B]	–	E	Y	Y	I	L	E	S	Y	G	T	Y	N	*P*	*S*	S	*G*	*L*	*T*	S	L	26.31% identity in 19 amino acid overlap
Saccharomyces cerevisiae [P38158]	–	M	G	Y	D	I	S	N	Y	E	*K*	V	W	*P*	T	Y	*G*	T	N	E	D	15.78% identity in 19 amino acid overlap
12 kDa endo-xylanase																						
Query sequences	12,000 Da	A	T	P	S	G	T	G	F	Y	N	G	F	Y	Y	S	F	F	T	T	G	
Chainia sp. [WO1990001060]	6,000±1,000 Da	–	–	–	–	–	–	–	–	*Y*	D	*G*	M	*Y*	*Y*	*S*	*F*	W	*T*	D	*G*	58.33% identity in 12 amino acid overlap
Neurospora crassa [Q7SDQ1]	23,717 Da	G	*T*	*P*	*S*	S	*T*	*G*	*F*	N	*N*	*G*	*F*	*Y*	*Y*	S	*F*	W	*T*	–	–	72.22% identity in 18 amino acid overlap
Aspergillus oryzae [Q9HFA4]	24,474 Da	S	*T*	*P*	*S*	S	*T*	*G*	Y	N	*N*	*G*	Y	*Y*	*Y*	*S*	*F*	W	*T*	D	*G*	65.00% identity in 20 amino acid overlap
Aspergillus clavatus [A1CCU0]	24,873 Da	G	*T*	*P*	*S*	S	*T*	*G*	W	N	*N*	*G*	Y	*Y*	*Y*	*S*	*F*	W	*T*	–	–	66.66% identity in 18 amino acid overlap
Aspergillus fumigatus [Q4WG11]	24,494 Da	G	*T*	*P*	*S*	S	*T*	*G*	W	N	*N*	*G*	Y	*Y*	*Y*	*S*	*F*	W	*T*	D	*G*	65.00% identity in 20 amino acid overlap
Neosartorya fischeri [A1DJ52]	24,341 Da	G	*T*	*P*	*S*	S	*T*	*G*	W	N	*N*	*G*	Y	*Y*	*Y*	*S*	*F*	W	*T*	D	*G*	65.00% identity in 20 amino acid overlap
Chaetomium globosum [Q2HCI0]	23,304 Da	G	*T*	*P*	*S*	*G*	*T*	*G*	T	H	E	*G*	*F*	*Y*	*Y*	*S*	*F*	W	*T*	–	–	72.22% identity in 18 amino acid overlap
Pyrenophora tritici-repentis [B2WLG7]	24,744 Da	S	*T*	*P*	A	*G*	*T*	*G*	M	N	*N*	*G*	*F*	F	*Y*	*S*	*F*	W	*T*	D	*G*	65.00% identity in 20 amino acid overlap
Phaeosphaeria nodorum [Q0U5W9]	24,904 Da	S	*T*	*P*	*S*	*G*	*T*	*G*	T	N	*N*	*G*	*F*	F	*Y*	*S*	*F*	W	*T*	D	*G*	70.00% identity in 20 amino acid overlap
Coccidioides immitis [Q1E5M2]	6,601 Da	–	–	–	*S*	S	A	K	I	F	Y	S	*F*	*Y*	*Y*	N	*F*	*F*	*T*	*T*	–	50.00% identity in 16 amino acid overlap

Amino acids indicated by italic type are those that are identical between the query sequence and sequences from different strains.

– unknown.

acid sequences identified with first 19 amino acid residues as: DPGMVYGFDKIAPSxGLTWT. The protein Basic Local Alignment Search Tool (BLAST) search was performed for its homology on universal protein database servers (National Center for Biotechnology Information, European Molecular Biology Laboratory, UniProt, ExPASy, Georgetown PIR), and pair wise local and global alignment (LALIGN/PLALIGN) was carried out individually for their identity according to percentage of identical amino acid present and overlap (Table 6) [46] and resulted with a maximum homology with hypothetical proteins of fungal as well as bacterial origin. It was found that 68.42% is similar with *Neosartorya fischeri* hypothetical protein coding for fungal lipases and colinesterases. *Aspergillus nidulan*, *Magnaporthe grisea*, *Candidatus Kuenenia stuttgartiensis*, and *Aspergillus terrus* all code for similar hypothetical protein with approximately more or less same molecular mass but with varying amino acid residues. Only *Aspergillus awamori* with 33.30% identity was observed to code for glucoamylase chain A from glycosyl hydrolase family 15.

Similarly, the purified endo-xylanase has the N-terminal amino acid sequences identified with first 20 amino acid residues as: ATPSGTGFYNGFYYSFFTTG. When protein BLAST search for *T. clypeatus* xylanase was performed, it resulted with high degrees of identities with hydrolase family-11 xylanase of fungal origin: 72.22% for *Neurospora crassa* Q7SDQ1 [47]; 72% for *Chaetomium globosum* (*Q2HCI0*); 58.33% for *Chainia* sp. (WO 1990001060); 65% for *Aspergillus oryzae* (Q9HFA4) and *A. fumigatus* (*Q4WG11*); and 65% for *N. fischeri* (*A1DJ52*) as enlisted in LALIGN/PLALIGN table (Table 6). The 12 kDa endo-xylanase upon alignment showed a maximum of 72% identity with *N. crassa* (23.7 kDa) [47] and *C. globosum* (23.3 kDa) xylanase. All the other amino acids aligned code for xylanases with respective molar mass as described in the table. The hypothetically coded low molecular weight xylanase (6.6 kDa) was obtained from an Ascomycota fungi *Coccidioides immitis* with 50% identity in 16 amino acid overlap. A similar industrially important low molecular weight xylanase (6.0± 1.0 kDa) by *Chainia* sp. was aligned with 58% identity. Thus, the N-terminal sequence analysis also revealed that the enzyme is an endo-xylanase. The conserved amino acid residues found among xylanase backbone are threonine, proline, serine, glycine, asparagine, and tyrosine. The amino acid composition analysis with amino acid conserved residues reveals the presence of highly conserved threonine and serine in *T. clypeatus* endo-xylanase enabling for its high consistent structural conformity and stability [45].

In conclusion, we purified the 12 kDa endo-xylanase from *T. clypeatus* using oat spelt xylan as a carbon source which could cleave the 1,4-β-xylopyranoside residue in main chain backbone of xylan, whereas previously using starch, a 56 kDa xylanolytic amyloglucosidase was produced which could cleave the side chain glucose residue present in side chain of xylan.

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