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Purification and Biochemical Characterization of Xylanase from *Sclerotium rolfsii*

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Xylanases have received great attention in the development of environment-friendly technologies in the paper and pulp industry. Their use could greatly improve the overall lignocellulosic materials for the generation of liquid fuels and chemicals. Fungi are widely used as xylanase producers and are generally considered as more potent producers of xylanases than bacteria and yeasts. After the purification steps, a purified xylanase enzyme was obtained with purification fold 14.3. The SDS-PAGE for enzyme protein gave a single band at about 32 kDa. The zymogram technique was performed to ensure that the purified enzyme was xylanase using 0.1% brichwood xylan. The optimum reaction temperatures of the xylanase enzyme were 50°C and 60°C, while optimum pH values were 5.0 and 6.0. From the above we can conclude that the optimum temperature lies between 50-60°C and optimum pH lies between pH 5.0 and 60. The activity of xylanase enzyme was stable at 50°C and 60°C, and pH 4.0 and 5.0 for at least 2 hrs. Mg^{2+} was the only metal, which enhanced the xylanase activity, while Cu^{2+} and Hg^{2+} showed the highest inhibitory effects on the activity of xylanase. The metal chelating agent EDTA had moderately inhibitory effect on enzyme activity.

Key words: Xylanase, SDS-PAGE, zymogram, inhibitors, *Sclerotium rolfsii.*

Cellulose, hemicellulose, and lignin are the major components of plant cell walls, with cellulose being the most abundant component followed by hemicelluloses[1, 2]. Plant biomass comprises on average 23% lignin, 40% cellulose, and 33% hemicellulose by dry weight[3]. Hemicellulose is composed of xylan as a major component[4] that constitutes about 20-40% of total plant biomass[5] and accounts for approximately one third of all renewable organic carbon on earth[6].

Xylan has a high potential for conversion to useful end products. Complete conversion of the hemicellulose requires the action of several main-chain- and side-chain-cleaving enzymes[7] including: endoxylanase (endo-1,4-β-xylanase, E.C.3.2.1.8), β-xylosidase (xylan 1,4-β-xylosidase, E.C.3.2.1.37), α-glucuronidase (α-glucosiduronase, E.C.3.2.1.139), α-arabinofuranosidase (β-L-arabinofuranosidase, E.C.3.2.1.55), and acetylxylan esterase (E.C. 3.1.1.72) (7, 8). Endo-²-1,4-xylanases catalyze the hydrolysis of thebackbone of xylan to produce xylooligosaccharides, which in turn can be converted to xylose by β-xylosidase[9].

Based on amino acid sequence homologies and hydrophobic cluster analysis, xylanases have been grouped mainly into two families of glycosyl hydrolases: family F or GH10 and family G or GH11[10-12]. However, other glycoside hydrolase families, 5, 7, 8, and 43, have also been found to contain distinct catalytic domains with a demonstrated endo-1,4-β-xylanase activity[13]. Xylanases of family G are of low molecular mass with pI 8–9.5 compared to the family F xylanases

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that are of high molecular mass with lower pI values[14]. The optimum pH for xylan hydrolysis is around 5 for most fungal xylanases, whereas pH optima of bacterial xylanases are generally slightly higher[15]. The xylanases and cellulases together with pectinases account for about 20% of the world enzyme market[16].

In a number of fungi, these various endoglycanases can be quite specifically induced. During the growth of *T. reesei* and *T. harzianum* on xylan-based media, mainly xylanase activities with low levels of endoglucanase are formed. Growth on cellulose or on heterogeneous native substrates containing both xylan and cellulose results in the concomitant production of both endo-glucanase and xylanase activities. This unspecific effect of cellulose could be explained by xylan impurities found in commercially available cellulose preparations[17].

The ability to produce xylanase by *Sclerotium rolfsii* isolated from diseased sugarbeet roots was studied[18] as well as the influence of different cultural conditions on enzyme production by this species in the laboratory[19]. This organism is known as an excellent producer of cellulolytic enzymes as well as of hemicellulolytic enzymes[20-22]. In this investigation, we will purify and study the biochemical characterizations of xylanase enzyme from *S. rolfsii*.

MATERIALS AND METHODS

Microorganism and culture conditions

S. rolfsii Sacc. was isolated from diseased sugarbeet roots[18] and maintained on a medium described by Johnson and Curl[23] and composed of (g/l): dextrose, 30; KH_2PO_4, 1; $MgSO_4.7H_2O$, 0.5; KCl, 0.5; KNO_3, 2; and 1 ml of each of stock solutions (1 g/l) of $FeSO_4.7H_2O$, $MnSO_4.7H_2O$, $ZnSO_4.7H_2O$, and thiamine; agar, 20 g.

Production of enzymes at optimized conditions

From the previous experiments, *S. rolfsii* was cultivated on the optimized medium according to Moussa[19] containing (g/l): peptone, 8.0; asparagines, 3.0; $MgSO_4.7H_2O$, 1.5; KH_2PO_4, 1.2; KCl, 0.6; xylan, 0.2; and trace element solution at 0.3 ml/l. The flasks were inoculated with 5-mm plug cut out from the margin of a 5-day-old culture. Incubation was carried out at 25 ± 2 °C under shaking at 100 rpm for 11 days. The culture filtrate was desalted and concentrated by ultrafiltration and used in purification.

Purification of xylanase enzyme

Filtrate proteins were fractionated by ammonium sulfate precipitation (0-80%). The precipitate was recovered by centrifugation at 10000 rpm for 30 min at 4ºC, then dissolved in 10 ml 50 mM acetate buffer (pH 6.8) and dialyzed overnight using 500 ml of the same buffer. This sample was chromatographed onto DEAE-Sephadex A-50 column, which has been equilibrated with 50 mM actate buffer (pH 6.8). The protein was eluted with gradient NaCl from 0-1 mol/l. Elution was done at a flow rate of 18 ml h^{-1}; 3.0-ml fractions were collected. Protein concentration and xylanase activity were determined. The fractions exhibiting significant activity were pooled. After desalting, the enzyme was loaded onto a Sephadex G-75 gel filtration column (18x600 mm column) equilibrated with the same buffer. Elution was done at a flow rate of 18 ml h^{-1}; 3.0-ml fractions were collected. All the steps were carried out at 4°C.

Enzymes assays

Xylanase activity was determined at 40ºC by using xylan from brichwood (Sigma) as substrate, in 50 mM acetate buffer, pH 4.5. Reducing sugars released were assayed by Somogyi method[24] modified from Nelson procedure[25] with xylose as standards. One unit of enzyme activity was defined as the amount of enzyme required to liberate 1 µmol/min of xylose equivalents.

SDS-PAGE electrophoresis

SDS-PAGE was performed according to the method of Laemmli[26] using 12.5% gel. Sample was dissolved in a solution containing 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol, and 15mM Tris-HCl (pH 6.8) and heated in a boiling water bath for 3 min. after electrophoresis; the gel was stained with Coomassie brilliant blue R-250 according to Merril[27].

Zymogram technique

Zymogram analysis was perforemed by the method of Morag *et al*.[28]. Sample was electrophoresed on SDS-12.5% polyacrylamide gel containing 0.1% brichwood xylan as described above. Then, the gel was washed four times for 30 min at 4°C in a 100 mM Na_2CO_3-$NaHCO_4$ buffer (pH 9.0) (the first two washes contained 25% [v/v]

isopropyl alcohol) to remove SDS and re-nature proteins in the gel and further incubated in the buffer for 10 min at 37°C. The gel was soaked in 0.1% Congo red solution for 15 min at room temperature and washed with 1M NaCl until excess dye was removed from the active bands. A zymogram was prepared after an introduction of the gel into 0.5% acetic acid. The background turned dark blue, and clear zones in areas exposed to enzymes activity were observed.

Optimum temperature, pH and thermal stability

Optimum assay temperature was determined by conducting a standard assay at 20-65°C. To determine optimum pH, the assay was carried out in acetate buffer (pH 3.0, 4.0, 5.0); phosphate buffer (pH 6.0, 7.0, 8.0), respectively. Thermal stability was estimated after incubation of the enzyme (10-120 min) in absence of the substrate determining the residual activity against time. For pH stability, the enzyme was incubated in the aforementioned buffer systems for 10-120 min in absence of substrate determining the residual activity against time. The enzyme assay was carried as mentioned above.

RESULTS

From the data presented in Table 1, we can conclude that the total activity and protein concentration decreased with progress of purification steps, which led to increase in specific activity of xylanase, this means that the purification steps used were highly efficient for xylanase enzyme. On the other hand, the purification fold increased by about 14 times in the final step of purification (sephadex G-75) than the crude enzyme (culture filtrate). The recovery percentage of protein was decreased dramatically with the purification processes were progressed.

After gel filtration step (sephadex G-75), The ezyme aliqute was freez dired and kept at -20ºC and used in the further experiments. The protein was applied to SDS-PAGE and stained with Coomassie brilliant blue R-250, which gave a single band at about 32 kDa (Fig. 1). The zymogram technique was performed to ensure that the purified enzyme was xylanase using 0.1% brichwood xylan. The zymogram test showed a colourless band corresponding to the protein band on gel (Fig. 1).

Table 1. Purification data obtained from different purification steps of xylanase from *S. rofsii*

Purification step	Total activity(U)	Total protein(mg)	Specific activity(U/mg)	Recovery (%)	Purification fold
Culture filtrate	25389.3	2613.1	9.7	100	1.0
Amm. Sulfate ppt.	17413.1	564.5	30.9	68.6	3.2
DEAE-Sephadex A-50	8792.7	87.3	100.7	34.3	10.4
Sephadex G-75	5939.4	42.9	138.5	23.4	14.3

Table 2. Effects of some metals and EDTA on xylanase activity from *S. rolfsii*

Compound	Concentration (mM)	Relative activity (%)
MgSO4	2.0	110
ZnCl2	2.0	99
FeSO4	2.0	95
AgNO3	2.0	85
CoCl2	2.0	87
MnSO4	2.0	75
HgCl2	2.0	15
CuSO4	2.0	35
EDTA	3.0	87

The reaction temperature of the xylanase enzyme was greatly affect the activity, where no activity at 10°C and gradually increased starting from 20°C to 50°C and no significant difference between the activity at 50°C and 60°C, then dramticaly the enzyme activity was completely inhibited at 70°C (Fig. 2). The effect of pH values on enzyme activity was shown in Fig. 3., where the xylanase activity was increased sharply from pH 3.0-5.0, and no significant differece between the activity at pH 5.0 and 6.0 and then also decreased sharply decreased till pH 8.0. From Fig. 4, the activity of xylanase enzyme was stable at 50°C and 60°C for at least 2 hrs. Also, the xylanase activity

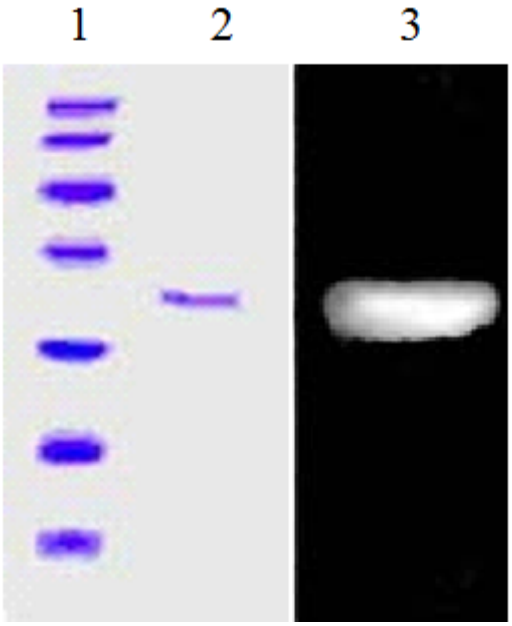

Fig. 1. SDS-PAGE and zymogram analysis of *S. rolfsii* xylanase. Lane 1: Molecular marker, Lane 2: Purified xylanase. The clear band in Lane 2 showed xylanase activity

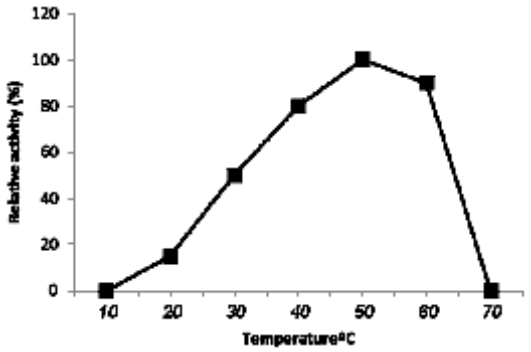

Fig. 2. Effect of different reaction temperatures on the activity of xylanase enzyme from *S. rolfsii*

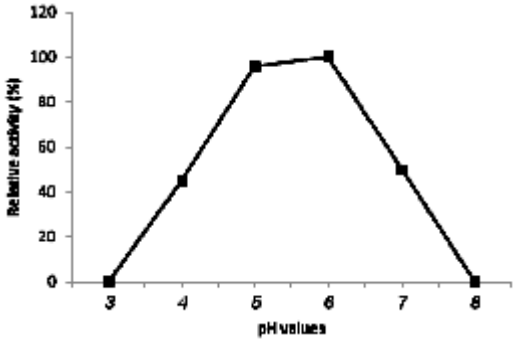

Fig. 3. Effect of different reaction pH values on the activity of xylanase enzyme from *S. rolfsii*

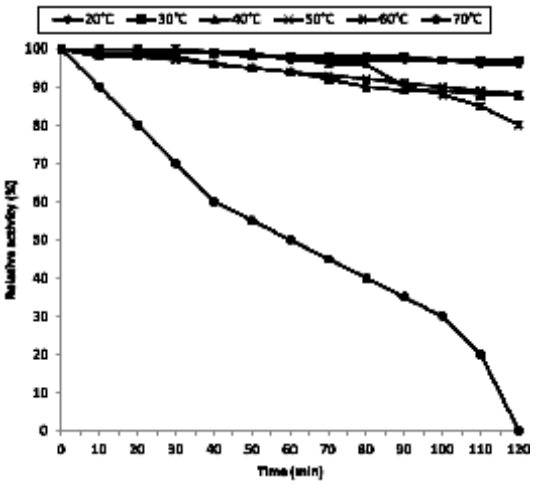

Fig. 4. Thermal stability of xylanase enzyme from *S. rolfsii* incubated at different temperatures for 2 hrs.

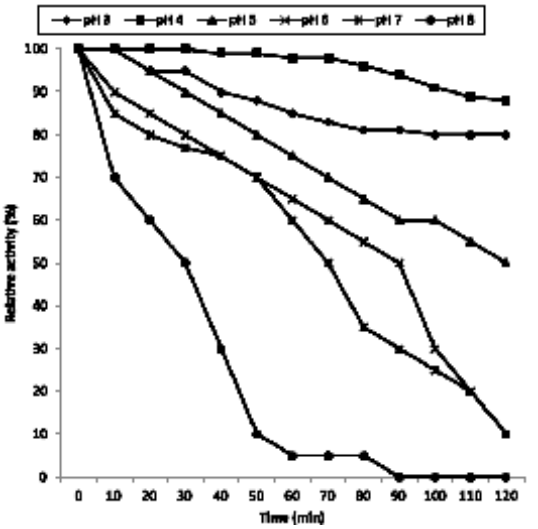

Fig. 5. pH stability of xylanase enzyme from *S. rolfsii* incubated at different temperatures for 2 hrs

was stable when incubated at pH 4.0 and 5.0 for at least 2 hrs (Fig. 5).

From the results in Table 2, Mg^{2+} was the only metal, which enhanced the xylanase activity, while Cu^{2+} and Hg^{2+} showed the highest inhibitory effects on the activity of xylanase (35 and 15%, respectively). Zn^{2+} and Fe^{2+} had neglecting inhibitory effects on the xylanase (99 and 95%, respectively), while Ag^{2+}, Co^{2+} and Mn^{2+} had moderate inhibitory effects on xylanase activity (85, 87 and 75%, respectively). The metal chelating

agent EDTA had moderately inhibitory effect on enzyme activity (87%).

DISCUSSION

Most of the microorganisms isolated from soil/waste/composting waste material are capable of producing a spectrum of cell wall-degrading enzymes [20, 21, 29]. Filamentous fungi have been used for more than 50 years in the production of industrial enzymes[30]. They are widely used as xylanase producers and are generally considered as more potent xylanase producers than bacteria and yeasts[16, 19, 31-33]. Species of fungal genera that are known to produce xylanases include *Aspergillus, Disporotrichum, Penicillium, Neurospora, Fusarium, Chaetomium, Trichoderma, Scelortium* etc.[20, 21, 34, 35].

The total activity and protein concentration decreased with progress of purification steps, which led to increase in specific activity of xylanase, this means that the purification steps used were highly efficient for xylanase enzyme. On the other hand, the purification fold increased by about 14 times in the final step of purification (sephadex G-75) than the crude enzyme (culture filtrate). The recovery percentage of protein was decreased dramatically with the purification processes were progressed.

The production of both xylanases and cellulases in media with xylan or cellulose as sole carbon source may be due to substrates contamination or substrate cross-specificity that can range from absolute for one polymer to about the same affinity for both of them[17, 36, 37]. Nevertheless, concurrent formation of cellulase and xylanase has been observed in several fungi using natural and synthetic substrates[38-40]. There are suggestions of an interaction between xylanase and cellulase induction[39] although the xylanolytic and cellulolytic systems in some filamentous fungi are likely to be under separate regulatory control[33, 38, 41]. In *S. rolfsii* there was a poor cross induction of cellulolytic and xylanolytic enzymes, as in *Aspergillus terreus* mainly induced by the respective synthetic dimmers[38].

The low molecular weight (32 kDa) of this enzyme was simillar to that of xylanases from *Aspergillus* (25 kDa)[42], from *Streptomyces lividans* 66 (31 kDa)[43]. Xylanases of family G are of low molecular mass with pI 8–9.5 compared to the family F xylanases that are of high molecular mass with lower pI values[14].

The optimum reaction temperatures of the xylanase enzyme were 50°C and 60°C, also it was stable at that temperatures. These properties should make it a good candidate in various industerial applications. With its pH stabiltiy and moderate thermal stability properties, it was most suitable for use in the animal feed industry[44]. The optimum pH for xylan hydrolysis is around 5 for most fungal xylanases, whereas pH optima of bacterial xylanases are generally slightly higher[15]. The xylanases and cellulases together with pectinases account for about 20% of the world enzyme market[16]. Many xylanases are active at acidic pH[45], the xylanase under study also had an acidic pH optimum (5.0-6.0) and was stable under that conditions.

The xylanase was optimally active at 50-60°C compared to 50°C from *P. varioti* and 65°C from *P. varioti* [46, 47]. The optimal temperature of xylanase in this study was similar to the xylanases from *T. emersonii*[48], *Gloeophyllum trabeum*[49] and *T. lanuginosus* strains ATCC 46882[50].

Xylanases produced by thermophilic fungi are usually more thermostable than those of mesophilic fungi. This xylanase in present investigation, like xylanases isolated from some thermophilic fungi, such as *T. lanuginosus* strains, the xylanase was also stable over a wide pH range[51, 52]. Thus, its thermal stability is an attractive feature for industrial applications.

Mg^{2+} was the only metal, which activated the xylanase activity, this was with the findings obtained by Yang et al.[42]. The Cu^{2+} and Hg^{2+} showed the highest inhibitory effects on the activity of xylanase, while the metal chelating agent EDTA had moderately inhibitory effect on enzyme activity, which was similar to those concerning the xylanase reported by others[53, 54].

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