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Production and Purification of Extreme Xylanase from *Aspergillus flavus* AUMC 10331 in Sub-merged Fermentation

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ABSTRACT: Xylan, extracted from oat spelts in a previous work, was assayed by HPLC and used as carbon source for the production of xylanase from *Aspergillus flavus* AUMC 10331. The produced xylanase was purified using ion exchange resin (IR-120 EP) and gel filtration column of Sephadex G-75 and Sephadex G-100. The purified xylanase showed total activity of 5.5 IU/ml and specific activity of 687.5 IU/mg, and the enzyme purified 156.75 fold with 4.43 % yield. The highest activity at pH 7.0 and 10.5 indicating two xylanases with the most interesting one with a maximum activity at pH 10.5 and 65 °C. The enzyme activity was greatly stimulated by 5 mM of $FeSO_4$ and $CuSO_4$, while slightly inhibited by other metal ions. K_m and V_{max} were determined as 8.36 mg/ml and 172.4 IU/min respectively.

Keywords: Xylanase; *Aspergillus flavus*; Fermentation.

1. INTRODUCTION

A great attention is being manipulated towards the development of renewable energy resources to meet future energy requirements with continued world energy exhaustion. In the plant cell walls, xylan is the most common hemicellulose comes next to cellulose [1] contributing up to 30% of the plant cell wall in angiosperms and up to 10% of the cell wall in gymnosperms [2], as well as (<30%) in annual plants and up to 35% of the renewable organic carbon on Earth, and strongly associated to cellulose microfibrils [3]. The degradation of xylan requires synergistic action of several hydrolytic enzymes acting together, from which endo-β-1,4-xylanase is the most important one which acts to cleave the internal bonds in xylan backbone as well as reducing the degree of polymerization of the polymer [4, 5]. Fungi are commonly used as source of xylanases, and their xylanolytic systems have been widely studied [5-13]. A variety of microorganisms have been reported to produce xylanase, in which fungi are the most potent producers [14]. However, xylanases are produced mainly by *Aspergillus* and *Trichoderma* spp. on an industrial scale [9]. A large number of *Aspergillus* species have been reported as good producers of xylanases [10, 12, 15].

This current investigation was designed to evaluate the production of extreme xylanase from extracted oat spelt xylan using *A. flavus* isolated from an extreme environment.

2. MATERIALS AND METHODS

2.1. Strain selection

Aspergillus flavus was isolated on Czapek's Dox agar + 10% NaCl from a newly reclaimed soil sample collected from around Lake Beida in Wadi-El-Natrun depression, Egypt [16]. The strain was identified using phenotypic characteristics and ITS region, and deposited in the culture collection of Assiut University Mycological Centre as AUMC 10331 and its sequence data were uploaded to GenBank as accession number KX531011. Among many fungal strains screened, this strain was potent for xylanase production [13], so it was selected for production of xylanase from oat spelt xylan in submerged fermentation.

2.2. HPLC assay of extracted xylan

Characterization of the prepared xylan using HPLC with fluorescent detection was conducted at the Analytical Chemistry Unit at the Faculty of Science, Assiut University, Assiut, Egypt. A wavelength of 295 nm was used as an excitation and 345 nm as an emission one. A 0.5 g of prepared xylan from oat spelts was dissolved in 5 ml Milli-Q water, and then 10, 25 and 50 ng/µl of this solution was injected at a retention time of 3.528 min (Figure 1).

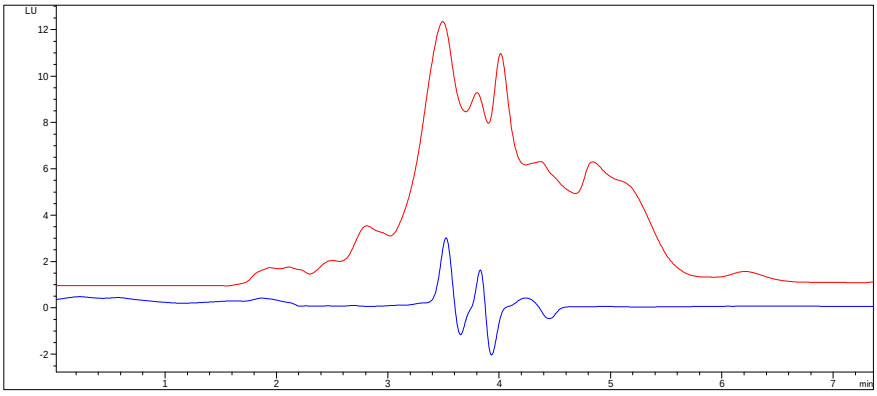

Figure 1. Standard 10 ppm of xylan in blue color and oat spelts (1:1) in red color.

2.3. Xylanase production

Xylan extracted from oat spelts [13] was used as a sole carbon source in sucrose-free Czapek's broth medium which has the composition of (g/l): oat spelts xylan, 10; Na_2NO_3, 2; KC1, 0.5; $MgSO_4.7H_2O$, 0.5; K_2HPO_4, 1; $FeSO_4$, 0.01; $ZnSO_4$, 0.01; $CuSO_4$, 0.005. Fifty ml of xylanase-production medium was dispersed into each Erlenmeyer conical flasks (250 ml). After autoclaving, the medium was inoculated with 1 ml suspension containing 1 x 10^6 (spore/ml) from 7-day-old culture of *A. flavus* AUMC 10331. The culture conditions were adjusted at pH, 9.0, 35 °C, 120 rpm for 96 hours incubation period [13].

2.4. Xylanase assay and protein determination

Xylanase activity was determined by mixing 0.9 ml of 1% birchwood xylan (prepared in 50 mM Na-citrate buffer, pH 5.0) with 0.1 ml of the enzyme and the reaction mixture was incubated at 50° C for 10 min [17]. The reaction was stopped by addition of 2.0 ml of 3, 5-dinitrosalicylic acid (DNS) and the contents were boiled at 100 °C in water bath for 10 min [18]. After cooling, the absorbance was detected at 540 nm (T60 UV-Visible spectrophotometer). The amount of reducing sugar liberated was quantified using xylose as standard. One unit of xylanase is defined as the amount of enzyme that liberates 1 µmol of xylose equivalents per minute under the standard assay conditions [19]. Protein content was estimated by the method of Bradford [20] using bovine serum albumin as standard. Xylanase specific activity corresponded to IU/mg protein.

2.5. Xylanase purification procedures

All purification procedures were performed at 4°C, unless otherwise specified.

2.5.1. Ammonium sulfate precipitation

The crude cell-free extracellular xylanase was obtained by ultrafiltration of the culture broth through 0.45 µm cellulose membrane filter. The clear supernatant was subjected to 60% ammonium sulfate precipitation, and the obtained protein was collected and lyophilized.

2.5.2. Dialysis

One g of the lyophilized enzyme was dissolved in 10 ml of citrate buffer (pH 5.0) and dialyzed against the same buffer for 8 h with replacement of the buffer every 2 h.

2.5.3. Ion exchange column

The dialyzed enzyme was further purified by IR-120 EPcation exchange column (2.4 × 20) cm. The bound proteins were eluted with 500 ml of (0.0 -1.0) M NaCl gradient at a flow rate of 0.25 ml/min. Xylanase fractions with the highest activity were collected and concentrated by lyophilization, and used as purified enzyme for subsequent purification steps.

2.5.4. Sephadex G-75 gel filtration column

The dialyzed xylanase was further purified by a Sephadex G-75 column (2.4 × 50) cm with elution using 500 ml of (0.0-1.0) M NaCl in the same buffer at a flow rate of 0.25 ml/min. The highly active fractions for xylanase activity were collected and concentrated by lyophilization, and used as purified enzyme for subsequent studies.

2.5.5. Sephadex G-100 gel filtration column

The concentrated xylanase fractions obtained from Sephadex G-75 column was further purified by a Sephadex G-100 column (2.4 × 50) cm and eluted with 500 ml of (0.0-1.0) M NaCl in the same buffer at a flow rate of 0.25 ml/min. The highly active xylanase fractions were collected, concentrated and used as purified enzyme for subsequent studies.

2.6. Effects of pH and temperature on xylanase activity

At pH 5.0, xylanase activity was determined between 30°C and 90°C in 5°C increment. For optimum pH determination, 1% birchwood xylan and the purified enzyme solution were prepared in 50 mM of different pH values ranging from 3.0 to 12.0 in 0.5 increment and incubated at the optimum temperature for 30 min. The reducing sugars liberated were determined [18] and the enzyme activity was calculated.

2.7. Kinetic parameters

The effect of birchwood xylan concentration on xylanase activity was evaluated under optimal assay conditions. 0.5 ml of diluted enzyme solution was incubated with 0.5 ml of various concentrations (0.1-1.0%) of soluble Birchwood xylan in 50 mM sodium citrate buffer at optimum pH and temperature for 10 min in water bath. Xylanase activity was assayed as described above. The kinetic parameters (Michaelis-Menten constant, K_m and maximal reaction velocity, V_{max}) were estimated by linear regression from double-reciprocal plots according to Lineweaver and Burk [21].

2.8. Substrate specificity

The specificity of xylanase was evaluated by replacing birchwood xylan in the standard colorimetric assay with a variety of xylan and non-xylan derived polymeric substrates (Birchwood xylan, oat spelt xylan, carboxymethyl cellulose, Avicell). The reducing sugars released were quantified by spectrophotometer at

wavelength of 540 nm and compared to those obtained for birchwood xylan.

3. RESULTS

3.1. HPLC assay of extracted xylan

To calculate the xylan concentration in the oat extract, calibration curve was constructed using a standard solution of xylan. A good linearity was obtained with R^2 = 0.9907. The xylan purity was found to be 56.92%.

3.2. Enzyme purification

Xylanase purification was performed using column chromatography technique including Sephadex G-75 and Sephadex G-100. The crude enzyme has a total activity of 9.768 IU/ml and a specific activity of 31.5 IU/mg proteins; during this step, the enzyme was purified 7.2 fold with 7.86 % recovery. The purified xylanase showed total activity of 5.5 IU/ml and specific activity of 687.5 IU/mg proteins and the enzyme was purified 156.75 fold with 4.43 % recovery. The purification fold revealed that the degree of the purified enzyme was higher after Sephadex G-75 and G-100 gel filtration columns (Table 1).

Table 1. Purification profile of xylanase from oat spelt xylan by *A. flavus.*

Sample	Volume ml	Activity (IU/ml)	Total protein (mg/ml)	Specific activity (IU/mg)	Purification fold	Yield (%)
Fermentation medium	1000	124.21	28.32	4.386	1	100
Ammonium sulfate (60 %)	10	9.768	0.31	31.5	7.2	7.86
Sephadex G-75 and G-100	100	5.5	0.008	687.5	156.75	4.43

3.3. Effect of pH on xylanase activity

The activity of xylanase at different pH values was measured using birchwood xylan as the substrate. Relatively high activity of the enzyme was detected at alkaline pH of 10.5-11.5 with existing of two peaks at pH 7.0 and 10.5 were found indicating two xylanases produced by *A. flavus.* The most interesting one is that enzyme which was active at pH 10.5 (Figure 2).

3.4. Effect of temperature on xylanase activity at pH 10.5

The optimum temperature for xylanase activity by the purified enzyme was determined by varying the reaction temperature at pH 10.5. The enzyme had an optimum temperature of 65°C (Figure 3).

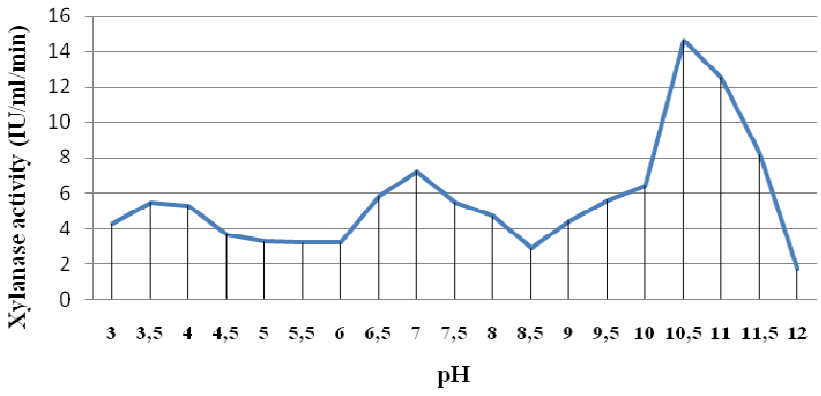

Figure 2. Effect of pH on the activity of the purified xylanase produced by *A. flavus* AUMC 10331.

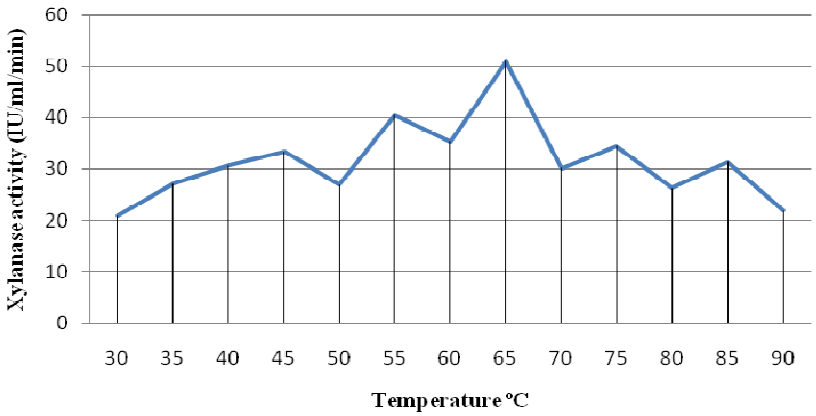

Figure 3. Effect of temperature on the activity of the purified xylanase by *A. flavus* AUMC 10331.

3.5. Effect of metal ions on xylanase activity

The enzyme activity was greatly stimulated by 5 mM of $FeSO_4$ and $CuSO_4$ achieving 341.88% and 240.1% respectively compared to control. In contrast, it was slightly inhibited by other metal ions (Table 2; Figure 4).

Table 2. Effect of some metal ions on xylanase activity.

Metal ion (5 mM/ml)	Xylanase activity	Residual activity (%)
Control	12.2988	100
Fe^{2+}	42.0468	341.88
Zn^{2+}	10.9668	89.17
Ca^{2+}	11.3664	92.42
Cu^{2+}	29.526	240.1
Ni^{2+}	11.4996	93.5
Co^{2+}	10.434	84.1
Mg^{2+}	11.6772	94.94
EDTA	12.12	98.54

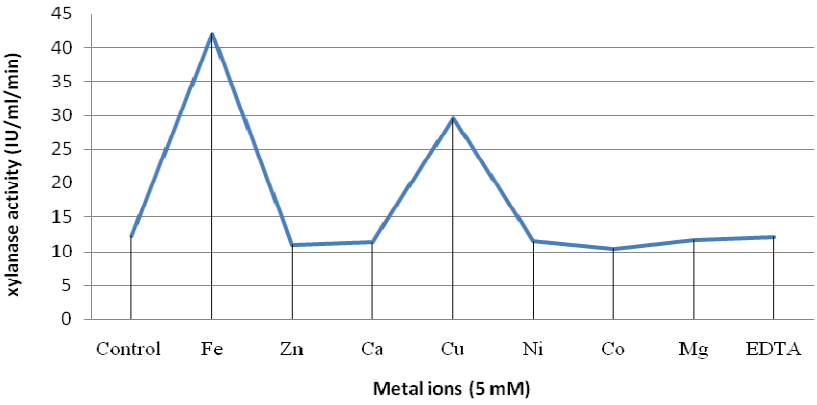

Figure 4. Effect of some metal ions on xylanase activity.

3.6. Km and Vmax of xylanase

When birchwood xylan concentration was used at 1 to 10 mg/ml, the purified xylanase was found to be compatible with Michaelis-Menten kinetics. K_m and V_{max} were determined as 8.36 mg/ml and 172.4 IU/ml respectively indicating a high affinity of the purified xylanase to birchwood xylan.

3.7. Substrate specificity of the crude and purified xylanase

The crude and purified xylanase were screened for their hydrolytic activity with xylan- (Birchwood xylan, oat spelt xylan) and non-xylan (carboxymethyl cellulose and Avicell), derived polymeric substrates. The highest activity was observed for oat spelt xylan followed by Avicell (Table 3).

Table 3. Crude and purified xylanase activity as affected by different substrates.

Substrate	Crude xylanase		Purified xylanase	
	Total protein = 0.31 mg/ml		Total protein = 0.008 mg/ml	
	Total activity IU/ml/min	Specific activity IU/mg protein	Total activity IU/ml/min	Specific activity IU/mg protein
Birchwood xylan	9.768	31.5	5.5	687.5
Oat spelt xylan	65.89	212.55	36.23	4528.75
CMC	0.011	0.035	0.00	0.00
Avicell	47.24	152.38	18.115	2264.4

5. DISCUSSION

Xylanase was purified by two step gel filtration chromatography using Sephadex G-75 and G-100 columns. The enzyme from the fermentation medium yielded 124.21 IU/ml which could be considered much higher than the xylanase activity produced in SmF by *A. flavus* K-03 (45 IU/ml) from birchwood xylan [7] and that produced by *A. terreus* UL 4209 (35 IU/ml) from oat spelt xylan [8] and *A. brasiliensis* ATCC 16404 (11.49 IU/ml) from wheat bran [22], however its activity was lower than that yielded by *Emericella nidulans* NK-62 (362 IU/ml) from wheat bran in SmF [23]. On the other hand, pure birchwood xylan was found to induce relatively high levels of xylanase production in *Aspergillus flavus* K-03[7], *Trichosporon cutaneum* [24], and *Thermomyces lanuginosus* [25], while oat spelt xylan was found to be more suitable than birchwood xylan for cellulose-free xylanase production by *A. terreus* UL 4209 [8]. Generally, xylanases are induced in most microorganisms during growth on substrates containing xylan because oat spelt and birchwood xylans were capable of playing a key role in the regulation of xylanase production [26].

In the current study, the enzyme was purified to 156.75 fold with 4.43% recovery. The high purification fold may be attributed to using of two types of Sephadex in the purification process. These types of Sephadex G-75 and G-100 have fractionation range of 3-80 KD and 4-150 KD respectively [27]. Thus, large amounts of proteins were excluded and eluted out yielding very low amount of total protein (0.008 mg/ml) and low yield (4.43%) and the purified enzyme using Sephadex reached a specific activity of 687.5 IU/mg protein which is much higher than that given by xylanase produced by *A. ficuum* AF-98 (288.7 U/mg) purified to 32.6 fold with 15.3% yield [9] using two step column chromatography of DEAE-Sephadex A-50 ion exchange resin and Sephadex G-100 column chromatography, and it was higher than the specific activity of xylanase produced by *E. nidulans* NK-62 (275 IU/mg) using wheat bran as a substrate partially purified by 80% ammonium sulfate [23].

In the present investigation, the activity of xylanase produced from oat spelt xylan by *A. flavus* was detected at a broad pH profile with two peaks being detected at pH 7.0 and 10.5 indicating that two xylanases

are produced. However, it showed residual activity of 85.6% at pH 11. The most interesting result is that it was active at pH 10.5 giving its optimum activity at 65°C. In this respect, xylanase produced using oat spelt xylan as a sole carbon source by *A. niger* Z1 gave its maximum activity at pH 7.5 and 60°C [28] and *A. fumigatus* MA-28 at pH 8.0 and 50°C [11], however, the enzyme of *A. fumigatus* MA-28 showed residual activity at 60-70°C (53-75%) and at alkaline pH 8-9 (56-88 %). The optimum xylanase activity was detected for *Fusarium proliferatum* NRRL 26517 at pH 5.0-5.5 and 55 °C using corn fiber xylan [6], for *A. niger* at pH 5.0 and 60°C using beechwood xylan [29], for *Trichoderma reesei* QM9414 at pH 5.3 and 50°C utilizing birchwood xylan [30] and for *A. niger* SCTCC400264 at pH 5.5 and 60°C on oat spelt xylan [30].

Regarding the effect of metal ions, the activity of xylanase produced by *A. flavus* from oat spelt xylan was greatly enhanced by addition of 5 mM of Fe^{2+} and Cu^{2+} to the reaction mixture and it reached 341.88% and 240.1% respectively. In harmony with the current results, the activity of xylanase produced by *A. fumigatus* MA-28 was enhanced by Fe^{2+} by 40% while EDTA and Mg^{2+} inhibited xylanase activity and resulted in loss of 65% and 58% of activity respectively [11]. The activity of xylanase produced by *A. ficuum* AF-98 was activated by Cu^{2+} up to 115.8 % while it was inhibited by Fe^{2+} [9]. However, the activity of xylanase from *A. awamori* 2B.361 U2/1 was activated by Mg^{2+} and inhibited by Cu^{2+} [31].

In the present study, the kinetic parameters for the enzyme produced from oat spelt xylan by *A. flavus* were calculated and the K_m and V_{max} were found to be 8.36 mg/ml and 172.4 IU/ml respectively for birchwood xylan. These values are in harmony with the values presented by other fungal xylanases which range from 0.09 to 40.9 mg/ml for $K_m$and from 0.106 to 6300 IU/min for V_{max} [32]. In the current results, K_m value for the purified xylanase of *A. flavus*was higher than thatreported for *A. fumigatus* MA-28 (K_m 4.9 mg/ml) [11], *A. foetidus* (K_m 3.58 mg/ml) [33], *A. ficuum* AF-98 (K_m 3.75 mg/ml) [9] and *Trichoderma harzianum* strain T4 (K_m 1.61 mg/ml) [34]. The K_m value of the current results showed that the purified xylanase has a high affinity for the substrate. This is of significance in industrial use of the enzyme, as conversion rate is high for the enzyme with low K_m value [35].

Regarding the substrate specificity of the enzyme of *A. flavus*, higher activity was observed for oat spelt xylan, a branched arabinoxylan (4528.75 IU/mg protein) followed by avicell (2264.4 IU/mg) than that on less branched birchwood xylan (687.5 IU/mg). The activity of the xylanase towards carboxymethyl cellulose and microcrystalline cellulose (avicell) indicates that this enzyme belongs to family 10 xylanases. The substrate specificity studies have revealed that family 10 xylanases may not be entirely specific for xylan and may also be active on cellulose substrates with low molecular mass [3, 36].

Author Contributions: AHM and MAI are supervisors of the study, they designed research and writing the manuscript and both authors read and approved the final production of the manuscript. RAM and OAAB carried out the experiments, analyzed the data, wrote and revised the manuscript, performed the extraction and purification methods of xylanase enzyme and carried out the research point by point. All authors read and approved the final manuscript.

Conflict of Interest: The authors declare no conflict of interest.

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