

# Synergistic production and purification of extreme xylanase produced by *Aspergillus flavus* AUMC 10331 and *A. oryzae* AUMC 10329 from rice husk in solid-state fermentation

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Received: 24 February 2019, Accepted: 22 March 2019, Published online: 24 March 2019

**Abstract.** *Aspergillus flavus* AUMC 10331 and *A. oryzae* AUMC 10329 were used in consortium culture to produce xylanase enzyme from rice husk using solid-state fermentation technique. The xylanase purification was performed using ion exchange resin IR-120 EP and Sephadex G-75. The purified xylanase showed a total activity of 293.0 IU and specific activity of 350.96 IU/mg protein, and the enzyme was purified to 8.1 fold with 2.7% recovery. The purified xylanase was active over a broad spectrum of pHs from 3-10, and the highest activity was obtained at pH 7.0, followed by 74% of xylanase activity at pH 9.5. At pH 9.5, the xylanase exhibited its optimal activity at 70 °C, indicating alkaliphilic and thermophilic xylanase. The xylanase activity was greatly increased by FeSO<sub>4</sub> and CuSO<sub>4</sub> up to 332.15% and 194.1%, respectively, and slightly inhibited by CoCl<sub>2</sub>. K<sub>m</sub> and V<sub>max</sub> for the purified xylanase were determined at pH 9.5 and 70 °C for birchwood xylan as 22.13 mg/ml and 135.13 IU/min, respectively. The crude and purified enzyme showed high specificity towards the xylans tested. The highest activity was observed for oat spelt xylan; it was three times the activity of birchwood xylan for the crude enzyme and more than six times for the purified enzyme. The specific activity of the xylanase towards birchwood xylan was lower than oat spelt xylan and avicell. The purified xylanase did not act towards carboxymethyl cellulose compared with the crude one.

**Key words:** extreme, xylanase, rice husk, SSF, *Aspergillus flavus*, *A. oryzae*

**Cite this as:** Moubasher, A.H., Ismail, M.A., Mohamed, R.A., Al-Bedak, O.A. (2019). Synergistic production and purification of extreme xylanase produced by *Aspergillus flavus* AUMC 10331 and *A. oryzae* AUMC 10329 from rice husk in solid-state fermentation. J. Multidiscip. Sci. 1(1), 1-7.

## 1. Introduction

Rice husk is considered one of the most widely available agricultural wastes in several rice-producing countries. Approximately 650 million tons of rice per year are produced, of which 20% are husks, giving a globally annual total production of 120 million tons [1]. 75-90% of the rice husk composition is an organic matter, mainly cellulose, hemicellulose, and lignin, and the rest are mineral components such as silica, alkalis, and some trace elements [2]. Rice husk is unusually high in ash compared to other biomass fuels range 10-20%. The ash is 87-97% silica [3]; highly porous and lightweight with a very high external surface area, and the silica content make it a valuable material for industrial applications.

Solid-state fermentation (SSF) has been defined as any fermentation process that involves a solid matrix acting as both physical support and a source of nutrition that allows the development of microorganisms in the absence of free liquid [4]. This system provides many advantages, such as higher volumetric yield, more excellent end-product stability, and fewer energy requirements than submerged cultures, reducing water activity, reducing microbial contamination [5]. Fungi are the most suitable microorganisms for SSF, while bacteria are considered unsuitable, according to the theoretical concept of water activity [6].

Production of biofuels from lignocellulosic biomass requires the hydrolysis of the biomass to its sugar units, glucose, and xylose, by enzymes. These enzymes include cellulase and xylanase that hydrolyze cellulose and xylan, respectively, in plant fibers. So, the present investigation was designed to utilize rice husk as a substrate for xylanase production in solid-state fermentation by consortium culture using *A. flavus* and *A. oryzae*.

## 2. Materials and methods

### 2.1. Fungal strains and agro-industrial substrate

Based on the results of SSF in previous works [7,8], rice husk was the best substrate for xylanase production by *A. oryzae* AUMC 10329 and came second after wheat bran by *A. flavus* AUMC 10331. Therefore, it was chosen for xylanase production by mixed culture of the two strains, and purification of the produced xylanase was also conducted.

### 2.2. Fermentation medium

The fermentation medium has the following composition (g/l): Na<sub>2</sub>NO<sub>3</sub>, 2; K<sub>2</sub>HPO<sub>4</sub>, 1; KCl, 0.5; MgSO<sub>4</sub>.7H<sub>2</sub>O, 0.5; FeSO<sub>4</sub>, 0.01; ZnSO<sub>4</sub>, 0.01; CuSO<sub>4</sub>, 0.005 and Birchwood xylan (Sigma).

### 2.3. Xylanase production in SSF

One liter-fermentation flask containing 50 g of ground rice husk moistened with 50 ml of the fermentation medium was inoculated with a spore suspension containing 1x10<sup>6</sup> spore/ml of 7-day-old cultures of each of *A. flavus* AUMC 10331 and *A. oryzae* AUMC 10329 (1 ml each). The inoculated flask was then incubated at 30 °C for 10 days in a static condition.

### 2.4. Enzyme production

After the incubation period, the crude enzyme was harvested in 200 ml sodium citrate buffer (50 mM, pH 5.0). The fermented slurry was filtered through double cheesecloth and then centrifuged at 10000 x g for 10 min at 4 °C. The clear cell-free supernatant was then completed to 500 ml and used for enzyme assay and subsequent purification.

### 2.5. Xylanase assay

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 [9]. The reaction was stopped by the addition of 2.0 ml of 3, 5-dinitrosalicylic acid (DNS), and the contents were boiled at 100 °C in a water bath for 10 min [10]. After cooling, the absorbance was detected at 540 nm (T60 UV-Visible spectrophotometer, at the Department of Botany and Microbiology, Faculty of Science, Assiut University). 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 [11].

### 2.6. Protein determination

Protein content was estimated by the method of Bradford [12] using bovine serum albumin as standard. Xylanase-specific activity corresponded to IU/mg protein.

### 2.7. Xylanase purification procedures

#### 2.7.1. Ammonium sulfate precipitation

All purification procedures were performed at 5 °C. The crude cell-free supernatant was subjected to 60% ammonium sulfate precipitation, and the obtained protein was collected and lyophilized.

#### 2.7.2. 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 a replacement of the buffer every 2 h.

#### 2.7.3. Ion exchange column

The dialyzed enzyme was purified by chromatographic column (2.4 × 20) cm containing IR-120 EP cation exchange resin. The bound proteins were eluted with (0.0-1.0) M NaCl gradient at a flow rate of 0.25 ml/min. Xylanase fractions with the highest activity were pooled, concentrated by Lyophilization, and partially purified xylanase for subsequent purification steps.

#### 2.7.4. Sephadex G-75 gel filtration column

The partially purified xylanase was subjected to Sephadex G-75 column (2.4 × 50) cm. NaCl solution (0.0-1.0) M, in the same buffer, was used in the elution of the bound protein at a flow rate of 0.25 ml/min. The highly xylanase-active fractions were pooled, concentrated, and used as a purified enzyme for subsequent steps.

#### 2.8. Effects of pH and temperature on xylanase activity

For optimum pH determination, 1% Birchwood xylan and the purified xylanase were prepared in 50 mM of different pH values ranging from 3.0 to 10.0 in 0.5 increments and incubated at 50 °C for 30 min. For determining the optimum temperature, xylanase activity was determined between 30 °C and 75 °C in 5 °C increments at the optimum pH value. The reducing sugar liberated was determined [10], and the enzyme activity was calculated.

#### 2.9. Kinetic parameters

The effect of various concentrations of birchwood xylan on xylanase activity was determined. The reaction mixture containing 0.5 ml of the enzyme solution and 0.5 ml of 0.1-1.0% birchwood xylan was incubated for 10 min in a water bath at the optimum pH and temperature. Xylanase activity was assayed as described above. The kinetic parameters  $K_m$  and  $V_{max}$  were estimated according to Lineweaver and Burk [13].

#### 2.10. Substrate specificity

A variety of xylan- (birchwood xylan, oat spelt xylan) and non-xylan-derived polymers (carboxymethyl cellulose, Avicell) were used in the standard spectrophotometric assay. The reducing sugars liberated were determined [10], and the enzyme activity was calculated.

### 3. Results and discussion

#### 3.1. Enzyme purification

The xylanase purification was performed using 2 step column chromatography technique, including ion exchange resin IR-120 EP and Sephadex G-75. During ammonium sulfate precipitation, the enzyme was purified to 2.4 fold with a 14.81% yield, and the enzyme has a total activity of 1607.28 IU and specific activity of 104.37 IU/mg protein. After ion exchange resin, the total enzyme activity was 1068.53 IU, it gained a specific activity of 146.9, and the enzyme was purified to 3.38 fold with 9.84% recovery. Sephadex G-75 gel filtration column purified the enzyme to 8.1 fold with a total activity of 293.05 IU and specific activity of 350.96 IU/mg protein, and the yield was 2.7% (Table 1).

**Table 1.** Purification profile of xylanase from rice husk by mixed culture of *A. flavus* and *A. oryzae* in SSF

Sample	Volume (ml)	Total activity (IU)	Total protein (mg)	Specific activity IU/mg Protein	Purification fold	Yield (%)
Fermentation medium	500	10850	250	43.4	1	100
Ammonium sulfate (60 %)	10	1607.28	15.4	104.37	2.4	14.81
Ion exchange IR-120 EP	30	1068.53	7.273	146.9	3.38	9.84
Sephadex G-75	50	293.05	0.835	350.96	8.1	2.7

Xylanase was purified by ion-exchange IR-120 EP and Sephadex G-75 column chromatography. The total activity of the enzyme produced in the fermentation medium containing rice husk was 10850 IU (= 217 IU/gds). Much lower xylanase activity was recorded in SSF by *Trichoderma* sp. (22.5 IU/gds) when grown on rice husk [14]. On the other hand, higher xylanase production was reported by *T. harzianum* 1073D3 with a maximum activity of 26.5 IU/mg in SSF using melon peel as a substrate [15]. In the current study, the enzyme produced from rice husk was purified to 8.1 fold giving a total protein of 0.835 mg/ml with a low yield (2.7%). The specific activity increased to 350.96 IU/mg compared to the crude enzyme, and it was higher than that obtained from partially purified xylanase (using 80% ammonium sulfate) of *E. nidulans* NK-62 (275 IU/mg) using wheat bran [16] and that from *Trichoderma* sp. FETL c3-2 (75 IU/mg) in SSF system using a mixture of sugarcane bagasse and palm kernel cake [17].

### 3.2. Effect of pH and temperature on xylanase activity

The activity of the purified xylanase from rice husk was screened over a broad spectrum of pHs from 3-10. Two peaks were detected at pH 7.0 and pH 9.5, indicating the presence of two xylanases; the most important of them was that giving its highest activity at pH 9.5, which is considered as an alkaliphilic enzyme (Figure 1). At pH 9.5, the xylanase exhibited its optimal activity at 70 °C, indicating that the xylanase was thermophilic (Figure 2).

The present investigation revealed that the purified xylanase was active over a wide range of pHs from 3-10, and the highest activity was obtained at pH 7.0. More than 74% of xylanase activity was also obtained at pH 9.5 at 70 °C, indicating that this xylanase was alkali-thermostable. In harmony with the current results, xylanase produced by *A. fumigatus* MA-28 gave its optimum activity at pH 8.0 and 50 °C; however, it showed residual activity at alkaline pHs 8–9 and at 60–70 °C [18]. Also, xylanase of *Thielaviopsis basicola* MTCC 1467 showed its activity in a wide pH range of 5–10 and retained more than 70% of its activity at pHs of 7–9 [19] and that produced by *Penicillium oxalicum*, which gave its maximum activity at pH 8.0 and 45 °C [20].

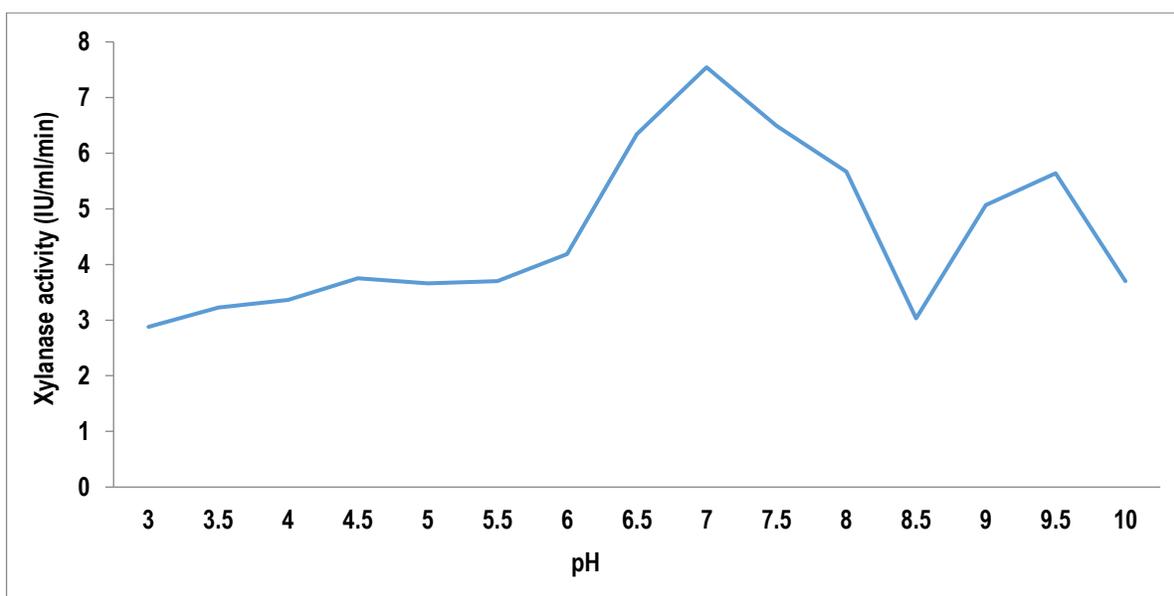


Figure 1. Effect of pH on the activity of xylanase from rice husk by mixed cultures of *A. flavus* and *A. oryzae*

Table 2. Effect of metal ions on xylanase activity

Metal ion (5 mM/ml)	Xylanase activity	Residual activity (%)
Control	15.0516	100
Fe <sup>2+</sup>	49.9944	332.15
Zn <sup>2+</sup>	15.1404	100.6
Ca <sup>2+</sup>	15.5844	103.54
Cu <sup>2+</sup>	29.2152	194.1
Ni <sup>2+</sup>	15.7176	104.42
Co <sup>2+</sup>	14.1192	93.8
Mg <sup>2+</sup>	15.3624	102.0
EDTA	15.1848	100.88

### 3.3. Effect of metal ions on xylanase activity

The xylanase activity was greatly increased by FeSO<sub>4</sub> and CuSO<sub>4</sub> up to 332.15% and 194.1%, respectively, and slightly inhibited by CoCl<sub>2</sub> (Table 2). Regarding the effect of metal ions on xylanase activity produced from rice husk, 5 mM of Fe<sup>2+</sup> and Cu<sup>2+</sup> to the reaction mixture enhanced the activity to reach 332.15% and 194.1% Co<sup>2+</sup> slightly inhibited it. In harmony with the current results, Bajaj and Abbass [18] recorded an enhancement in the xylanase activity of *A. fumigatus* MA-28 by 40% with Fe<sup>2+</sup> while EDTA and Mg<sup>2+</sup> moderately inhibited its activity. On the contrary to our results, Fe<sup>2+</sup> inhibited the xylanase activity of *A.*

*ficuum* AF-98, but similar to our results, Cu<sup>2+</sup> was found to enhance its activity to 115.8% [21], and reversely, Cu<sup>2+</sup> inhibited xylanase activity of *A. awamori* in the study of Umsza-Guez et al. [22].

### 3.4. K<sub>m</sub> and V<sub>max</sub> of xylanase produced from rice husk at pH 9.5 and 70 °C

K<sub>m</sub> and V<sub>max</sub> for the purified xylanase produced from rice husk were determined at pH 9.5 and 70 °C for birchwood xylan as 22.13 mg/ml and 135.13 IU/min, respectively. The kinetic parameters for the enzyme produced in the current work from rice husk were calculated, and the K<sub>m</sub> and V<sub>max</sub> were found to be 22.13 mg/ml and 135.13 IU/min, respectively, for birchwood xylan. The double reciprocal graphic representation was linear, confirming the Michaelian behavior of the rice husk xylanase. The K<sub>m</sub> and V<sub>max</sub> values of the enzyme from rice husk remained in the previously reported range of 0.09 to 40.9 mg/ml for K<sub>m</sub> and of 0.106 to 6300 IU/min for V<sub>max</sub> [23]. K<sub>m</sub> value reported in this study was almost similar to that previously obtained for *Talaromyces thermophilus* (22.5 mg/ml) [24] and *Penicillium sclerotiorum* (23.4 mg/ml) [25], indicating that the investigated xylanase presented a higher affinity for birchwood xylan and high catalytic efficiency to hydrolyze the substrate [24,25]. On the other hand, the K<sub>m</sub> value reported here for the purified xylanase from the mixed culture of *A. flavus* and *A. oryzae* was higher than that reported for other xylanases from *A. fumigatus* MA-28 [18], *A. foetidus* [26], *A. ficuum* AF-98 [21] and *Trichoderma harzianum* T4 [27].

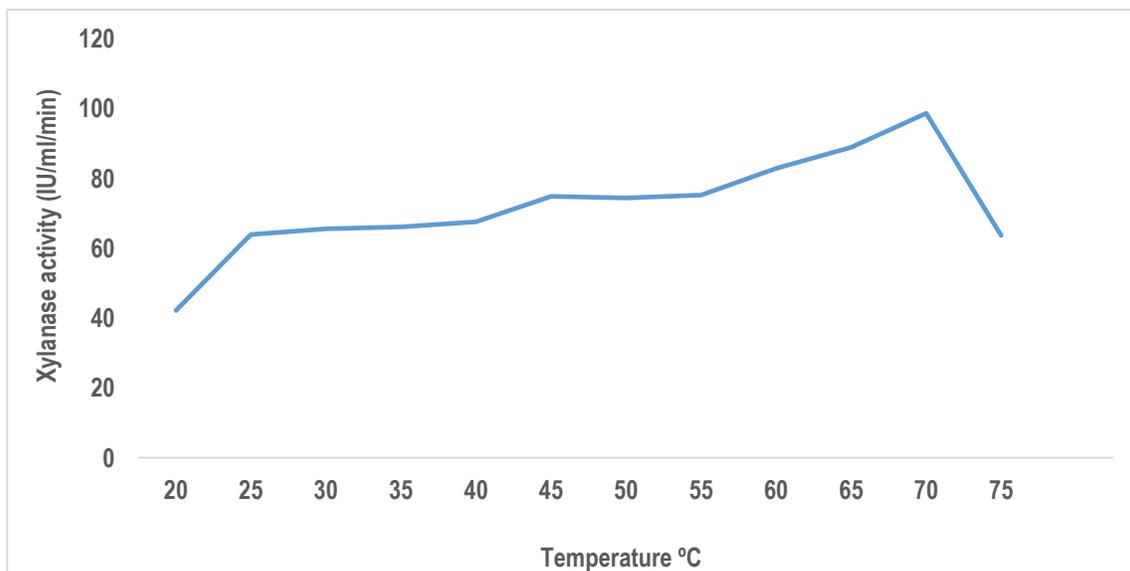


Figure 2. Effect of temperature on the activity of xylanase from rice husk at pH 9.5

Table 3. Substrate specificity of the crude and purified xylanases produced from rice husk in SSF

Substrate	Crude xylanase		Purified xylanase	
	Total protein = 1.54 mg/ml		Total protein = 0.0167 mg/ml	
	Total activity IU/ml/min	Specific activity IU/mg protein	Total activity IU/ml/min	Specific activity IU/mg protein
Birchwood xylan	160.728	104.37	5.86	350.9
Oat spelt xylan	521.43	338.6	39.42	2360.48
CMC	37.47	24.33	0.00	0.00
Avicell	41.2	26.75	33.92	2031.14

### 3.5. Substrate specificity of the crude and purified xylanases

The crude and purified enzyme showed a high specificity towards the xylans tested (Table 3). The highest activity was observed for oat spelt xylan; it was three times the activity of birchwood xylan for the crude enzyme and more than six times for the purified enzyme. The specific activity of the xylanase towards birchwood xylan was lower than oat spelt xylan and avicell.

The purified xylanase did not act towards carboxymethyl cellulose compared with the crude one (Table 3). Regarding the substrate specificity of xylanase produced from rice husk, the enzyme showed higher activity on oat spelt xylan than birchwood xylan, and it was three times the activity of birchwood xylan for the crude enzyme and about six times for the purified enzyme. Also, the specific activity of the xylanase towards birchwood xylan was lower than the ramified oat spelt xylan and avicell. The purified xylanase did not act on carboxymethyl cellulose compared with the crude.

**Conflicts of interest.** There is no conflict of interest.

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