



Agro-industrial residues as alternative sources for cellulases and xylanases production and purification of xylanase produced by *Aspergillus flavus* AUMC 10331 isolated from extreme habitat

Ismail MA^{1,2}, Moubasher AH^{1,2,*}, Ramadan A Mohamed¹ and Al-Bedak OA²

¹Botany and Microbiology Department, Faculty of Science, Assiut University, 71526, Assiut, Egypt

²Assiut University Mycological Centre (AUMC), Assiut University, 71526, Assiut, Egypt

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Abstract

Agro-industrial residues, namely wheat bran (WB), red sawdust (RSD), white sawdust (WSD), corn cobs (CC), rice husk (RH) and sugarcane bagasse (SB) were used as alternatives of pure xylan for production of xylanase, carboxy methyl cellulase (CMCase) and avicellase in solid state (SSF) and submerged (SmF) fermentation using *Aspergillus flavus* AUMC 10331. In SSF, WB was the most utilizable yielding the highest concentration of xylanase (792.4 IU.gds⁻¹) and CMCase (101.8 IU.gds⁻¹) and RH was the best for avicellase (152.4 IU.gds⁻¹). In SmF, *A. flavus* regularly utilized all substrates to produce the three enzymes. From the six plant residues, CC was the superior source for xylanase production (9660 IU.gds⁻¹) and CMCase production (307 IU.gds⁻¹), while RH registered the highest avicellase (5554 IU.gds⁻¹). Xylanase from CC was purified using ion exchange (IR-120 EP) and Sephadex G-75 column chromatography. The purified xylanase showed activity of 32.77 IU.ml⁻¹.min⁻¹ and a specific activity of 273.1 IU.mg⁻¹ proteins. The enzyme was active over a pH range of 4.5–8.0, and its highest activity was detected at pH 7.0 and 65 °C. Xylanase activity was stimulated by FeSO₄ up to 121.97 %. K_m and V_{max} were 12.18 mg.ml⁻¹ and 204.1 IU.min⁻¹, respectively.

Key words – avicellase – cellulose – solid state fermentation – submerged fermentation – Wadi-El-Natrun region – xylanase

Introduction

Agro-industrial residues are produced in huge amounts worldwide. In Egypt, they still remain unexploited and causing severe pollution problems and health hazards during their burning in fields. However, such residues usually have a composition rich in sugars, minerals and proteins, and therefore, they should not be considered “wastes” but “raw materials” for other industrial processes (Mussatto et al. 2012) and they could be used as a good source for enzyme production. The majority of these residues contain (30–40) % cellulose, (20–40) % hemicellulose and (20–30) % lignin (Deschamps & Huet 1985), so their biodegradation by microorganisms needs the action of more than one enzyme. Microorganisms can utilize these residues to produce valuable products and chemicals (Kango et al. 2003). Fungi are excellent enzyme producers, especially

cellulases and xylanases, because of their high productivity comparing with bacteria and yeasts (Haltrich et al. 1996). They are produced by a great number of bacteria and fungi (Kulkarni et al. 1999, Subramaniyan & Prema 2002). The global market of specialty enzymes is expected to reach about \$4 billion by 2018 and will continue to grow because of advancements in the biotechnology industry, the continued need for cost-efficient manufacturing process, and calls for greener technologies (Sarmiento et al. 2015). Xylanases show great interest for a wide spectrum of industrial applications such as the bioconversion of lignocellulosic biomass into sugars and ethanol, clarification of juices, wines and beer finishing, improvement of nutritional quality of silage and green animal feedstock, production of xylo-oligosaccharides as food additives, baking industry, paper and pulp industry, xylitol production, and the de-inking processes of waste papers (Viikari et al. 2001).

This study was focused on the production of xylanases and cellulases and purification of the xylanase from *A. flavus* strain.

Materials & Methods

Strain selection

The strain *Aspergillus flavus* AUMC 10331 (KX 531011) used in this investigation was isolated on 10% NaCl-Czapek agar from a soil sample cultivated with barley and collected from El-Beida Lake having a pH of 8.22 and sodium ions of 10.98 g/kg dry soil (an extreme habitat of Wadi-El-Natron, Egypt) (Ismail et al. 2017). The strain was deposited in the culture collection of the Assiut University Mycological Centre as AUMC 10331 and its ITS sequence was uploaded to the GenBank as accession number KX531011 and it was chosen for its ability to produce xylanases and cellulases (Moubasher et al. 2017).

Xylanase production from agro-industrial residues

Substrate pretreatment

Six different agro-industrial residues, namely wheat bran (WB), red sawdust (RSD), white sawdust (WSD), corn cobs (CC), rice husk (RH) and sugarcane bagasse (SB) were chosen for enzyme production in solid state (SSF) and submerged fermentation (SmF). All substrates were obtained from public markets in Assiut Governorate, Egypt. They were washed with distilled water, oven dried at 50 °C to a constant weight and ground to fine particles to pass through 2 mm sieve.

Fermentation medium

Sucrose-free Czapek's broth was used as fermentation medium. The medium has the following composition (g.L⁻¹): Na₂NO₃, 2; K₂HPO₄, 1; KCl, 0.5; MgSO₄.7H₂O, 0.5; FeSO₄, 0.01; ZnSO₄, 0.01; CuSO₄, 0.005 and Birchwood xylan (Sigma), 1.

Xylanase production in solid state fermentation (SSF)

Triplicate Erlenmeyer flasks (250 ml); each containing 10 g of each of agro-industrial residue, were prepared. Each was moistened with 10 ml of the fermentation medium. The fermentation conditions were adjusted at pH, 9.0; sodium nitrate as nitrogen source (Moubasher et al. 2017). The flasks were then autoclaved for 20 min at 121 °C. After cooling, each flask was inoculated with 1 ml spore suspension containing 1x10⁶ (spore/ml) from 7-day-old culture of *A. flavus* AUMC 10331. The inoculated flasks were incubated for 10 days in a static condition at 35 °C.

Xylanase production in submerged fermentation (SmF)

A. flavus AUMC 10331 was cultivated in triplicates Erlenmeyer flasks (500 ml) each containing 100 ml fermentation medium supplemented with 1 g of each of agro-industrial residue.

After autoclaving, each flask was inoculated with 2 ml spore suspension containing 1×10^6 (spore/ml) from 7-day-old culture and incubated at 35 °C and 120 r.p.m for 10 days.

Enzyme extraction

In SSF, the flask content was collected and harvested in 100 ml of 50 mmol sodium citrate buffer (pH 5.0) and the fermented slurry was filtered through double cheese cloth. In SmF, the fermented medium was filtered through filter paper (Whatman No. 3). The filtrates were centrifuged at 10000 xg for 20 min at 4 °C. The cell-free supernatants were used for xylanase and cellulase assay.

Xylanase and cellulase assay

Xylanase, CMCase and avicellase activities were assayed (Bailey et al. 1992), where the reaction mixture contained 0.9 ml of 1.0 % birchwood xylan or carboxymethyl cellulose (CMC) or avicell, respectively (dissolved in 50 mmol acetate buffer pH 5.0) and 0.1 ml of the enzyme solution. The reaction was carried out in water bath at 50 °C for 10 min. The amount of reducing sugar liberated was determined by the dinitrosalicylic acid (DNS) method using xylose (for xylanase) and glucose (for CMCase and avicellase) as standards (Miller 1959) at wavelength of 540 nm. One unit of xylanase or cellulase activity was defined as the amount of enzyme that catalyzes the release of 1 μ mol of xylose or glucose, respectively equivalent in one minute under the assay conditions (Ghose & Bisaria 1987). The protein concentration was determined using bovine serum albumin as a standard (Bradford 1976).

Xylanase purification procedures

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

Ammonium sulfate precipitation

The clear cell-free supernatant was subjected to 60 % ammonium sulfate precipitation. The precipitated protein was collected and lyophilized (VirTis, Model 6KBTES-55 Freeze Dryer, USA).

Dialysis

The lyophilized protein was dissolved in citrate buffer (pH 5.0) and dialyzed against the same buffer for 8 h and the buffer was replaced every 2 h to exclude small molecules and obtain a buffered solution in this pH.

Ion exchange column

The dialyzed enzyme was further purified by anion exchange column (2.4 x 20) cm. The bound proteins were eluted with (0–1.0) M NaCl gradient at a flow rate of 0.25 ml.min⁻¹. The highly active xylanase fractions were collected, concentrated by lyophilization and used as purified enzyme for subsequent purification steps.

Sephadex G-75 gel filtration column

The xylanase from ion exchange column was further purified by Sephadex G-75 column (2.4 x 50) cm with (0–1.0) M NaCl gradient at a flow rate of 0.25 ml.min⁻¹. The highly active xylanase fractions were collected, concentrated by lyophilization and used as purified enzyme for subsequent purification steps.

Effect of pH on xylanase activity

1 % birchwood xylan (Sigma) and purified enzyme solutions were prepared in 50 mmol of different pHs ranging from 3.0 to 10.0 in 0.5 of increment. The buffers used for the required pH range were citrate buffer (pH 3–6), sodium phosphate (pH 6.5–8), and glycine-NaOH (pH 8.5–10). The reaction mixture (0.9 ml of 1 % birchwood xylan and 0.1 ml of the purified enzyme) was

incubated in water bath at 50 °C for 30 min and the liberated xylose was determined as described by Miller (1959).

Effect of temperature on xylanase activity

At pH 5.0, xylanase activity was assayed between 40–80 °C (in 5 °C increment). The reducing sugars liberated were determined (Miller 1959), and the enzyme activity was calculated.

Effect of metal ions and EDTA on the enzyme activity

The effect of some metal ions (in concentration of 5 mmol/ml) on the activity of the purified enzyme was investigated by adding the divalent metal ions (Ca^{2+} , Co^{2+} , Ni^{2+} , Cu^{2+} , Fe^{2+} , Mg^{2+} , Zn^{2+} and EDTA) to the reaction mixture. The activity of the enzyme in the absence of metal ions or EDTA was determined under the standard assay conditions to define 100 % activity.

Determination of kinetic constants (K_m and V_{max})

K_m (Michaelis–Menten constant) and V_{max} (maximum velocity of the reaction) values of the xylanase were determined by measuring enzyme activity at different concentrations of birch wood xylan (0.1–1.0) %, and using the equation of Lineweaver & Burk (1934).

Substrate specificity of xylanase

The substrate specificity of the xylanase was studied using 1 % (birchwood xylan, oat spelt xylan) and non-xylan (carboxy methyl cellulose and avicel) derived polymeric substrates. The activities were assayed at 50 °C for 10 min, and the amount of reducing sugars released was quantified by DNS reagent (Miller 1959) and compared to those values obtained from birchwood xylan.

Xylanase production in SSF

As alternatives of pure xylan, for production of xylanase along with CMCase and avicellase by *A. flavus* AUMC 10331, wheat bran in SSF was the most utilizable substrate yielding the highest concentration of xylanase ($792.4 \text{ IU.gds}^{-1}$) and CMCase ($101.8 \text{ IU.gds}^{-1}$) followed by rice husk which gave $436.2 \text{ IU.gds}^{-1}$ xylanase and 87.8 IU.gds^{-1} CMCase while rice husk was the best for avicellase production yielding $152.4 \text{ IU.gds}^{-1}$ followed by wheat bran ($140.6 \text{ IU.gds}^{-1}$) (Fig. 1).

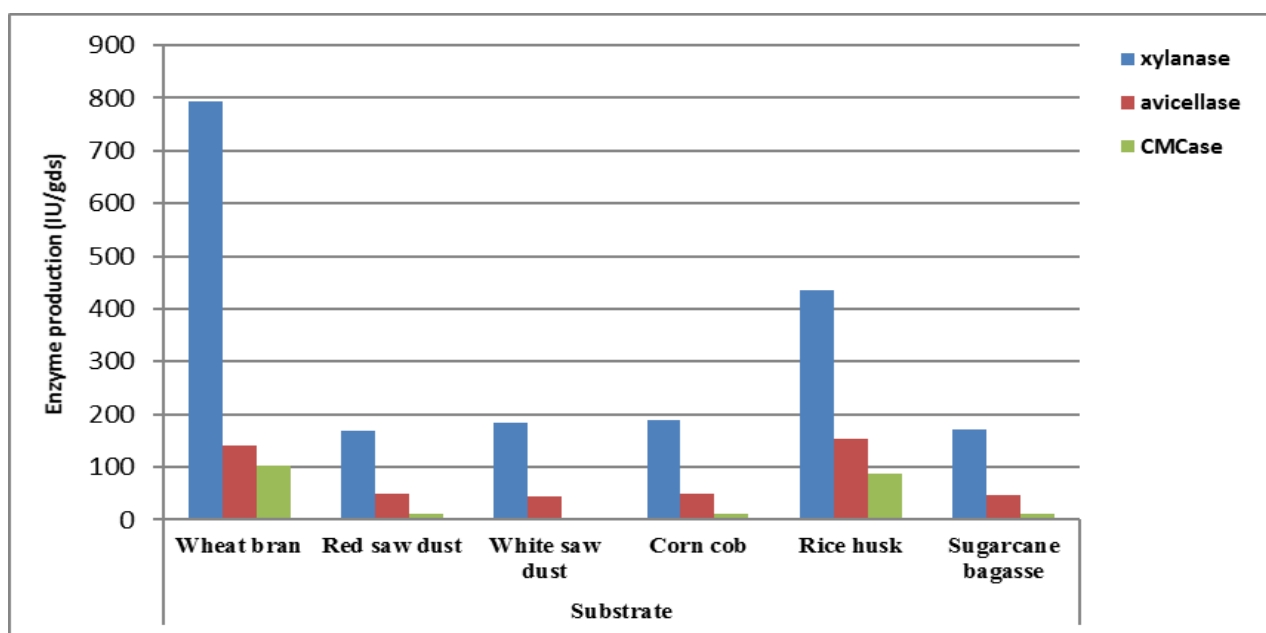


Fig. 1 – Xylanase, avicellase and CMCase production from some agro-industrial residues by *A. flavus* in SSF

Xylanase production in SmF

In SmF, *A. flavus* AUMC 10331 regularly utilized all substrates to produce the three enzymes. Xylanase and CMCase productivity (9660 IU.gds⁻¹ and 307 IU.gds⁻¹ respectively) from corn cobs was the highest amongst all tested substrates while rice husk gave the highest avicellase production (5554 IU.gds⁻¹) (Fig. 2).

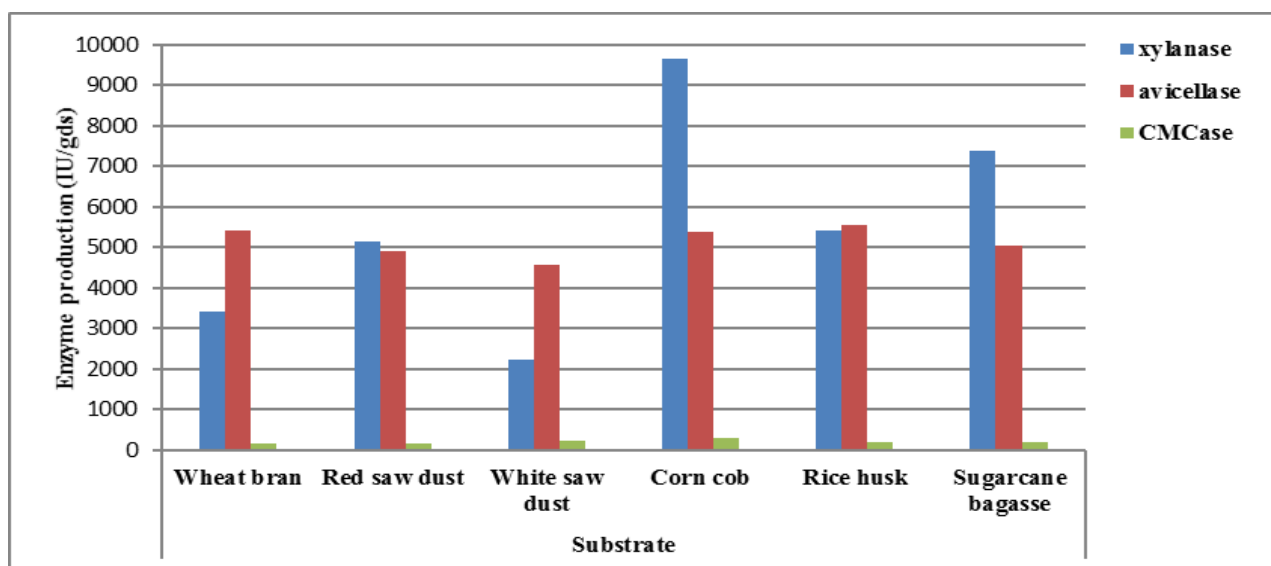


Fig. 2 – Xylanase, avicellase and CMCase production from some agro-industrial residues by *A. flavus* in SmF

Purification of xylanase from corn cobs by *A. flavus* AUMC 10331 in SmF

Enzyme purification

The crude enzyme exhibited a total activity of 20.7 IU.ml⁻¹.min⁻¹ and specific activity of 5.47 IU.mg⁻¹ proteins. The enzyme was purified to 3.1-fold during this step. The purified xylanase using ion exchange resin and Sephadex G-75 showed a total activity of 32.77 IU.ml⁻¹.min⁻¹ and a specific activity of 273.1 IU.mg⁻¹ protein thus, the enzyme was purified to 155-fold. The summarized data of xylanase purification has been shown in Table 1.

Table 1 Purification profile of xylanase produced from corn cobs by *A. flavus* AUMC 10331 in SmF

Samples	Volume (ml)	Activity (IU.ml ⁻¹ .min ⁻¹)	Total protein (mg)	Specific activity (IU.mg ⁻¹)	Fold
Fermentation medium	100	96.60	54.83	1.762	1
(NH ₄) ₂ SO ₄ (60 %)	10	20.7	3.78	5.47	3.1
Ion exchanger IR-120 EP	30	24.27	0.316	76.8	43.6
Sephadex G-75	50	32.77	0.12	273.1	155

Effect of pH on xylanase activity

The activity of xylanase at different pH values was measured using birchwood xylan as the substrate. The enzyme was active over pH range from 4.5 to 8 with slightly higher activity detected at pH 7.0 (Fig. 3).

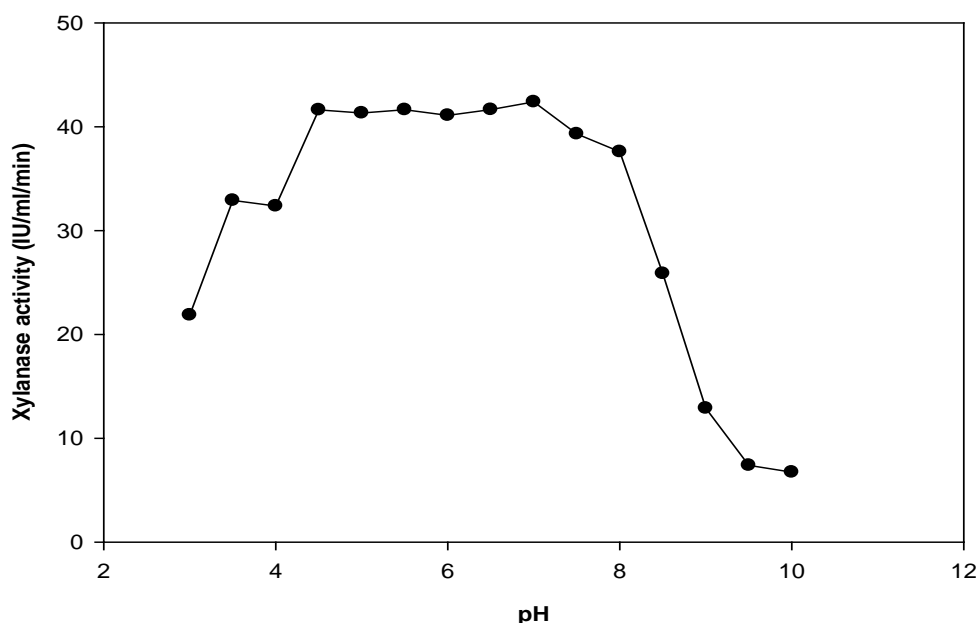


Fig. 3 – Effect of pH on activity of xylanase from corn cobs

Effect of temperature on xylanase activity

At pH 7.0, the purified xylanase exhibited its optimal activity at 65 °C indicating that the xylanase was thermotolerant (Fig. 4).

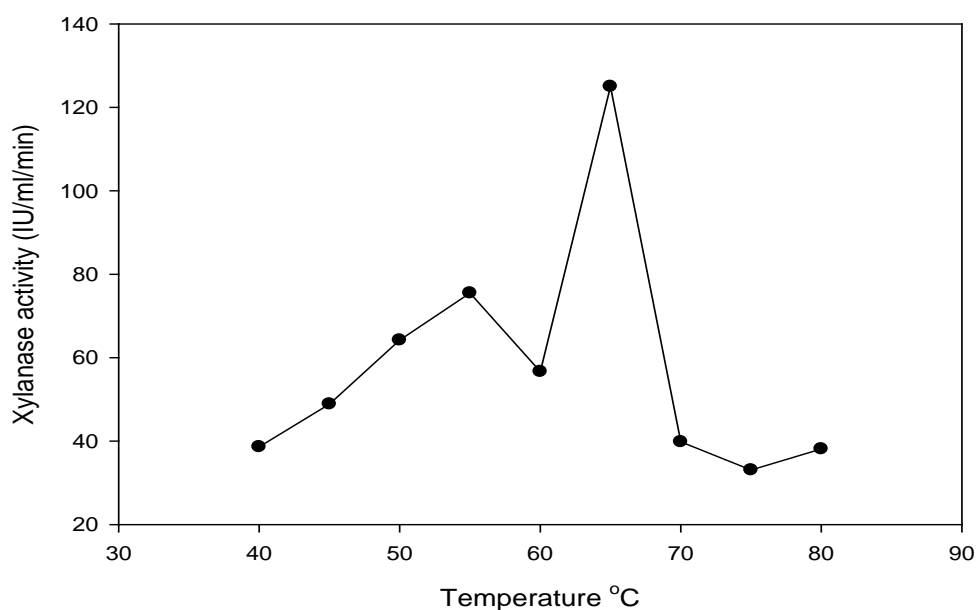


Fig. 4 – Effect of temperature on the activity of xylanase from corn cobs

Effect of metal ions on the activity of xylanase from corn cobs

The xylanase activity was stimulated by FeSO₄ up to 121.97 % while the remaining metal ions tested slightly increased the enzyme activity (Table 2).

Kinetics of xylanase from corn cobs at pH 7.0 and 65 °C

Within xylan concentration from 1 to 10 mg/ml, the purified xylanase from corn cobs was found to be compatible with Michaelis–Menten kinetics. K_m and V_{max} were calculated as 12.18 mg.ml⁻¹ and 204.1 IU.min⁻¹, respectively.

Table 2 Effect of metal ions on the activity of xylanase produced from corn cobs

Metal ion (5 mmol.ml ⁻¹)	Xylanase activity (IU.ml ⁻¹ .min ⁻¹)	Residual activity (%)
None	126.096	100
Fe ²⁺	152.8248	121.97
Zn ²⁺	131.1576	104.0
Ca ²⁺	138.528	109.86
Cu ²⁺	134.532	106.7
Ni ²⁺	132.2232	104.86
Co ²⁺	130.6248	103.6
Mg ²⁺	132.8448	105.35
EDTA	130.1808	103.24

Substrate specificity of crude and purified xylanase from corn cobs

The crude and purified enzymes showed high substrate specificity towards oat spelt xylan compared with birchwood xylan. The highest activity was obtained from oat spelt xylan for both enzymes. The purified enzyme did not act on CMC compared with the crude (Table 3).

Table 3 Substrate specificity of the crude and purified xylanase produced from corn cobs in SmF

Substrates	Crude xylanase		Purified xylanase	
	Protein = 2.153 mg.ml ⁻¹		Protein = 0.12 mg.ml ⁻¹	
	Activity IU.ml ⁻¹ .min ⁻¹	Specific activity IU.mg ⁻¹ protein	Activity IU.ml ⁻¹ .min ⁻¹	Specific activity IU.mg ⁻¹
Birchwood xylan	20.7	9.61	57.54	479.5
Oat spelt xylan	67.4	31.3	65.712	547.6
CMC	3.39	1.57	0.00	0.00
Avicell	17.9	8.3	26.46	220.5

Discussion

In the present study, low-cost agro-industrial residues were used for production of xylanase along with CMCase and avicellase. In SSF, wheat bran was the most utilizable substrate by *A. flavus* AUMC 10331 yielding the highest concentration of xylanase (792.4 IU.gds⁻¹) and CMCase (101.8 IU.gds⁻¹) followed by rice husk which gave lower levels of xylanase (436.2 IU.gds⁻¹) and CMCase (87.8 IU.gds⁻¹); however, rice husk was the best for avicellase production yielding 152.4 IU.gds⁻¹ followed by wheat bran giving 140.6 IU.gds⁻¹. In agreement with the current results, maximum xylanase production was observed in 0.5 % of each of wheat bran and sugarcane bagasse (129.8 IU.gds⁻¹) followed by 0.5 % of each of wheat bran and corn cob (94.0 IU.gds⁻¹) in SmF by *A. flavus* (Silva et al. 2015). In this respect, wheat bran gave 38.9 IU.gds⁻¹ of xylanase by *P. oxalicum* (Muthezhilan et al. 2007) and 32.4 IU.gds⁻¹ by *A. flavus* (Mostafa et al. 2014). However, different concentrations of xylanase (191-738 IU.gds⁻¹) along with cellulase (1-25 IU.gds⁻¹) were obtained from wheat bran by different isolates of *A. flavus* in solid state fermentation (Nair et al. 2008). On the other hand, much higher xylanase (2830.7 IU.gds⁻¹) was obtained by *A. oryzae* using wheat bran in SSF (Pirota et al. 2013). The inducing effect of wheat bran for xylanase production in solid state fermentation is attributed to the fact that the cell-wall polysaccharides of wheat bran contained 40 % xylans (Sanghi et al. 2008) and its particles were decomposed to form soluble compounds that are used by the fungus (Muthezhilan et al. 2007). Rice husk also is a rich source of xylan (28 %) and xylose (23 %). Therefore, it is an attractive substrate for production of xylanase enzyme (Wong et al. 1988).

In SmF, *A. flavus* was regularly superior for the production of the three enzymes from all utilized substrates. Xylanase and CMCase productivity (9660 IU.gds⁻¹ and 307 IU.gds⁻¹

respectively) was the highest from corn cobs amongst all tested substrates while rice husk gave the highest avicellase production of 5554 IU.gds⁻¹. It was also found that submerged fermentation was superseded for the production of xylanase, avicellase and CMCase despite the amount of each substrate in SSF was 10 times greater than in SmF. The current results disagreed with those obtained by Dutt & Kumar (2012) who found that CMCase production by *A. flavus* and *A. niger* in SSF was higher than that in SmF. *A. flavus* was also found to produce high levels of CMCase in Cz broth medium using wheat bran as substrate in SmF (Gomathi et al. 2012).

Agro-industrial residues are considered as the best substrates for xylanase production by microorganisms (Poorna & Prema 2006, Pandey et al. 2000). Thus *A. flavus* AUMC 10331 is considered a promising strain which can be used in co-production of xylanase, avicellase and CMCase from the agro-industrial residues. In the present study, it was found that SmF was the best for production of xylanase, avicellase and CMCase than SSF where the amount of enzymes was duplicated in xylanase and was more than 10 times in avicellase and CMCase. The higher productivity of SmF than SSF may be attributed to the higher availability of the substrate in SmF due to the better aeration and the surface of the substrate particles is regularly more subjected to the enzyme action than in solid state. Also, the heat transfer and the homogeneity of the submerged fermentation medium render it more favourable than the non-homogenous solid state (Al-Bedak 2017).

In the current study, xylanase was produced from corn cobs, the superior agro-industrial residue, and was purified using two steps column chromatography; ion exchange and Sephadex G-75. The enzyme from the fermentation medium yielded xylanase activity of 96.6 IU.ml⁻¹.min⁻¹ (= 9660 IU.gds⁻¹) which is much higher than those reported from *A. fumigatus* MA-28 (845 IU.gds⁻¹) (Bajaj & Abbass 2011), and from *A. brasiliensis* ATCC 16404 (11.49 IU.ml⁻¹ = 172.35 IU.gds⁻¹) when grown in submerged fermentation using wheat bran as substrate (Ho & Iyilia 2015), from *A. flavus* (259.6 IU.gds⁻¹) (Silva et al. 2015) and from *A. flavus* K-03 (less than 300 IU.gds⁻¹) when corn cobs were used as substrate in SmF (Kim 2005).

In the current study, the enzyme was purified to 155-fold. The purified enzyme after Sephadex G-75 reached a specific activity of 273.1 IU.mg⁻¹ protein which was slightly lower than that given by xylanase produced by *A. ficuum* AF-98 (288.7 U.mg⁻¹) purified to 32.6-fold using DEAE-Sephadex A-50 ion exchange resin and Sephadex G-100 column chromatography (Fengxia et al. 2008), and it was almost equal to the specific activity of partially purified xylanase produced by *E. nidulans* NK-62 (275 IU.mg⁻¹) using wheat bran as a substrate (Kango et al. 2003).

The present results revealed also that, the enzyme was active over a pH range from 4.5 to 8 with slightly higher activity at pH 7.0 and 65 °C. In this respect, using wheat bran and sugarcane bagasse, *A. flavus* gave its highest xylanase activity in a wide range of pH (3.0–8.0) with an optimum pH of 5.5 and temperature of 45 °C (Silva et al. 2015), and *A. ficuum* AF-98 at pH 5.0 and 45 °C (Fengxia et al. 2008), using wheat bran by *A. terreus* UL 4209 at pH 6.0 and 35 °C (Chidi et al. 2008) and *A. fumigatus* MA-28 at pH 8.0 and 50 °C (Bajaj & Abbass 2011), barley and rice bran by *A. flavus* K-03 at pH 6.5 and 25 °C (Kim 2005), tomato pomace by *A. awamori* strain 2B.361 U2/1 at pH 5.0 and 50 °C (Umsza-Guez et al. 2011). However, the enzyme of *A. fumigatus* MA-28 showed residual activity of (53–75) % at 60 °C–70 °C and (56–88) % at alkaline pH (8–9) (Bajaj & Abbass 2011).

Regarding the effect of metal ions and EDTA on xylanase activity produced by *A. flavus* from corn cobs, Fe²⁺ stimulated the activity up to 121.97 % while the remaining metal ions (Ca²⁺, Co²⁺, Ni²⁺, Cu²⁺, Mg²⁺ and Zn²⁺) and EDTA slightly enhanced the enzyme activity. In harmony with the current results, the activity of xylanase produced by *A. fumigatus* MA-28 was enhanced by Fe²⁺ by 40 %; however EDTA and Mg²⁺ moderately inhibited its activity up to 65 % and 58 % respectively (Bajaj & Abbass 2011). Reversely, the activity of xylanase produced by *A. ficuum* AF-98 was inhibited by Fe²⁺ (Fengxia et al. 2008), however, the activity of xylanase from *A. awamori* was stimulated by Mg²⁺ and inhibited by Cu²⁺ (Umsza-Guez et al. 2011).

The kinetic parameters for the enzyme produced from corn cobs by *A. flavus* AUMC 10331 were calculated using birchwood xylan and the K_m and V_{max} were found to be 12.18 mg.ml⁻¹ and

204.1 IU.min⁻¹ respectively. This K_m value was higher compared with other xylanases of *A. fumigatus* MA-28 (Bajaj & Abbass 2011), *A. foetidus* (Shah & Madamwar 2005), *A. ficuum* AF-98 (Fengxia et al. 2008) and *Trichoderma harzianum* strain T4 (Franco et al. 2004) and it was near to that of *Trichoderma inhamatum* (14.5 mg/ml) on oat spelt xylan (Silva et al. 2015).

Regarding substrate specificity of the enzyme produced by *A. flavus* AUMC 10331 from corn cobs in SmF, the crude and purified enzymes showed high specificity towards oat spelt xylan compared to birchwood xylan. The purified enzyme did not act on CMC compared with the crude and this may be due to that the purification process of xylanase excluded other enzymes found in the crude extract. Further study should make use of these results to produce xylanase enzyme on a large and industrial scales for different applications.

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