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Fermentation and saccharification of agro-industrial wastes: A cost-effective approach for dual use of plant biomass wastes for xylose production

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ABSTRACT

Industrial enzymes particularly xylanase have more demand due to its vast commercial application in paper and pulp, food, animal feed industries as well as for bioconversion of lignocellulosic waste into valuable chemical products. Xylanases are hydrolase enzyme that catalyzes the β-1,4 linkages in heteropolysaccharide xylan which ultimately yields xylose monomers Microbes mainly fungi are used to synthesize xylanase to catalyze xylan into its component sugars that are further utilized by other microorganisms for consecutive fermentation into biofuel. The current study was focused on the dual use of different agro-industrial waste matrices under submerged fermentation to synthesize xylanase using *Aspergillus niger* KIBGE-IB36 that also produce xylose after enzymatic pretreatment process. Among all agricultural wastes, high titer of xylanase production (3071 Umg^{-1}) was observed when 1% wheat bran was used. The zymographic analysis revealed multiple xylanase protein bands. To pre-treat agricultural wastes partially purified enzyme was used for different intervals of time showed increasing pattern of reducing sugars. After enzymatic saccharification, agricultural wastes were analyzed for thin layer chromatography (TLC) and scanning electron microscopy (SEM) that showed xylose production and maximum change in the surface morphology of all agricultural wastes, respectively. The present study revealed the use of fungi in low cost saccharification process which successfully developed the dual use of agricultural waste to produce xylanase and xylose after enzymatic pretreatment of xylanase that have potential to be use in food and pharmaceutical industry. Xylose can also serve as the most energy-efficient source for biofuel production.

1. Introduction

Each year a huge amount of lignocellulosic wastes is spawned through different industrial processes which includes paper and pulp, breweries, timber and textile industries. But the clearance of these wastes is a flattering problem because these wastes are causing environmental pollution, taking huge space and are mostly disposed-off by burning (Akhavan Sepahy et al., 2011). The use of wide variety of industrial waste to produce hydrolytic enzymes into fermentable sugars and biofuel is an appropriate method specially to produce xylanases and cellulases. This technique favors the natural environment and reduces the release of toxic compounds (Facchini et al., 2011). Lignocellulosic biomass is usually composed of cellulose, hemicellulose, and lignin (Mamma et al., 2008). The main hemicellulosic heteroglycan is xylan contain a long chain of β-1,4 linked xylopyranose residues (Senthilkumar et al., 2005). For the degradation of xylan, two main xylanolytic enzymes (endoxylanase and β-xylosidase) are employed to break the xylan backbone into xylooligomers and xylose units (Behnam et al., 2016). Xylanase have various industrial applications including animal feed, food, biofuel, textile, pharmaceutics and bio-bleaching of paper and pulp (Izidoro and Knob, 2014; Kheng & Omar 2005).

To obtain fermentable carbohydrate residues it is necessary to pre-treat lignocellulosic biomass. For this purpose, different pretreatment technologies are used which includes mechanical pre-treatment, physical pre-treatment and chemical pre-treatment (El-Tayeb et al., 2012). The main drawback of these treatments is delignification by splitting ester bonds crosslinking lignin and xylan which in turn increase the polarity of biomass (Safari Sinegani et al., 2005). On the other hand, biological pre-treatment of agricultural waste using microbial enzymes is more appropriate technique for improved digestibility of agricultural

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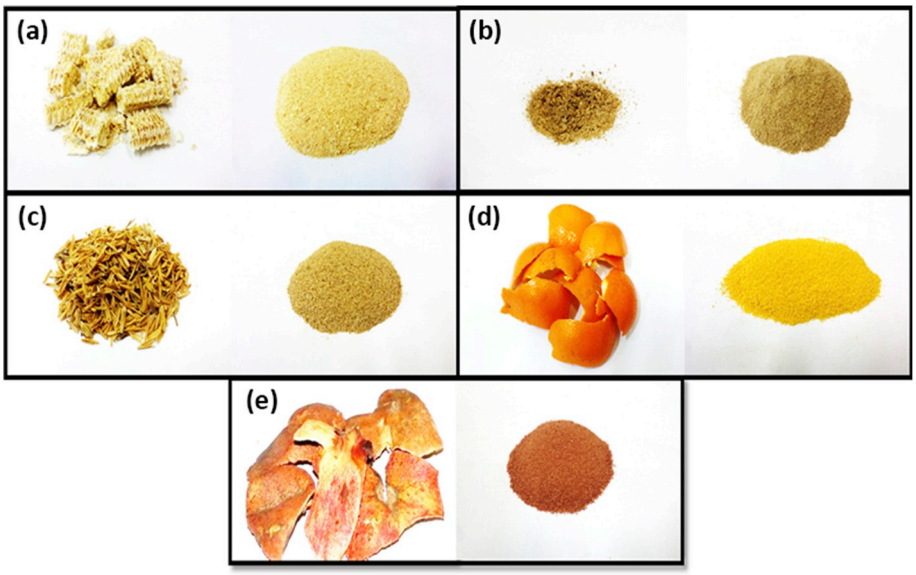

Fig. 1. Agricultural wastes used as a substrate to produce xylanase and saccharification. (a) Corncob (b) Wheat bran (c) Rice husk (d) Orange peel (e) Pomegranate peel.

Table 1
Production of xylanase using different agricultural waste from *Aspergillus niger* KIBGE-IB36.

Agricultural Waste	Specific activity (Umg^{-1})		
	($0.05gL^{-1}$)	($0.1gL^{-1}$)	($0.2gL^{-1}$)
Corncob	1434 ± 28.68	1824 ± 36.48	2187 ± 43.74
Wheat bran	2476 ± 49.52	3071 ± 61.42	1245 ± 24.9
Rice Husk	408 ± 8.16	501 ± 10.02	978 ± 19.56
Orange Peel	308 ± 6.16	378 ± 7.56	891 ± 17.82
Pomegranate Peel	391 ± 7.82	439 ± 8.78	721 ± 14.42
Potato Starch	Nil	Nil	Nil

All the experiments were performed in triplicates and the results expressed are the mean values of all experimental setup (n = 3). Mean ± S.D.

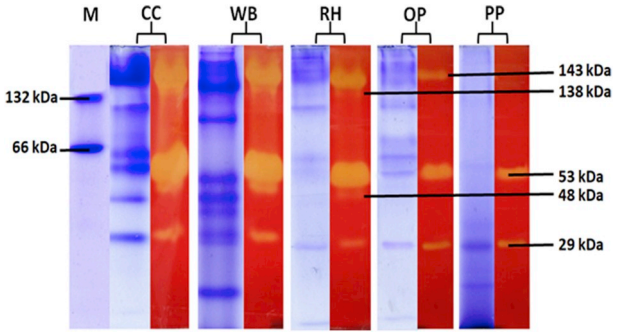

Fig. 2. Protein profile and zymographic analysis of xylanase. M = Marker BSA (bovine serum albumin), Monomer = ~66 kDa, Dimer = ~132 kDa; CC= Corn cob; WB= Wheat bran; RH = Rice husk; OP= Orange peel; PP= Pomegranate peel.

waste (Lakshmi et al., 2009).

To produce xylanolytic enzymes several microorganisms like bacteria, yeast and fungi are used. Among them, filamentous fungi (mainly *Aspergillus niger*) are considered as a promising candidate to produce extracellular enzymes due to its abundant nature, less dietary requirements and high yield of xylanase production (Pullan et al., 2014). The genome of *Aspergillus niger* translates and expresses many carbohydrate active enzymes (CAZymes) to break down plant cell wall polysaccharides after cultivation on lignocellulosic substrates which are significant feedstock for biofuel production, such as wheat bran, corncob, rice husk, sugar cane bagasse etc. (van Munster et al., 2017).

Currently, there has been no reliable technology is investigated so far for the pretreatment of wheat bran using different process in a large or laboratory scale for making industrial material. As, lignocellulosic biomass are biodegradable and serve as an economical source for enzyme production, the current study is designed to analyze the production of xylanase by *Aspergillus niger* KIBGE-IB36 using different industrial wastes and to determine the best lignocellulosic material for optimum production of xylanase and its applicability of saccharification into xylose that can be use in biofuel production and pharmaceutical industry.

2. Materials and methods

2.1. Microorganism

This study was carried out using filamentous fungi i.e. *Aspergillus niger* KIBGE-IB36 [GenBank: KF905650] which was isolated previously (Pervez et al., 2015). The *Aspergillus niger* KIBGE-IB36 was sub cultured routinely on PDA agar medium and stored at 4 °C for further use.

2.2. Agricultural wastes

To produce xylanase five different agricultural waste substrates were used namely corncob (CC), wheat bran (WB), rice husk (RH), orange peel (OP), pomegranate peel (PP). In addition, potato starch was also used as a non-xylan carbon source (control). All agricultural products were purchased from local market in Karachi, Pakistan. Initially, all the wastes were air dried, milled in domestic blender and sieved to get small sized substrate (0.075–80 mm) for maximum production of xylanase (Fig. 1).

2.3. Inoculum size and submerged fermentation (SMF)

Spores were transferred aseptically from PDA slant of five days old culture using sterile needle in 10.0 ml sterile distilled water containing 0.1% Tween-20. Standardized spore suspension of 10^6-10^8 spores ml^{-1} was serially diluted up to 10^{-5}. From this diluted suspension, 0.1 ml was transferred into 10.0 ml starter culture and incubated at 30 °C for 3 days.

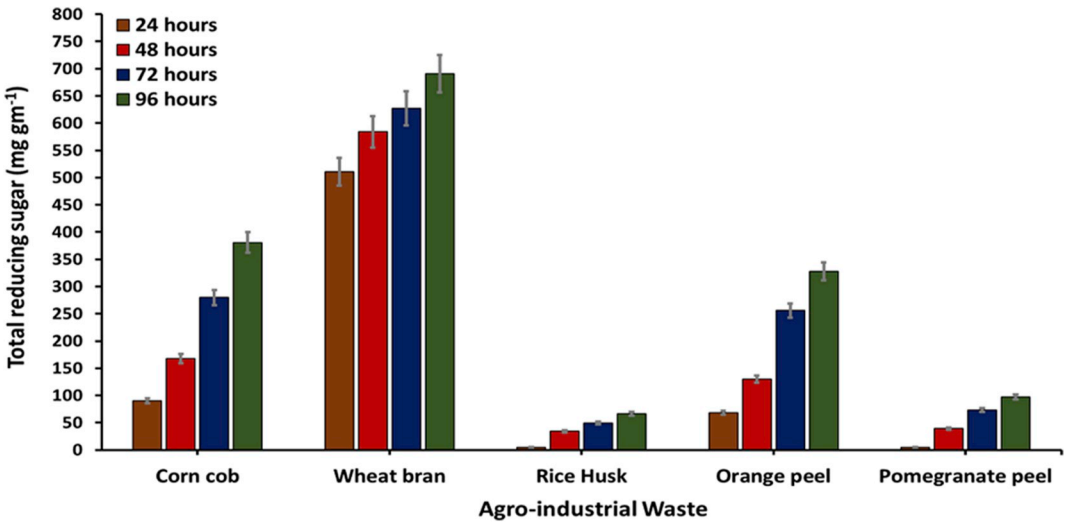

Fig. 3. Production of reducing sugars using enzymatic pretreated ago-industrial waste. All the experiments were performed in triplicates and the results expressed are the mean values of all experimental setup (n = 3). Mean ± S.D.

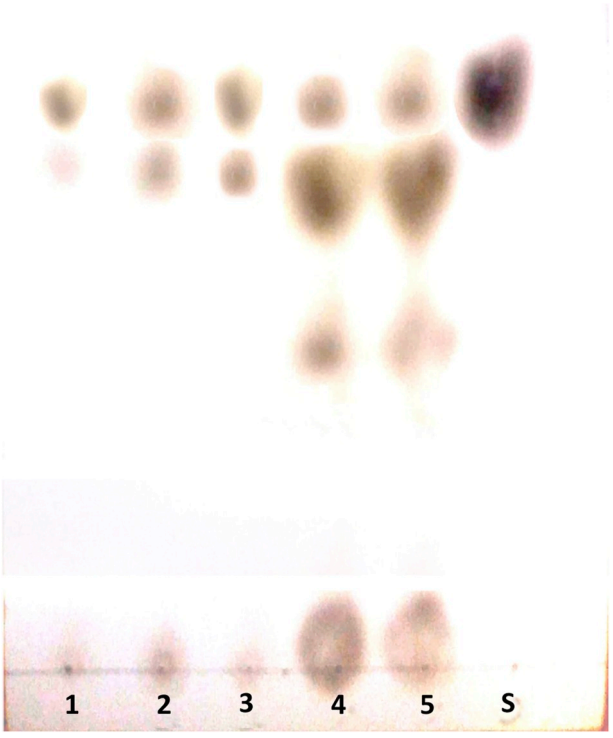

Fig. 4. Thin layer chromatography of agro-industrial wastes after enzymatic pretreatment, 1: corncob; 2: wheat bran; 3: rice husk; 4: orange peel; 5: pomegranate peel; S: standard xylose.

The fermentation was carried out using modified Czapek medium (Javed et al., 2017) with 5,10 and 20gL^{-1} concentration of each substrate. The fermentation medium was autoclaved at 121 °C for 15 min and fungal spores were incubated at 30 °C, under static condition for six days.

2.4. Enzyme separation

The extracellular enzyme was separated after six days of incubation period by centrifugation at 4000 rpm for 30 min (4 °C) and filtered through 0.45 μm Whatman No.1 filter paper. Finally cell free filtrate was used for enzyme activity and total protein estimation (Javed et al., 2017).

2.5. Enzyme assay

The enzyme activity of xylanase was determined according to the method of Miller (1959) using 3,5, dinitrosalicylic acid taking xylose as a standard. One unit of xylanase is defined as the amount of enzyme that released 1 μmol of xylose in unit time under standard assay condition.

2.6. Total protein determination

The total protein of culture filtrate was estimated by Lowry's method using bovine serum albumin (BSA) as standard (Lowry et al., 1951).

2.7. Partial purification of enzyme

After fungal biomass removal, the cell free filtrate was partially purified using 40% saturation of ammonium sulfate and dialyzed against the buffer (citrate phosphate pH 5.0) at 4 °C left overnight at same temperature using dialysis tubing (12,000 KDa cut off, Servapore). The dialyzed enzyme was kept at 4 °C for further use. (Bibi et al., 2019).

2.8. Electrophoretic analysis

Partially purified enzyme was subjected to native polyacrylamide gel electrophoresis (12%, w/v) by the method of Laemmli (1970) using bovine serum albumin as standard. Protein bands were detected by coomassie brilliant blue R-250 staining method. Xylanase activity in PAGE gels was detected by Congo red method with slight modifications. Briefly, 0.1% beech wood xylan was incorporated in 12% native PAGE gel. After electrophoresis, the gel was rinsed with deionized water and incubated in citrate phosphate buffer (pH 5.0) for 30 min at 50°C. The gel was then washed with deionized water, stained with Congo red dye (0.8%) and finally de-stained with NaCl (1 M) until clear hydrolyzing bands were visualized. The molecular weight of xylanase was estimated by using Quantity One software.

2.9. Application of partially purified enzyme on lignocellulosic biomass

To investigate the effect of xylanase on lignocellulosic substrate 1 ml of 2.5gL^{-1} of each substrate (CC, WB, RH, OP, PP) in 10 mM citrate phosphate buffer (pH 5.0) was used to react 0.1 ml of enzyme at 50 °C

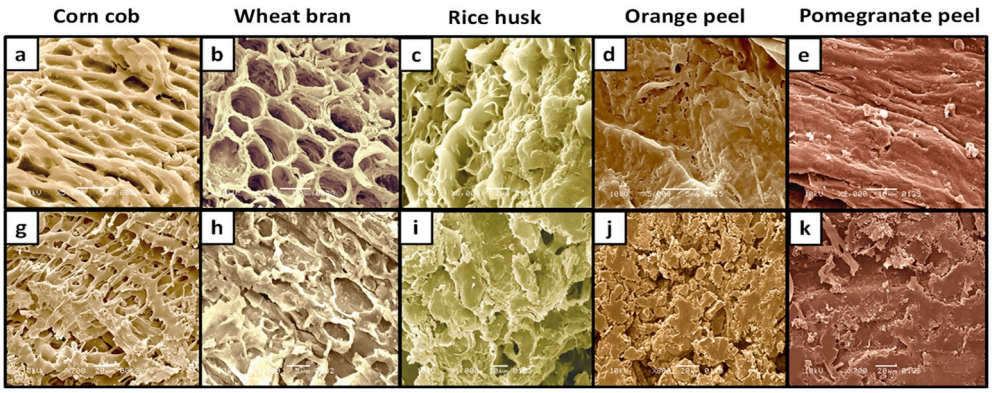

Fig. 5. SEM analysis of agro-industrial wastes. (a–e) before enzymatic pretreatment (f–k) after enzymatic pretreatment.

with mild shaking. The total reducing sugar were measured after every 24 h i.e. 24, 48, 72, and 96 h of incubation by DNS method (Eken-Saraçoğ;lu et al., 1998).

2.10. Thin layer chromatography (TLC)

Xylanase hydrolyzed end-product was analyzed by thin layer chromatography (TLC) using 96 h enzymatic pre-treated agricultural wastes sample. After enzymatic pre-treatment all the samples were centrifuged at 3000×*g* for 15 min to obtain clear hydrolyzed sample. Samples were further concentrated after air drying. Sample (10 µl) were spotted on thin layer chromatography (TLC) plate (TLC Silica gel 60 F_{254} aluminum sheets, Merck) using micropipettes and air dried. The solvent system used for resolving sugars comprised of *n*-butanol- acetic acid-water (5:3:1, v/v/v) and were detected after spraying H_2SO_4 (20%, v/v) and visualized after heating the plates at 100 °C (Kumar et al., 2018).

2.11. Scanning electron microscopy (SEM)

SEM was carried out according to Ramarajan and Manohar (2017) using treated and untreated agricultural wastes. Briefly, enzymatic pre-treated agricultural waste was centrifuged and air dried. The air-dried samples were then sputter-coated with gold, using an Auto cutter (Model JFC-1500 Jeol, Japan) for SEM (JSM 6380A Jeol, Japan) analysis.

3. Results and discussion

3.1. Production of xylanase

The increasing expansion of agricultural wastes activity has led to the accumulation of a large quantity of lignocellulosic residues all over the world. The structural composition of hemicelluloses is varying from plant to plant. This diversity stimulates remarkable variation in the catalysis of xylan (Chen, 2014). Choosing suitable substrate is a main step for high production of xylanase in submerged fermentation. In addition, the substrate not only worked as a carbon and energy source but also generating essential inducing compounds for the organism (Ghoshal et al., 2015). Different substrates have been utilized by different researchers to produce xylanase. Previously, *Aspergillus niger* KIBGE-IB36 was found to be the most promising candidate for high production of xylanase in submerged fermentation using corncob xylan as a substrate (Javed et al., 2017). In this study corncob, wheat bran, rice husk, orange peel, pomegranate peel and potato starch were augmented into the medium which showed different spectrum of xylanase synthesis except potato starch. Xylanase production was not observed in the presence of potato starch. It is reported that potato starch has less amount of hemicellulose (7%) which is not enough for fungi to utilize for xylanase production (Hoff and Castro, 1969). On the other hand, among different agricultural wastes high yield of xylanase was produced using wheat bran as a substrate. Xylanase activity was found to be maximum (3071 Umg^{-1}) when 1% wheat bran was used as a substrate while at high concentration lower yield of xylanase was achieved (Table 1). It might be due to inability of fungi to assimilate high concentration of agricultural substrate. Previously, de Alencar Guimaraes et al. (2013) also reported that 1% wheat bran was the best carbon source for xylanase production by *A. niger* that produced 12.76 U/mg of enzyme.

Wheat bran is an abundant and cheap dietary source of minerals, fiber, vitamins and bioactive compounds. It contains 53% of dietary fiber which comprise of xylan, lignin, cellulose, galactan and fructans (Reisinger et al., 2013; Andersson et al., 2014). It served as broad substrate source of carbon and nitrogen for microorganisms. It has been reported that wheat bran has better characteristic air flow, free particle binding and have ability to penetrate by mycelia (El-Shishtawy et al., 2014). Therefore, it showed maximum xylanase production which is a better prospect economically in fermentation processes. On the other hand, corn cob is the most important renewable raw material (Bahcegul et al., 2013). It is generally composed of hemicellulosic arabinoglucuronoxylan which has the potential to be use in the production of biofuels (Pointner et al., 2014). Hence, it is an attractive substrate for production of xylanase enzyme. While rice husk is mainly comprised of xylan (28%) and xylose (23%) (Wong et al., 1988). High value products can be produced by consuming orange peel and pomegranate peel waste as a possible valuable cheap cost resource (Balu et al., 2012). It is reported that orange peel waste contains high content of soluble sugars, cellulose, hemi-cellulose, and pectin as the most important components (Rivas et al., 2008) while, pomegranate peel contains pectin, hemicellulose, cellulose and lignin (Ibrahim, 2013).

It is reported that in submerged fermentation, enzymes synthesized by microorganisms are highly influenced by different parameters such as carbon sources, temperature, pH, and operating parameter such as incubation time. In the present study, the result indicates that carbon is the main influential factor to produce xylanase. Therefore, wheat bran is a convenient substrate to produce xylanase by *Aspergillus niger* KIBGE-IB36.

3.2. Detection of xylanase by in situ electrophoresis

After partial purification of xylanase using 40% ammonium sulfate xylanolytic activities were analyzed using *In-situ* gel electrophoresis which revealed multiple xylanase protein bands. In this study, corncob, wheat bran and rice husk induced maximal number of xylanases (142,138, 53, 48, 29 kDa) as compared to 3 bands (143, 53, 29 kDa) and 2 (53, 29 kDa) bands induced in orange peel and pomegranate peel respectively (Fig. 2). Although, three different xylanases were commonly observed in all agricultural wastes except in pomegranate peel where only two types of xylanases expressed, but higher intensity in corncob, wheat bran and rice husk indicated better expression of these

multiple xylanases that also reflect the high activity observed in enzyme extract. It has been reported that several filamentous fungi can degrade xylan efficiently as these microorganisms have multiple xylanases of various families. In addition, the type of substrate also influenced the number and intensity of expressed multiple forms of xylanase (Liao et al., 2012; Pollet et al., 2009). Moreover, comparative number of different xylanases produced was reliant on the nature of carbon source used (Badhan et al., 2004). This is because plant have a diverse structure and composition of xylan and xylanases are not equally accessible to all the xylosidic linkages present in heteroxylan. Hence, to completely and efficiently cleave the complex xylan polysaccharide, multiple xylanases with distinguish biochemical characters and specific activities are required (Liao et al., 2015).

3.3. Application of partially purified enzyme on lignocellulosic biomass

The global climate change alarming the subsequent requirement to reduce greenhouse gases emissions by using bioethanol as an energy source. To address this problem exploitation of lignocellulosic biomass can be considered as a cheap and eco-friendly material to produce bioethanol. Hemicellulose mainly xylan is a branched hetero-polymer comprising of pentose (D-xylose and D-arabinose) and hexose (D-mannose, D-glucose and D-galactose) sugars (Uday et al., 2016). For bioethanol production, it is necessary to convert plant cell wall carbohydrates into fermentable sugars using different pretreatment technologies (MOSIER, 2005). It is reported that enzymatic pretreatment processes are mild and environment friendly (Maurya et al., 2015). Precise knowledge and research are required about specific ligno-cellulytic enzymes that are involved in efficient hydrolysis of agricultural wastes. In the present study, five different agricultural wastes (CC, WB, RH, OP, PP) were pretreated with partially purified xylanase at 50 °C for 24, 48, 72, and 96 h with initial concentration of 2.5gL^{-1}. The samples were withdrawn periodically for the analysis of reducing sugars. After enzymatic pretreatment increasing pattern of reducing sugars was observed (Fig. 3). Xylanases are important hydrolyzing enzymes that saccharifies xylan into xylo-oligosaccharides and xylose units (Chanwicha et al., 2015). Xylose is the second-most abundant sugar after glucose and are widely used in the production of diabetic sweetener, biofuels, xylitol and other useful chemicals (Banka et al., 2014).

All the agricultural wastes used for saccharification were prone to enzymatic hydrolysis as shown by a significant increase in the production of reducing sugars. The high saccharification yields was observed in wheat bran whereas, the low yields of saccharification were obtained from rice husk and pomegranate peel. It might be due to the high hemicellulose and low lignin content of wheat bran compared with rice husk and pomegranate peel. Moreover, lignin may limit the access of the hemicelluloses to hydrolytic enzymes due to its less susceptibility to chemical and enzymatic hydrolysis (Sakakibara et al., 2009).

Previously, lignocellulosic biomass such as wheat straw, rice straw, sugarcane bagasse, barley, and timothy grass, woody raw materials, forest residues, softwoods and paper pulps have been widely studied for biofuel production after pretreatment (Loow et al., 2015; Akhtar et al., 2016). It is reported that the saccharification rate of lignocellulosic biomass using fungal isolates were found to be 2-fold (Taha et al., 2015). Whereas, in another study the hydrolysis yield of 82% was achieved using white-rot fungi *Irpex lacteus* after 28 days of biological pretreatment of corn stalks (Du et al., 2011).

3.4. Thin layer chromatography (TLC)

To detect the end-product of xylanase after pre-treatment, thin layer chromatography was performed that showed xylose as a major end-product of all agricultural waste's hydrolysis. These results indicated that xylanase have efficiently hydrolyzed the substrate that ultimately breakdown the internal linkages to produce xylose that can be use in various industrial applications (Fig. 4).

3.5. Scanning electron microscopy (SEM)

After saccharification of 96 h of all five agricultural wastes, the reaction mixture was centrifuged and air dried for scanning electron microscopy (SEM). The topographical structure of all agricultural wastes without pretreatment was smooth, ordered and the texture was relatively hard. While after enzymatic pretreatment the SEM of corncob, wheat bran and orange peel revealed clear degradation of structure due to efficient hydrolysis by xylanase with maximum change in the surface morphology with cracks, loosened, and micro-porous surface. On the other hand, rice husk and pomegranate peel showed partial degradation with slight crimp on the surface (Fig. 5). Hence, the present qualitative study established the association of xylanase not only as a xylan hydrolytic enzyme but also attributed in the modification of structure and surface of agro-industrial wastes.

4. Conclusions

Agricultural wastes are the cheap source to produce valuable products and enzymes. Unfortunately, a large amount of feedstock is disposed of without any processing. In the current study, dual use of agro-industrial wastes was successfully developed to produce enzyme as well as fermentable sugars. It was observed that by using cheap source of substrate like wheat bran and corn cob we can synthesize xylanase that can also utilized in the pretreatment of agricultural wastes to obtain xylo-oligosaccharides that can be potentially used for biofuel production. Further research needs to be done for biofuel production using these xylo-oligomers.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101341.

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