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Production of antioxidant xylooligosaccharides from lignocellulosic materials using *Bacillus amyloliquifaciens* NRRL B-14393 xylanase

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ABSTRACT

The present study aimed to evaluate the enzymatic production of XOS, antioxidant activities together with total phenolic contents from different lignocellulosic materials and birchwoodxylan using both crude and pure forms of *Bacillus amyloliquifaciens* NRRL B-14393 xylanase respectively. The mode of action of the pure xylanase was studied by HPLC and the end products analysis of birchwoodxylan revealed that xylose, xylobiose and xylotriose were the only end products. The resulted XOS mixture exhibited potent antioxidant activity of 87.36 % and high total phenolic content of 182.88 mg GAE/ liter of extract. XOS were obtained from different alkali pretreated agricultural residues by the crude xylanase action with a yield in a range from 54.4% to85.5%.Maximum scavenging abilities and total phenolic contents of XOS mixtures of all the tested materials were determined. XOS mixtures of orange and mango peels revealed relatively higher antioxidant activities (96% and 76.84%, respectively) and total phenolic acid contents (156.32 and 133.74 mg GAE/ liter of extract, respectively) compared to the other XOS mixtures. In conclusion, *Bacillus amyloliquifaciens* NRRL B-14393 xylanase could be a promising source for production of XOS with high phenolic contents and antioxidant activities.

INTRODUCTION

Currently, research on converting the agricultural byproducts into value added products is actively undertaken to produce different economically important biomolecules; one of them isxylooligosaccharides (XOS). These oligomeric carbohydrates, a class of non-digestible food ingredients having a degree of polymerization (DP) of 2-6, is produced during the hydrolysis of xylan, the most abundant component of the plant hemicelluloses (Brienzo *et al.,* 2010). XOS are indigestible by gastric or pancreatic enzymes, but able to be utilized by selected group of beneficial gut microflora associated with several health physiological benefits including reduction of blood cholesterol, maintenance of gastrointestinal health, reducing the risk of colon cancer, beneficial to type 2 diabetes mellitus, increase of mineral absorption (Ando *et al.,* 2004; Swennen *et al.,* 2006; Mussatto and Mancilha, 2007; Sheu *et al.,* 2007). Additionally, XOS are valuable food sweetener of prebiotic, non-carcinogenic and antifreezing activities so it could be incorporated as additive in beverages and fermented dairy products (Crittenden and Playne, 1996; Kabel *et al.,* 2002; Yang *et al.,* 2005). XOS are mainly produced by acid hydrolysis of hemicellulosic materials, which is considered environment unfriendly (Vazquez *et al.,* 2000). The other approaches involve trans-glycosylation reactions viaglycosyltranferases and glycosynthase β-xylosidases. These processes are expensive due to purification steps involved and need pure monosaccharides as raw materials. Due to high cost of production, its use in functional foods is uneconomical (Mussatto and Mancilha, 2007). A more developed process for obtaining purified XOS are necessary.

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The enzymatic production of XOS is considered a promising alternative strategy compared to chemical processes due to the production of easily recovered, highly pure XOS with a fewer undesirable byproducts (Akpinar *et al.,* 2007; Zhao *et al.,* 2012). Xylanases (E.C. 3.2.1.8) are hemicellulases responsible for the degradation of β-1,4xylan in a random fashion, yielding a series of linear and branched oligosaccharide fragments (Bhat and Hazlewood, 2001). It has been shown in our previous papers (Rashad *et al*, 2015 a,b) that the crude and pure enzymes from *Bacillus amyloliquifaciens* NRRL B-14393 are endowed with a number of promising properties that enhanced their industrial potential. Therefore, this study was aimed to produce XOS from different lignocellulosic materials and birchwoodxylan. Furthermore, the antioxidant activities and total phenolic contents of the produced XOS were evaluated.

MATERIALS AND METHODS

Plant material

Water hyacinth plant (WH) was collected in winter season from Mansoureya canal, Giza, Egypt. Its roots were cut off and the green parts (stem and leaves) were thoroughly washed with tap water, sliced, crushed in a mixer to small pieces 0.5-1 cm and store at 4°C till use. Then this substrate was used as a sole carbon source for the production of the xylanase.

The agroindustrial wastes

Mango peels, orange peels and sugarcane bagasse were obtained from juice processing shops in the local market. Corn cobs, wheat bran, rice straw and saw dust were purchased from the local market. All these materials were dried in an oven at 60 °C till constant weight for each one was achieved (A.O.A.C. 1995), then mechanically ground (except wheat bran) in an electric miller and sieved to a particle size ranged from 10 to 200 mesh.

Experimental organism

Bacillus amyloliquifaciens NRRL B-14393was obtained from Agricultural Research Service, Peoria, Illinois, USA. The strain was maintained on nutrient agar slant containing (3g/l beef extract, 5g/l peptone, 8g/l sodium chloride and 15g/l agar), then stored at 4 ℃ and sub-cultured monthly. Inoculum was developed by transferring a loopful of stock culture into a sterile nutrient medium and incubated at 37 °C on a shaker at 200 rpm for 24 h.

Production and purification of xylanase

Ten gram of fresh water hyacinth plant (leaves and stems) supplemented with 0.5 % (w/w) sucrose (initial pH 6; 95% moisture level)was placed in 100-ml Erlenmeyer flasks and autoclaved at 121 °C for 15 min, cooled and then inoculated with 7% of *B. amyloliquifaciens* inoculum. The inoculated medium was then incubated statically for 24 h. at 35°C as previously prepared (Rashad *et al.,* 2015a).

The enzyme was extracted from the fermented matter with 10-fold (v/w) distilled water by shaking (200 rpm) at 30ºC for 60 min (Yang *et al.,* 2006). The resultant slurry was filtered through a wet muslin cloth and centrifuged at 10,000xg for15 min. Finally, the extracts were collected and considered as a source of crude enzyme.

The crude enzyme purification was carried out on DEAE-Sepharose ion-exchange column followed by gel filtration chromatography on Sephadex G-100 (Rashad *et al*., 2015b) and used in this study.

Xylanase assay

Xylanase activity was measured by incubating 1.0% (w/v) birch wood xylan in 0.05 M acetate buffer (pH 5.5) and an appreciable amount of diluted enzyme extract (0.1 ml) in a total volume of 0.3ml at 50°C for 60 min (Rashad *et al*., 2002). The release of reducing sugar was measured as xylose by Somogyi-Nelson method (Nelson, 1944; Somogyi, 1952) using xylose as a standard. One unit (U) of xylanase is defined as the amount of enzyme that releases 1 µmol xylose/min under the assay conditions. The specific activity is expressed as units per mg of protein.

Protein determination

For specific activity determination, protein was measured by Lowry *et al.*(1951) method using a bovine serum albumin as a standard.

Mode of action and end product analysis

Purified xylanase (0.25 mg/ml) was incubated with substrate (10 mg / ml) in5 mMtris- HCl buffer (pH 9.5) at 50 °C for various time intervals (1/2 h, 2.5h, 6 h and 24h), then aliquots were withdrawn, boiled for 5 min and filtered through a 0.45 µm membrane. The hydrolyzed reaction products were analyzed by HPLC , Shimadzu Class-VPV 5.03 (Kyoto, Japan) equipped with refractive index RID-10A Shimadzu detector, LC-16ADVP binary pump, DCou-14 A degasser and Shodex PL Hi-PlexPb column (Sc 1011 No. H706081), Guard column Sc-LcShodex, and heater set at 65^{o}C.

Twenty-microliter samples from both reaction mixtures and standards (xylose, xylobiose, xylotriose and arabinose at concentration of 0.2 mg/20 µl except for xylose which was at 4mg/µl) were injected and eluted on an amino-bonded column with acetonitrile: water (80:20) as a mobile phase at a flow rate of 1ml/min (A.O.A.C. 1997)

Xylooligosaccharides production from birchwoodxylan by pure *B. amyloliquefaciens* xylanase

Purified xylanase (0.25 mg/ml) was incubated with birch wood xylan (10 mg / ml) at 5 m Mtris-HCl buffer (pH 9.5). Reaction mixture were incubated at 50 °C for 150 min. The hydrolysis was repeated several times to obtain sufficient XOS-containing liquor then concentrated by lyophilization. The concentrated XOS were dissolved in distilled water to obtain concentrations of (0.5- 20) mg ml^{-1} for further studies.

Alkali treatment of different wastes

Alkali treatment was conducted by suspending 25 g of different substrates (corn cobs, wheat bran, sugarcane bagasse, rice straw, water hyacinth, mango peels, saw dust and orange peels) in 500 ml of 0.5% sodium hydroxide solution (0.1 g NaOH/g substrate) in a 1 liter conical flask and boiling for 1 h in a water bath (Choudhury *et al.*, 1980; Rashad *et al.*, 2015a).

Xylooligosaccharides (XOS) production from agricultural lignocellulosic wastes by crude *B. amyloliquefaciens* xylanase

Pretreated corn cobs, wheat bran, sugarcane bagasse, rice straw, water hyacinth, mango peels, saw dust and orange peels (10 mg dry wt.) were dispersed in acetate buffer (pH 5.5, 0.05 M), treated with the crude xylanase in a final volume of 10 ml and incubated for 72 h at 50 ˚C followed by centrifugation (3,000×g) (Rashad *et al.*, 2013a).

The hydrolysis was repeated several times to obtain sufficient XOS-containing liquor. To the supernatant, three volumes of ethanol were added to precipitate the unhydrolyzed hemicelluloses (Bian *et al.*, 2013). Ethanol was removed from the filtrate by rotary evaporation under reduced pressure at 45 °C, and the solid fraction was freeze-dried. The concentrated XOS were used for the further studies.

DPPH radical-scavenging assay

Antioxidant activity of XOS was measured by the effect of scavenging 2,2-diphenyl-1-picrylhydrazyl radicals according to the modified methods of Mensor *et al.*(2001) and Veenashri and Muralikrishna (2011). One ml of 0.1 mM DPPH in ethanol was added to 1 ml of the XOS solution at different concentrations. The mixture was shaken vigorously and kept for 30 min in the dark at room temperature (30 ± 1°C). The absorbance was measured at 517 nm. The control was carried out with water instead of the sample solution (1:1dilution of DPPH), while ethanol was used as the blank. The capability of the sample to scavenge the DPPH radicals was calculated using the following equation:

Scavenging activity (%) = [1 - (absorbance sample / absorbance control)] x 100%

Total phenolics assay

Total phenolics were estimated as gallic acid equivalents essentially according to that described by Quettier-Deleu *et al.*(2000)with minor modification. An aliquot of 0.5 ml of the XOS solution at different concentrations was added to 7.0 ml deionized water and 0.5 ml Folin-Ciocalteu phenol reagent. After 3 min, 2.0 ml of 20% Na_2CO_3 were added and heated in a boiling water bath for 1 min comparatively to gallic acid standard. Absorbance was measured at 750 nm after cooling in darkness and the results expressed in mg of gallic acid equivalent per liter of extract.

Statistical analysis

The results are reported as Mean ± Standard error (S.E.) for at least four times experiments.

RESULTS AND DISCUSSION

Xylooligosaccharides (XOS) production from birchwoodxylan by purified *B. amyloliquefaciens* xylanase

Xylanases have attracted considerable attention because of their industrial application in the production of XOS (Vazquez *et al.*, 2000; Moure *et al.*, 2006). The mode of action of only a few homogeneously purified endoxylanases of microbial origin has been studied, either by research on kinetics, determining K_m and V_{max} values (Hiromi, 1970) or by end product analysis (Thoma *et al.*, 1970). Of the two techniques, end product analysis is more suitable for endotype hydrolyzing enzymes, while the kinetic method is particularly useful for exo-type hydrolases (Suganuma *et al.*, 1978). The hydrolysis products of birchwoodxylan by purified *B. amyloliquefaciens* xylanase were analyzed by HPLC. Figure (1a-d) illustrated the HPLC pattern of standard sugars (xylose, xylobiose, xylotriose and arabinose, respectively).

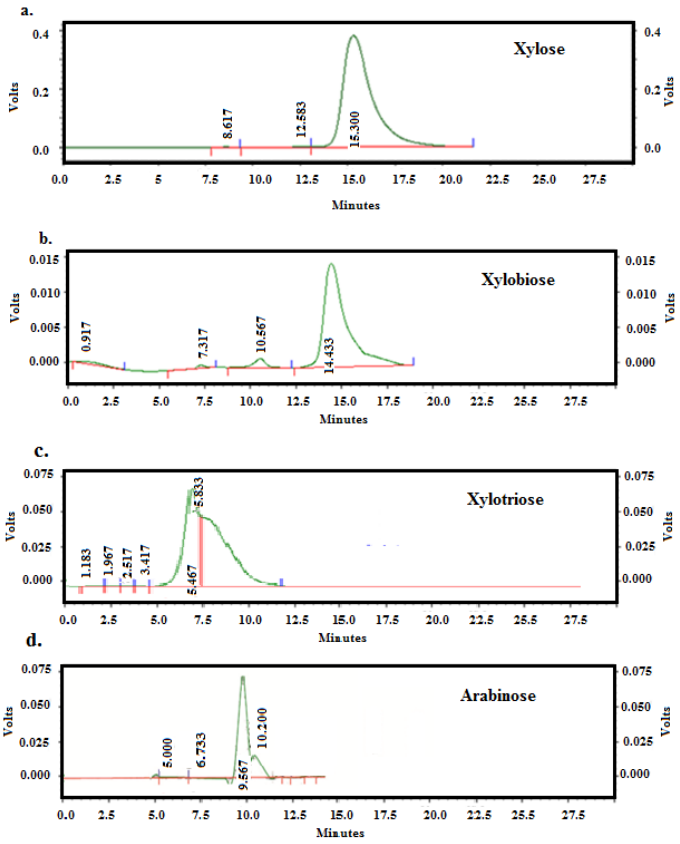

Fig. 1: Typical HPLC chromatograms of standards (a- xylose, b-xylobiose, c-xylotriose and d- arabinose standards).

The use of pure *B. amyloliquefaciens* xylanase allowed for the production of value added compounds, such as XOS from birchwoodxylan. In the reaction of xylan with pure *B. amyloliquefaciens* xylanase (Rashad *et al.*, 2015b), the end products of xylan hydrolysis in the early stage are xylose, xylobiose (Fig.2a).After 150 min, xylotriose was formed reaching to 41.94%, whereas xylose and xylobiose represented 13.22% and 44.84%, respectively (Fig.2 b).With time course of hydrolysis, the amount of xylose and xylobiose increased slowly reaching to

15.25% and 45.35%,respectively. While the amount of xylotriose decreased to 39.40% of the products produced in 6 h (Fig. 2c).When the reaction time was further extended to 24 h, the XOS (X –X3) content increased with xylobiose as the major end product (45.66%), xylose (20.67%) and xylotriose (33.67%) suggesting the applicability of the enzyme in bioconversion of birchwoodxylan into XOS (Fig.2 d).

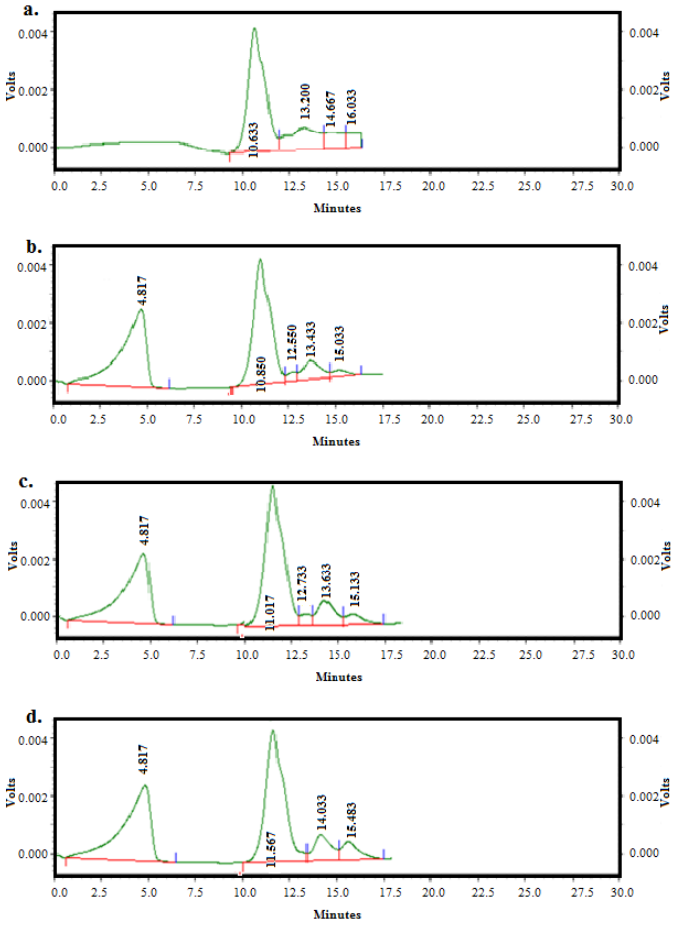

Fig. 2: Typical HPLC chromatograms of birchwoodxylan hydrolysis produced by *B. amyloliquefaciens* pure xylanase after various time interval (a-30 min, b-150 min, c- 6h , d- 24 h).

These results were in accordance with xylanases isolated from *Pleurotus ostreatus* (Fathy *et al.,* 2003), *Clostridium thermocellum* (Mandelli *et al.*, 2014) and *Streptomyces* sp. CS624 (Mander *et al.,* 2014). In the case of *Bacillus* sp. strain SPS-0 xylanase, the major products of hydrolysis of birch wood xylan were xylose and xylobiose (Bataillon *et al.,* 2000). Rashad *et al*.(2015b) reported that the *B. amyloliquefaciens* NRRL B-14393 pure xylanase molecular mass (29 kDa), substrate versatility, thermal stability in addition the mode of action in this study suggests that the enzyme is an endoxylanase of the glycoside hydrolase family 11. Thus, the *B. amyloliquefaciens* NRRL B-14393 xylanase could be used to produce XOS, which can be used as functional food additives for the selective growth of human-beneficial intestinal microflora (Laskowski *et al.,* 1993).These characteristic is common to *Penicillium griseofulvum* and *Penicillium funiculosum* GH11 xylanases that yield xylobiose and xylotriose as the main end reaction products (Tison *et al.,* 2009; Driss *et al.,* 2011).However, Kumar and Satyanarayana (2011) reported that *B. halodurans*TSEV1 xylanase hydrolyzed xylan to xylobiose-xyloheptaose (X2-X7). Also, the results in Figs.2A-D showed the absence of arabinose as hydrolysis end products. This enzyme cannot act on L-arabinosyl initiated points at β-(1→4) linkages and produce xylotriose, xylobiose and xylose as the major end products. This endo-xylanase enzyme can break down XOS as small as xylobiose (Bastawde, 1992; Fathy *et al.,* 2003).While, The products of hydrolysis of the birch wood xylan by *Gracilibacillus* sp. TSCPVG were arabinose, xylose–xylotetraose (X–X4) (Giridhar and Chandra, 2010).Moreover, Toshio *et al*.(1990), reported that because of its less calorific value, xylobiose, a derivative of XOS was used as sweetener.

Antioxidant activity and total phenolic content of the xylooligosaccharides (XOS) produced from birchwoodxylan by pure *B. amyloliquefaciens* xylanase

Scavenging ability and total phenolic content of XOS obtained after 150 min enzymatic hydrolysis at various concentrations are shown in Figs. 3 and 4. The DPPH assay showed that the antioxidant activity of XOS exhibited a dose-dependent behavior. The scavenging effect of XOS was gradually increased and reached 87.36 % at a concentration of 5 mg/ml, after which it gradually decreased to 81.16% at 15 mg/ml concentration and upto 20 mg/ml it was stabilized.

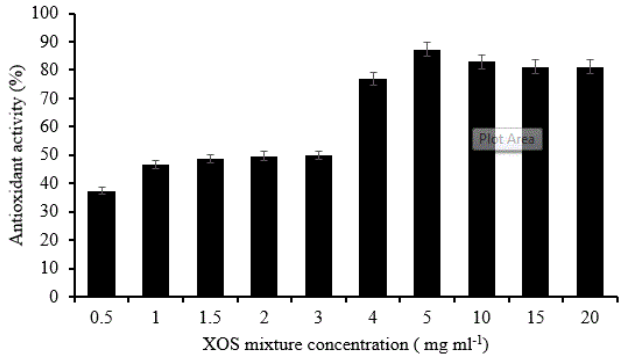

Fig. 3: Antioxidant activity against DPPH of XOS obtained by enzymatic hydrolysis for 150 min.

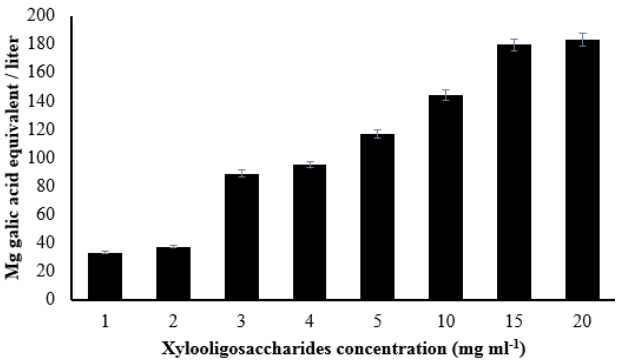

Fig. 4: Determination of total phenolic content of XOS produced from birchwoodxylan by pure *B. amyloliquefaciens* xylanase.

Even though the concentration of the total phenolic content of XOS mixture at 5 mg/ml was of 116.86 mg GAE/ liter of extract which is lower than the values (144.17-182.88 mg GAE/ liter of extract) attained at higher concentrations (10-20 mg/ml concentration), its antioxidant activity was comparatively highest (87.36 %). From this it can be assumed that the type of phenolic compounds are important more than the total phenolics for the effective antioxidant activity (Reddy and Krishnan, 2010)

Production of Xylooligosaccharides (XOS) by enzymatic degradation of agricultural lignocellulosic wastes

Considering the demand for XOS in pharmaceutical, food and other industries finding renewable, cheap xylan sources, instead of the hardwood xylan, are necessary for the preparation of XOS. Utilization of agricultural biomass as raw material provides economic and ecological benefits because it reduces the amount of waste and makes possible to recover high value added compounds. XOS were obtained from the pretreated wheat bran, orange peels, rice straw, mango peels, sugarcane bagasse, corn cobs, saw dust and WH plant by crude xylanase treatment for 72h at 50 ˚C (Fig.5).

The profile of the XOS yield was different for each substrate used, where the highest amount was obtained from enzymatic hydrolysis of wheat bran (85.50%) followed by orange peels (85%), rice straw (84.6%), mango peels (84%), sugarcane bagasse (69%), corn cobs (58%), saw dust (56%) and WH plant (54%).

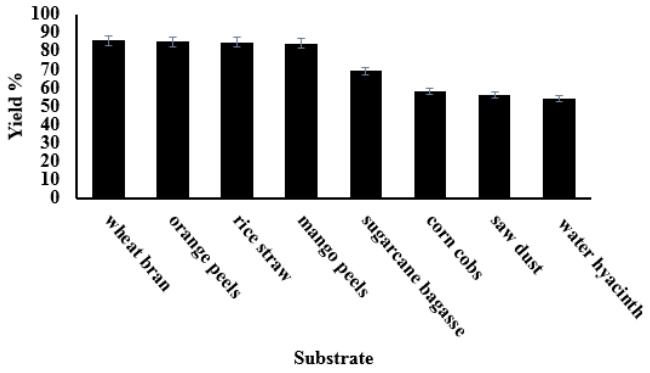

Fig. 5: Xylooligosaccharides (XOS) production from pretreated agricultural lignocellulosic wastes by crude *B. amyloliquefaciens* xylanase.

These results indicate that the greatest enzymatic conversion yield correlated to the lowest structural substrate complexity (Mandelli *et al.,* 2014). Xylanase, an endolytic enzyme cleaves the backbone of xylan randomly at the places where there are two or more contiguous unsubstituted xylose residues that serve as easy access point for cleavage. The resulting product vary in degree of polymerization ranging from xylose, xylobiose, xylotriose, to higher XOS (Yuan *et al.,* 2005).

From the results we can further generalize that wheat bran is rich in sugars compared to the other substrates resulting in high amount of XOS. Lower values of wheat bran XOS were produced by xylanases of *P. pastoris*, *B. subtilis* and *T. lanuginosus* (31, 44.25 and 40%) (Jun *et al.,* 2009; Reddy and Krishnan, 2010; Veenashri and Muralikrishna, 2011), respectively.

Antioxidant activities and total phenolic contents of the xylooligosaccharides (XOS) produced from agricultural lignocellulosic wastes by crude *B. amyloliquefaciens* xylanase

The in vitro antioxidant activity and total phenolic acid contents of the XOS mixtures obtained by enzymatic hydrolysis of all the residues used in this study are shown in Figs.6 and 7. The sensitivity of the scavenging abilities is determined by the strong absorption of DPPH. The best results for antioxidant activity and phenolics production content were observed for orange peels XOS mixtures (96% and 156.32 mg GAE/ liter of extract, respectively) followed by mango peels XOS mixtures (76.84% and 133,74 mg GAE/ liter of extract ,respectively) at 1000µg/ml concentration. The antioxidant activities and total phenolic contents exhibited by the other XOS mixtures ranged between (10-20%) and (11.59-49.72 mg GAE/ liter of extract) at various concentrations. Although, the percentage of XOS conversion is more in the case of wheat bran ,but the antioxidant activity and total phenolics were less because lignin phenolics are not contributing to the yield (Reddy and Krishnan, 2010).

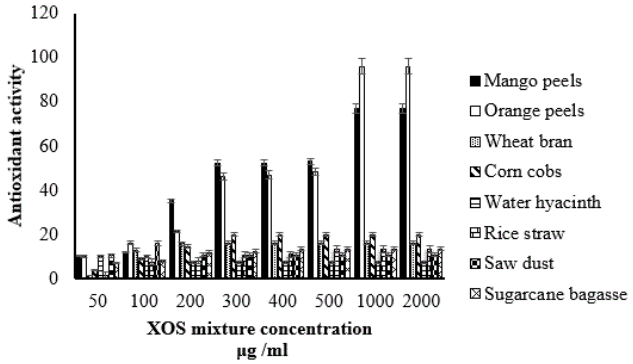

Fig. 6: Antioxidant activity against DPPH of XOS obtained by enzymatic hydrolysis of agricultural lignocellulosic wastes for 72h.

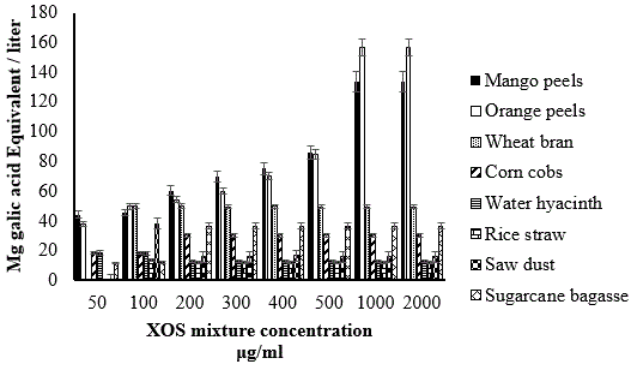

Fig. 7: Estimation of total phenolic content of the XOS produced from agricultural lignocellulosic wastes by crude *B. amyloliquefaciens*xylanase.

The XOS exhibited concentration dependent antioxidant activity. A similar behavior has been reported for other XOS, from cereal, millet brans and sugarcane bagasse **(**Veenashri and Muralikrishna, 2011; Bian *et al.,* 2013;Rivas *et al.,* 2013; Lin *et*

al.,2014).The total phenolic acid content and in addition its nature plays a significant role on the overall anti-oxidant capacity of the XOS mixtures (Veenashri and Muralikrishna, 2011).

The relatively higher antioxidant activities of XOS mixtures derived from orange peels and mango peels compared to other XOS mixtures could be explained by the presence of higher amount of total phenolic acid contents. Veenashri and Muralikrishna (2011) reported that the activity exhibited by the XOS mixture from ragi has been justified by the contents of ferulic and syringic acids.

The antioxidant activity exhibited by XOS from orange peels was higher than that of wheat bran, rice, maize, sugarcane bagasse, rice husk and bamboo bagasse XOS mixtures **(**Reddy and Krishnan, 2010; Veenashri and Muralikrishna, 2011; Bian *et al.*, 2013).The above results indicated that XOS from orange peels and mango peels can be exploited in preparing nutritional health foods.

CONCLUSIONS

Our findings demonstrated the biotechnological potential for use of both crude and pure *B. amyloliquefaciens* xylanases in the hydrolysis of agro-industrial residue by-products and birch wood xylan respectively. The bi-functionality of *B. amyloliquefaciens* xylanase allowed for simultaneous extraction of XOS and antioxidant compounds from plant biomass.

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