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Research Article

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Alkaline extraction of xylan from agricultural waste, for the cost effective production of xylooligosaccharides, using thermoalkaline xylanase of thermophilic *Anoxybacillus* sp. Ip-C.

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ABSTRACT

Microbial production of xylanase is gaining attention commercially, due to its wide industrial as well as biotechnological applications, specially in the form of thermostable xylanase. The production cost is one of the major limiting factor indicating the need for low cost production systems. Anoxybacillus sp. Ip-C, a thermophilic xylanolytic bacteria isolated from hot spring of Ladakh, was used to produce thermostable, thermoalkaline and cellulose free xylanase from cheap agricultural sources such as sugarcane bagasse, wheat husk and corn cobs. Extractions of xylan from agricultural wastes under dilute sulphuric acid and alkaline conditions were performed, and this xylan was used as substrate for production of thermoalkaline xylanase as well as valuable xylooligosaccharides. The maximum xylan recovery of 49% was found from sugar cane bagasse (10% sodium hydroxide along with steam application) in comparison to wheat husk (42%) and corn cobs (40%). Analysis of hydrolyzed culture broth showed significant amount of xylooligosaccharides (xylose 1.2 mg/ml; xylobiose 0.95 mg/ml; xylotriose 0.97 mg/ml & xylotetrose 0.78 mg/ml) production. This study not only indicated the significance of sugarcane bagasse in cost effective production of xylanase by Anoxybacillus sp. Ip-C, that may be used potentially for increasing productivity and nutrient digestibility of farm animals as well as in various industrial applications but also established its potentiality as a raw material for extraction of xylan and its conversion into xylooligosaccharides, an emerging prebiotic.

Keywords*: Sugar cane bagasse, Anoxybacillus sp. Ip-C, xylanase, cost effective, xylooligosaccharides.*

INTRODUCTION

In India, availability of agricultural waste is approximately 625 million tons annually including sugarcane bagasse, ground nut cake, rice bran, rice straw, wheat bran, cotton leaf scraps, fruits and vegetable wastes, etc[1]. Among these sugarcane bagasse (SCB) is the second most commonly used non-wood fiber plant material (content 28-30% xylan) for pulp and paper production as well as a cheap carbon source for production of industrially important enzymes, bio fuels, food supplements and others[2]. The management of these wastes effectively and economically must be given a prime priority ensuring not only in reducing the detrimental impact of the wastes to environment, but most importantly in the transformation of these wastes into useful raw materials or the production of value added products of industrial and commercial potential[3]. The use of waste agro materials as carbon sources in fermentation media is therefore become significant as a cost effective strategy for production of potential enzymes. Many bacterial as well as fungal species are reported to produce potential xylanases[4,5,6] when cultivated in presence of xylan. Biodegradation of xylan using xylanases [Endo-1,4-b-D- xylanohydrolase, EC 3.2.1.8] produces xylooligosaccharides, can be used as ingredients of functional food, cosmetics, biofuel, pharmaceuticals or agricultural products[7,8], without environmental pollution[8]. In the last decade, xylanases have

widespread potential applications in different industrial areas such as pulp bleaching, baking and brewing, animal feeding, waste-treating and bioenergy conversion[10,11].

The aim of this study is to evaluate the potentiality of some agro-wastes to serve as low-cost substrate (xylan) for production of xylooligosaccharides by a thermoalkaline xylanase of wild type *Anoxybacillus* sp. Ip-C.

Microorganism and culture media

The thermophilic *Anoxybacillus* sp. Ip-C (JF968627) was used for the production of xylanase. It's a rod shaped gram positive bacteria having growth optima of 60 °C at pH 7.0. This strain was isolated from hot spring of Ladakh, produce highly thermostable cellulase free alkaliphilic endoxylanase having optimum xylanolytic activity at 70°C temperature, pH 9.0 (Hauli *et al*. 2013)[11].

The strain *Anoxybacillus* sp. Ip-C was grown and maintained in Xylan agar plate. The mineral medium used for the production of xylanase contained gram per liter: KH_2PO_4 3.0; K_2HPO_4 2.0; $MgSO_4 \cdot 7H2O$ 0.5; $CaCl_2 \cdot 2H_2O$ 0.1; $FeSO_4 \cdot 7H_2O$ 0.05; $MnSO_4 \cdot 4H_2O$ 0.02; $ZnSO_4 \cdot 7H_2O$ 0.01; $CoCl_2 \cdot 6H_2O$ 0.02 and NH_4Cl 10.0, supplemented with 1% oat spelt xylan (2% (w/v) agar in case of xylan agar plate). The cultures were grown for 1 day at 60°C until the culture enters into its log phase, ready to use as seed culture for xylanase production. It is a 45 kDa molecular weight endoxylanase, having optimum activity at pH 9.0 and 70ºC temperature; the enzyme retain 90% of its original activity for 96 hrs at 70 ºC with a specific activity of 189.054 U/mg. V_{max} and K_m of the enzyme were found to be 13.5 µmol min^{-1} mg^{-1} protein and 4.59 mg ml^{-1}, and enhances its activity in presence of Ca^{+2}, Fe^{+2} and Mg^{+2}; whereas SDS and Hg^{+2} completely inhibit the enzyme activity, as reported by Hauli *et al.,* (2013)[11].

Alkaline extraction of xylan from lignocellulosic samples

Alkaline extraction of xylan from lignocellulosic samples was performed following a modified protocol of Hauli *et al.* (2013)[8]. The milled lignocellulosic powder samples (sugar cane bagassae, wheat husk, corn cobs) were soaked in 10% sodium hydroxide (with a ratio of 1: 10) and, kept for overnight in constant agitation at 60° C, and then were steamed at 100 °C for 3 hours. After alkaline treatment, the supernatant was recovered by centrifugation (10,000 rpm for 15 minutes) and was acidify with 12N HCL to pH 5.0. Then 1.5 volume of 95% ethanol was added to precipitate the xylan. After centrifugation, the xylan was allowed to air dry before drying in hot air oven for 4 hours at 55°C. The pellets were weighed and powdered in a mixer and stored at room temperature for further analyses. The true recovery of xylan was calculated using the following formula:

True recovery (%) = dry weight of extracted xylan (g) / weight of the sample (g) × 100.

Enzyme Production using different agro-waste extracted xylan

The enzyme production was carried out in 500 ml Erlenmeyer flasks containing 100 ml of the production medium, mineral salt medium (Hauli *et al*. 2013)[11] supplymented with 1% (w/v) of xylan, extracted from sugar cane bagasse, wheat husk and corn cobs. In the control medium, commercially available Oat spelt xylan (HiMedia) was used as carbon source. The flasks were inoculated with 100 µl of fresh culture and kept under shaking at 80 rpm, at 60 °C, for 2 days. The samples were removed at different time intervals for determining time course growth and enzyme production. The culture supernatent was collected for determination of enzyme activity. Among this three agro wastes extracted xylan sources (i.e. SCB xylan, wheat husk xylan and corn cobs xylan), the potential one was selected depending on the growth, enzyme production and protein content of these growth broth in comparison to the control production broth content pure oat spelt xylan (HiMedia).

Enzyme assay

Xylanase activity was assayed following the method of Bailey *et al.*(1992)[12] using 1% soluble oat spelt xylan as the substrate at 70°C (pH 9.0), and the reducing sugars were determined according to DNS method [13]. One unit of enzyme activity was defined as the amount of enzyme required produced 1 µmol of xylose per minute under standard assay condition.

Time course growth and xylanase production using SCB extracted xylan

The time course of growth and the production of extracellular xylanase were studied in two sets of

production broth, one is supplemented with pure oat spelt xylan (1%) and another one with sugar cane bagasse extracted xylan (1%), to determine the significance of the extracted xylan as a cost effective xylan source. Culture supernatants ware collected at different time intervals over the period of cultivation for analyzing xylanase production (through enzyme activity) along with culture growth. The culture growth was monitored my measuring OD at 600 nm. Protein content was also measured by the method of Bradford[14] using bovine serum albumin as a standard, for each production broth.

Detection and estimation of produced xylooligosaccharides from SCB extracted xylan

To detect the production of xylooligosaccharides in production broth (minimal medium supplemented with 1% sugar cane bagasse extracted xylan), small volume (100ul) of production broth was collected at periodic intervals of 24, 48 and 72 hrs. The unused xylan was precipitated with isopropanol and the precipitate was removed by centrifugation (10,000 rpm, 10 min) along with cells, and the collected supernatant containing xylooligosaccharides was concentrated in half using vacuum evaporator. 20µl of these products were applied on TLC plate (Merck) along with xylooligosaccharides (xylose, xylobiose, xylotriose, xylotetrose) as the reference standards. Chromatography was performed in duplicate by the ascending method on silica gel TLC plates (Merck) with a solvent system consisting of n-butanol, acetic acid and water (2:1:1). Xylooligosaccharides on one plate were detected by heating the plates at 120°C for about 10 min after they were sprayed with 5% (v/v) sulfuric acid in ethanol (Hauli *et al.*, 2013)[11] and from the other untreated plate the corresponding portions on TLC plate were scalped and residual sugar content was detected through standard DNS method (Miller, 1959)[13] for estimating the amount of produced xylooligosaccharides.

RESULT & DISCUSSION

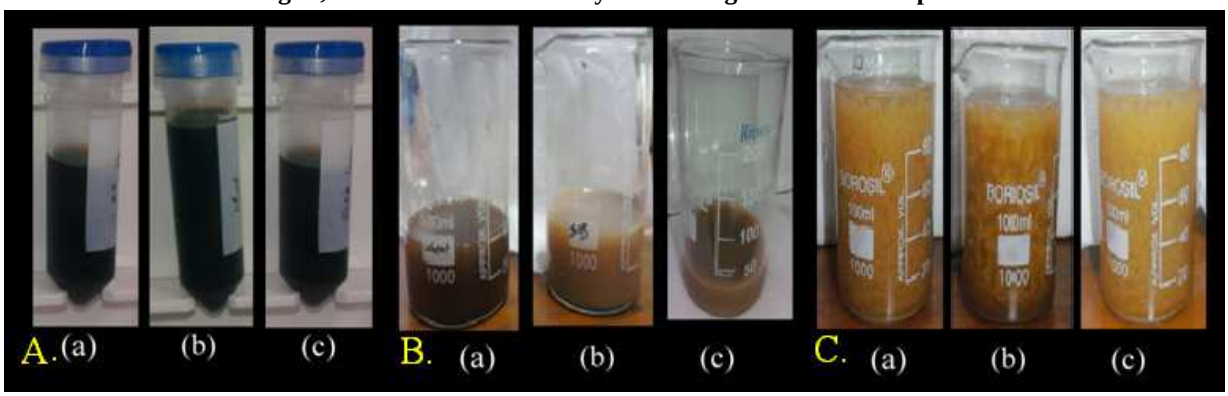
Alkaline extraction of xylan from lignocellulosic samples:

During alkaline extraction, blackish red extracts were generated after centrifuging the sodium hydroxide (along with steam) treated milled lignocellulosic powder samples (sugar cane bagasse, wheat husk, corn cobs) Fig 1A. An intense colour change (from blackish red to milky tea) was observed during acidification with 12 N HCL for lowering the extract pH to 5.0 (Fig.1B). During ethanol precipitation a notable precipitation was observed (Fig.1C). After collecting the precipitated xylan by centrifugation, it was allowed to air dry before drying in hot air oven for 4 hours at 55°C. The pellets were weighed and powdered (Fig.2) for using as a substrate for the production of xylooligosaccharides. The true recovery of xylan from different agro-waste was given in Table 1.

Table 1. Alkaline extraction of xylan from lignocellulosic samples:

Alkali treatment (%NaOH)	True recovery of xylan from different xylan source (%)		
	Sugar Cane bagasse	Wheat Husk	Corn cobs
10% with steam	49	42	40

Fig: 1; Alkaline extraction of xylan from lignocellulosic samples



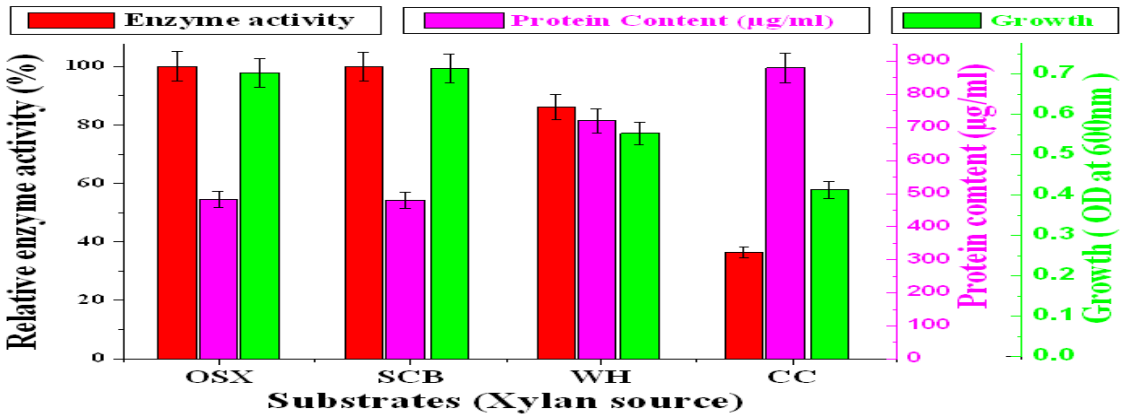
A. Alkaline extract(blackish red) from different agro-waste; B. changes in colour during pH change; C. Clear visible precipitate during ethanol precipitation; (a) refers to wheat husk extracted xylan, (b) for sugar cane bagasse and (c) refers to corn cob.

Fig: 2. Dried extracted xylan powders. (a) refers to sugar cane bagasse extracted xylan, (b) for wheat husk and (c) refers to corn cob

Production of enzyme

Among the three agro waste extracted xylan source, maximum enzyme production was found in production broth containing sugar cane bagasse extracted xylan, similar to the control production media containing oat spelt xylan (fig.3). In respect to growth and enzyme production, sugar cane bagasse extracted xylan was selected as better source of xylan for the production of xylanase.

Fig.3. Utilization of agricultural wastes for xylanase production by *Anoxybacillus* sp. Ip-C grown at 9.0 pH and 60°C for 2 days

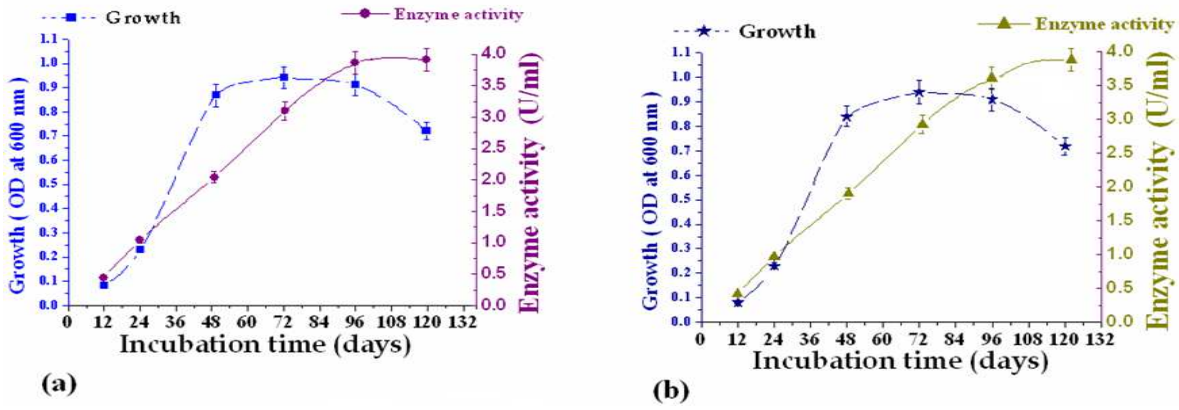


(OSX= Oat spelt xylan; HiMedia (control); SCB= Sugar cane bagasse; WH= wheat husk; CC= corn cob)

Time course growth and xylanase production

Time course growth and xylanase production was performed in two different sets using SCB extracted xylan in one and pure oat spelt xylan in another (fig.4) to determine the potentiality and applicability of SCB extracted xylan as a raw source of xylan for growth and enzyme production by *Anoxybacillus* sp. Ip-C. As the result shows the same production of enzyme in respect to growth at different time intervals in both pure xylan as well as in SCB extracted xylan, indicates the suitability of SCB extracted xylan as a substrate for xylanase production.

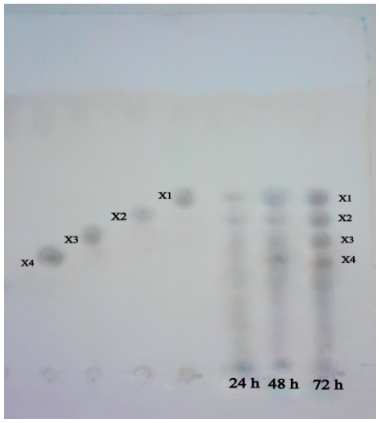
Fig. 4: Time course growth and xylanase production using SCB extracted xylan (b) and pure oat spelt xylan (a)



Time course Production and detection of xylooligosaccharides

Production of xylooligosaccharides through hydrolyzing SCB xylan by *Anoxybacillus* sp. Ip-C, during growth was detected by TLC method. Four different spots corresponds to four xylooligosaccharides was detected with respect to xylooligosaccharide standards (fig.5). Concentrations of these produced xylooligosaccharides were determined using quantitative TLC method (Table 2). Amount of produced xylooligosaccharides after 72 hours of growth (table 2), indicates the satisfactory xylooligosaccharides production by *Anoxybacillus* sp. Ip-C using SCB extracted xylan.

Fig. 5.



Thin-layer chromatography of hydrolysis products (xylooligosaccharides) obtained from SCB extracted xylan during growth at different time (24, 48 and 72 hours) interval. X1, xylose; X2, xylobiose; X3, xylotriose and X4, xylotetrose.

Table 2. Amount of produced xylooligosaccharides after 72 hours

Produced xylooligosaccharides (mg/ml) after 72 hours of growth*			
xylose	Xylobiose	Xylotriose	Xylotetrose
1.2	0.95	0.97	0.78

* All experiments are performed in triplicate and the data represented are the mean of three.

CONCLUSION

The results show that *Anoxybacillus* sp. Ip-C produces significant xylanase when cultured in media containing agro-wastes (sugarcane bagasse) extracted xylan as sole carbon sources, and produces satisfactory amount of xylooligosaccharides, an emerging prebiotic. Thus it can be concluded that sugar cane bagasse extracted xylan is an excellent carbon source for the production of thermo alkaline xylanase as well as for the production of valuable xylooligosaccharides by *Anoxybacillus* sp. Ip-C, which not only helps in recycling of agricultural organic waste but also reduces the production cost.

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REFERENCES

1. Bhosale, J.H., Sukalkar, S.R., Uzma, S.M.Z, Kadam, T.A., Production of xylanase by *Streptomyces rameus* grown on agricultural wastes: *Biotechnol. Bioinf. Bioeng.*, **1(4):** 505-512 (2011)
2. 2. Kundu, A., Karmakar, M and Ray, R.R., Simultaneous production of animal feed enzymes (endoxylanase and endoglucanase) by *Penicillium janthinellum* from waste jute caddies: *International Journal Of Recycling of Organic Waste in* Agriculture, **1:**1-13 (2012)
3. Kumar, A., Bohra, C.P., Singh, L.K., Environment, Pollution and Management. APH Publishing Co, New Delhi, 2003.

4. Beg QK, Bhushan B, Kapoor M, and Hoondal, G.S., Production and characterization of thermostable xylanase and pectinase from *Streptomyces* sp.QG-11-3: *J. Ind. Microbiol. Biotech*., **24:** 396-402 (2000)
5. Ellis, J.T and Magnuson, S.T., Thermostable and Alkalistable Xylanases Produced by the thermophilic bacterium *Anoxybacillus flavithermus* TWXYL3: *ISRN Microbiology*, doi:10.5402/2012/517524
6. Nascimento, R.P., Marques, S., Alves, L., Girio, F.M., Amaral-Collaço, M.T., Coelho, R.R.R., A novel strain of *Streptomyces malaysiensis* isolated from Brazilian soil produces high endo-β-1,4-xylanase titres: *World J. Microbiol. Biotechnol*.,**19:** 879–881 (2003)
7. Dhillon, A., Gupta, J.K., Jauhari, B.M., Khanna, S., A cellulase poor, thermostable, alkali-tolerant xylanase produced by *Bacillus circulans* AB 16 grown on rice straw and its application in biobleaching of eucalyptus pulp: *Bioresour. Technol*., **73:** 273–277 (2000)
8. Hauli, I., Sarkar, B., Roy, A and Mukhopadhyay, S.K., Ethanol production from xylose and enzymatic hydrolysate of hemicelluloses by a newly isolated yeast strain: *J. Microbiol. Biotech. Res*., **3 (4):** 54-58 (2013)
9. Sunna, A., Gibbs, M.D and Bergquist, P.L. A novel thermostable multidomain 1,4-bxylanase from '*Caldibacillus cellulovorans*' and effect of its xylan-binding domain on enzyme activity: *Microbiol*. **146:** 2947– 2955(2000)
10. Li, N., Shi, P.J., Yang, P.L., Wang, Y.R., Luo, H.Y., Bai, Y.G., Zhou, Z.G., Yao, B., A xylanase with high pH stability from *Streptomyces* sp. S27 and its carbohydrate-binding module with/without linker-regiontruncated versions: *Appl. Microbiol. Biotechnol*. **83:** 99–107 (2009)
11. Hauli,I., Sarkar, B.. Mukherjee, T., Mukhopadhyay, S.K., Purification and characterization of a thermoalkaline, cellulase free thermostable xylanase from a newly isolated *Anoxybacillus* sp. Ip-C from hot spring of Ladakh: *Research in Biotechnology*, **4(4):** 30-43 (2013)
12. Bailey, M.J., Baily, P., Poutanen, R., Interlaboratory testing of methods for assay of xylanase activity: *J. Biotechnol*., **23:** 257–270 (1992)
13. Miller, G.L., Use of dinitrosalicylic acid reagent for the determination of reducing sugar: *Anal. Chem*., **31:** 538–542 (1959)
14. Bradford, M.M., Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding: *Anal. Biochem.*, **72:** 248–254 (1976)