JEB
Website : www.jeb.co.in
E-mail : editor@jeb.co.in

Journal of Environmental Biology

ISSN: 0254-8704 (Print)
ISSN: 2394-0379 (Online)
CODEN: JEBIDP

Production, partial purification and characterization of xylanase using *Nicotiana tabacum* leaf dust as substrate

Komal P. Acharya* and Prateek Shilpkar
Department of Microbiology, M.D. Gramseva Mahavidyalaya, Gujarat Vidyapeeth, Sadra-382 320, India
*Corresponding author E-mail : **komal.ach13@gmail.com**

Publication Info

Paper received:
27 October 2014

Revised received:
21 April 2015

Accepted:
09 June 2015

Abstract

Isolated *Bacillus sp.* was used in the present study for production of xylanase from *Nicotiana tabacum* leaf dust. The strain was able to give a maximum of 1.77 Uml^{-1} xylanase activity under optimized fermentation conditions which was further increased upto 2.77 Uml^{-1} after extraction and partial purification of enzyme. After partial purification, the enzyme was characterized and it gave the highest xylanase activity at pH 7.0, when 0.2 ml enzyme was incubated with 2.0% substrate (*Nicotiana tabacum* leaf dust) for 60 min at 60^0C. Saccharification study of *Nicotiana tabacum* leaf dust with partially purified enzyme revealed that 18.4% reducing sugar was released in 20 hrs incubation, and TLC and HPTLC analysis showed that xylose and glucose sugars were obtained after hydrolysis of substrate. FTIR analysis confirmed decomposition of substrate.

Key words

Characterization, *Nicotiana tabacum,* Saccharification, Submerged fermentation, Xylanase.

Introduction

Plant cell wall is composed of cellulose, hemicellulose and lignin. While cellulose is crystalline and resistant to hydrolysis, hemicellulose has amorphous structure and is easily hydrolysed by variety of enzymes. Mixture of xylan, xyloglucan, glucomannan, galactoglucomannan, arabinogalactan or other heteropolymers is present in hemicelluloses (Verma and Satyanarayane, 2012). The above composition shows that complete degradation and waste management require number of enzymes, and xylanase is an important one among them. Xylanase is a class of enzyme which degrades linear polysaccharide β-1,4-xylan into xylose sugar. Xylanase has variety of applications like pretreatment of lignocellulosic biomass, improvement of nutritional properties of cereal-based diets in pig and poultry, flour improvement for bakery products, saccharification of wastes originated from agriculture, industrial and municipal areas, processing of pulp and fibres, enhanced bleaching of cellulose pulps (Beg *et al.*, 2001); extraction of coffee, plant oils and starch, and in clarification of fruit juices in combination with pectinase and cellulase (Aysegul, 2008); and in production of rayon, cellophane and several chemicals like cellulose esters and cellulose ethers (Subramaniyan and Prema, 2002). Xylanase release sugars from plant parts used in various industrial fermentative productions. Xylanases are produced by diverse genera and species of bacteria, like, *Bacillus subtilis* (Irfan *et al.*, 2012); *Pseudomonas* sp. XPB-6 (Sharma and Chand, 2012); *Bacillus pumilus* ASH (Battan *et al.*, 2007); *Paenibacillus curdlanolytican* strain B-6 (Pason *et al.,* 2006) and *Arthrobacter* sp. MTCC 6915 (Murugan *et al.*, 2011).

Industrially important compounds like enzymes can be produced by submerged and solid state fermentation. Each system has its advantages and limitations but submerged fermentation is preferred for production of more purified enzymes because it is easy to control, produces high yields and the cost and risk of contamination is lower (Krishna, 2005).

Choice of substrate for enzyme production ultimately governs the cost of production. For industrial production of enzymes, production cost is very important thus, solid agricultural wastes can be used as substrates to make the process cost effective. Tobacco belongs to genus *Nicotiana* and family *Solanaceae.* Leaves are economically important part of plant which are used for in manufacturing cigarettes, cigars, gutka and other smoking products. Bacteria use tobacco leaf dust as substrate, so it has to degrade the complex plant cell wall polyschharides by producing a wide range of hydrolases such as cellulases, hemicellulases and amylases (Raheman *et al*, 2014). Various interactions occurs between these enzymes and their substrates, is also required to enhance hydrolysis of substrate (Bayer *et a*l, 2000). Apart from that, now a days, demand of cellulose free xylanase enzyme has increased thus, ability of bacteria to produce various enzymes is required. In view of the above, the present study aimed at partial purification of xylanase enzyme produced by isolated bacteria, estimation of metabolites produced after degradation of substrate and to evaluate out effect of degradation on chemical structure of tobacco leaf dust.

Materials and Methods

All chemicals used during the work were of analytical grade. Brichwood xylan was purchased from Sisco Research Laboratory Pvt. Ltd., India. *Nicotiana tabacum* leaf dust was collected locally. Physico-chemical characteristics of *Nicotiana tabacum* leaf dust were analysed by standard methods and the results revealed that, the tobacco leaf dust contains total organic carbon (21.5 g%), total nitrogen (2.78 %), total phosphorus (2.65%), total potassium (32.4 ppm), total sodium (1.2%), total sulfur (0.5 %), cellulose (2.3 %), hemicellulose (15 %), lignin (11 %) and nichotin (2 %).

Xylanase producing bacterial strain: The bacterial strain of *Bacillus* sp. used in the present study was isolated and screened out from composting and dumping sites of Department of Microbiology, Gujarat Vidyapith, Sadra, Gandhinagar, Gujarat on the basis of zone of clearance produced on xylan containing agar plate and by estimation of xylanase activity using DNSA method (Miller, 1959). Xylanase production by isolated bacterial strain was enhanced by optimizing fermentation conditions and nutritional parameters, which was determined by DNSA method.

Enzyme extraction and partial purification: Enzyme produced by potent bacterial strain was extracted from fermentation sets run under optimized conditions. After fermentation, liquid medium was centrifuged at 10,000 rpm for 15 minutes. 50 ml of this supernatant was mixed with 150 ml methanol and continuously stirred for 6-7 hours followed by overnight cooling. Next day the mixture was again centrifuged at 10,000 rpm for 15 min., supernatant was discarded and remaining partially purified enzyme was dissolved in 0.1 M citrate buffer and stored under cooled condition.

Enzyme assays and protein estimation: CMCase (Ghose, 1987), Fpase (Ghose, 1987), β-Glucosidase (Mahadevan and Sridhar, 1986), amylase (Bernfield, 1955), xylanase assay (Maria and Samia, 2006) and protein content (Lowry *et al,* 1951) in partially purified enzyme was determined.

Enzyme characterization: Xylanase enzyme activity was characterized for incubation temperature (30, 40, 50 and 60^{0}C), pH (citrate buffer having pH 3,4,5 6; phosphate buffer of pH 7 and 8; and glycine-NaOH buffer of pH 9,10 and 11), concentration of enzyme (0.2, 0.4, 0.6, 0.8 and 1.0 mL), concentration of Birch wood xylan substrate (0.5, 1.0, 1.5, 2.0 and 2.5 ml of 1% substrate solution), and incubation time (10, 20, 30, 40, 50 and 60 min). At each stage xylanase activity was measured in which, the reaction mixture contained 1 ml of 1 M citrate buffer (pH 4.8), and 1.0 ml of 1 % Brichwood xylan, as substrate was added in both test and blank. Both the sets were incubated at 50^{0}C for 5 min. followed by addition of 0.5 ml active enzyme in test and incubation at 50^{0}C for 60 min. Enzyme was inactivated by heating it at 100^{0}C for 15 minutes and 0.5 ml of inactivated enzyme was added to blank and 1 ml DNSA reagent was added in both the mixtures. Mixtures were boiled at 100^{0}C for 10 min, diluted to 10 ml with distilled water, cooled to room temperature and O.D. was measured at 540 nm.

Saccharification study: Sacharification was carried out to check the efficiency of characterized enzyme to quantitative and qualitative release of reducing sugars from Nicotiana tabacum leaf dust. For this study, the substrate (1% w/v Nicotiana tabacum leaf dust) was amended as sole carbon source in the fermentation medium (composition g/L: peptone-10, NaCl-5, meat extract-3, pH-5), followed by addition of 2% of 12hr old inoculum of isolated bacterial culture and incubated at 25^{o}C for 28 hrs. At different time intervals of 4hrs the sample of culture broth was taken and centrifuged at 10,000 rpm for 15 min at 4^{0}C and the clear supernatant was used to measure the amount of reducing sugars released following DNSA method (Miller, 1959).

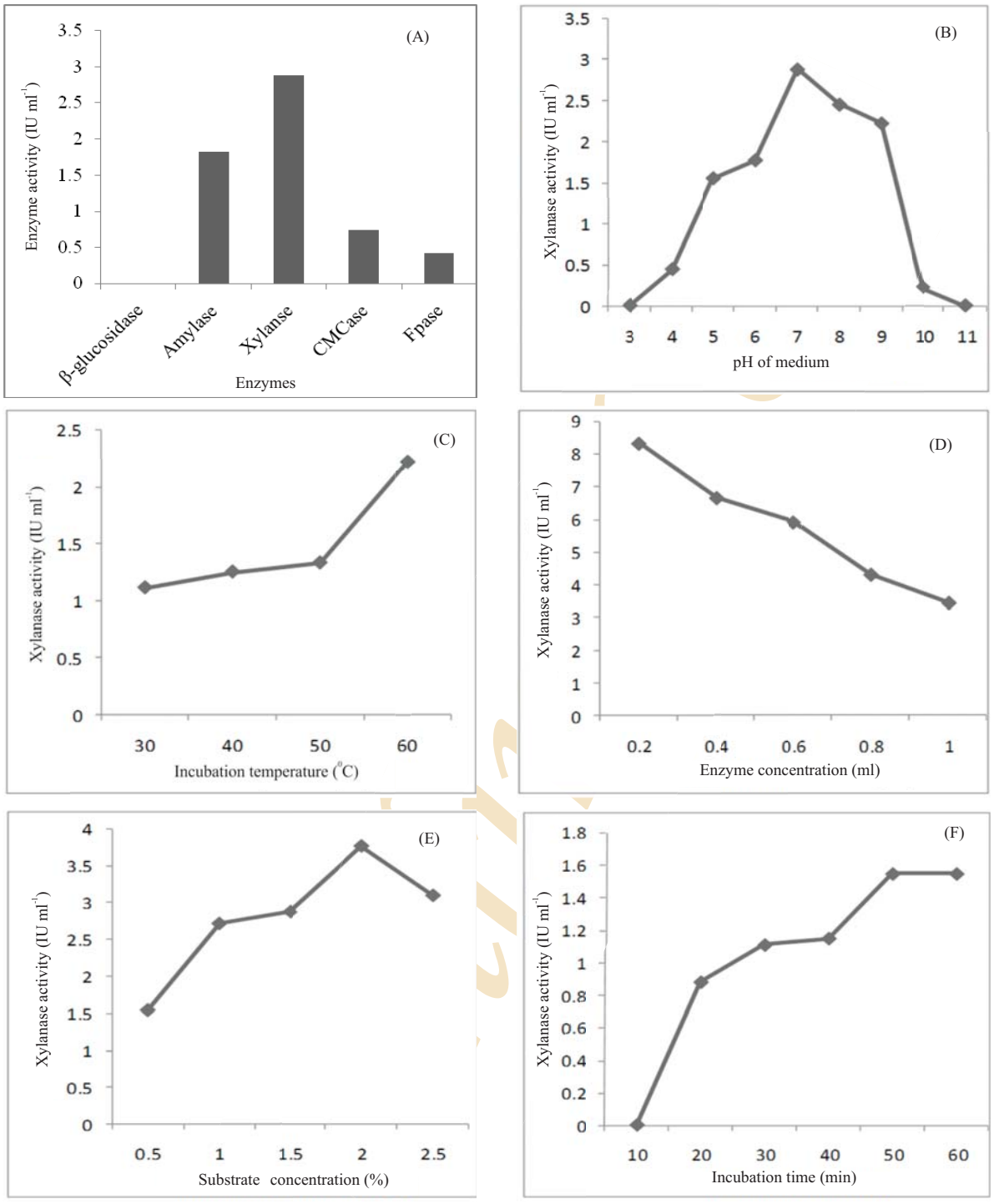

Fig 1: (A) Enzymatic profile of partially purified enzyme; (B) changes in xylanase activity with pH; (C) changes in xylanase activity with temperature; (D) changes in xylanase activity with enzyme concentration; (E) changes in xylanase activity with substrate Concentration; (F) changes in xylanase activity with incubation time

FT-IR analysis: To confirm the decomposition of substrate (Nicotiana tabacum leaf dust) by bacterial isolate FTIR (Fourier Transform InfraRed) spectroscopy was performed. Two milligram of well grind Nicotiana tabacum leaf dust was pressed with (FT-IR) grade (1:100) to make pellet. It was then dispersed in a liquid specifically, a mineral oil to from a paste which was placed in spectrometer. Estimation was carried out in the mid-infrared range, 4000 to 400 cm^{-1}, with Perkin Elmer FT-IR system spectrum BX at the Centre of Excellence (Quality Testing Facility and R & D Centre), Vapi, Gujarat, India. Resolution was set to 4 cm^{-1}, 16 scans were recorded, averaged for each spectrum and corrected against ambient air as background. All degraded samples were compared with undegraded tobacco leaf dust by software Perkin Elmer 'Spectrum Lite' software.

Determination of reducing sugars by TLC and HPTLC: Detection of reducing sugars was carried out using TLC and HPTLC. In TLC, detection of xylose was carried out using hydrolysate on TLC plate and chloroform: methanol: water (35:15:4 by volume) as solvent system. High performance thin layer chromatography (HPTLC) was carried out basically as described by Matsufuji et al, (1998) using CAMAG thin layer chromatography system.

Results and Discussion

The partially purified enzyme produced by isolated potent bacterial culture showed protein content of 368 µg ml^{-1}, amylase activity 1.83 $IUml^{-1}$, CMCase activity 0.741 $IUml^{-1}$, Fpase activity 0.417 $IUml^{-1}$, xylanase activity 2.881 $IUml^{-1}$ and while no β-glucosidase activity (Fig 1a).

Data presented in Fig 1b shows that xylanase activity increased with pH value and reached maximum at pH 7 (2.86 IU ml^{-1}) and decreased thereafter. No xylanase activity was recorded at pH 3 and 11. A remarkable drop in xylanase activity was observed at highly alkaline and acidic condition. Decrease in enzyme activity at higher pH could be attributed to denaturation of enzyme or modification of its active site residues under the influence of H^+ ion concentration (Pandey and Pandey, 2002). Latif *et al.,* (2006) reported maximum xylanase activity in *Chaetomium thermophile* NIBGr between pH 6.5 and 7, whereas *Bacillus pumillis* (Nagar *et al.,* 2010) and *Arthrobacter sp.* MTCC 5214 (Khandeparkar and Bhosle, 2006) gave maximum activity at pH 6.

The effect of temperature on xylanase activity was determined in the range of 30^0 – 60^0C. As shown in Fig. 1c, gradual increase in activity was observed with temperature rise from 30 to 60^0C, and maximum xylanase activity was observed at 60^0C (2.22 $IUml^{-1}$). This clearly revealed that enzyme is thermostable and requires higher temperature for maximum activity. Xylanases produced by *Aspergillus sp*. (Camacho and Aguilarm, 2003), *Aspergillus foetidus* (Shah *et al*., 2005) and *Bacillus sp.* (Sapre *et al.,* 2005) also showed highest activity between 60^0C and 100^0C.

Activity of enzyme depends on concentration of enzyme. Results (Fig. 1d) revealed that at 0.2 ml enzyme concentration, xylanase activity was maximum and decreased sharply and gradually with increased concentration of enzyme. Results clearly indicate that for a given amount of substrate and fermentation conditions 0.2 ml enzyme was sufficient to react, higher than this would inhibit the activity of enzyme itself.

A gradual increase in xylanase activity was observed with increase in substrate concentration from 0.5 to 2.0%, and thereafter the activity decreased sharply with further increase in concentration of substrate to 2.5% (Fig. 1e). This might be due to improper mixing of substrate at higher concentration and it formed a thick suspension which reduced the contact of substrate with enzyme and resulted in lower enzyme activity *(*Sephay *et al.*, 2011). Maximum xylanase activity of at 0.25-2% substrate was reported by Battan *et al*., (2007) in *Bacillus pumilus* ASH.

Xylanase activity increased continuously with incubation time upto 50 min, and thereafter the activity becames constant at 60 minute incubation time (Fig 1f), which might be due to for binding of substrate to active sites of enzyme required specific duration and further increase in incubation time after saturation of active sites with substrates failed to bind the substrate and hence there no gain in activity was noted.

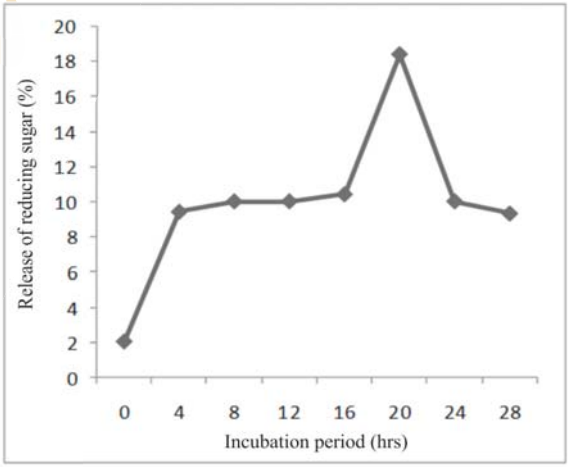

Fig. 2: Release of reducing sugar with incubation period

Results of saccharification study revealed that release of sugar increased with incubation time and reached maximum at 20 hrs and then decreased sharply at 24 hrs, and further decreased at 28 hrs (Fig 2) which clearly show that substrate was found to susceptible for enzyme hydrolysis.

Types of sugar released during microbial hydrolysis of tobacco leaf dust were judged by TLC and HPTLC. Results revealed that two types of sugars were released during hydrolysis and these sugars were xylose and glucose. The present results indicated that xylanase cleaved the substrate to liberate mainly xylooligosaccharides, and able to act on resulting oligosaccharides to form xylose.

The peaks of FT-IR spectrum of tobacco leaf were compared with spectra library of pure substances or components (Smidt and Schwanninger, 2005; Jacox, 2003). Remarkable changes were observed in substrate ranging between 1623.55 – 1033.91 cm^{-1}. If the peaks of FT-IR spectra in tobacco leaf dust before and after enzymatic hydrolysis (Fig 3 a, b) are compared then it can be conclude that many peaks present in leaf dust before enzymatic hydrolysis disappeared after hydrolysis like peaks at 1493.70 cm^{-1} (aromatic compound having C=C bond), 1420.15 cm^{-1} (alkene group having C-H bond and aromatic compounds having C=C bond) and 1287 cm^{-1} (alkylhalide group, amine, ether and ester group) were observed in non-degraded tobacco leaf dust sample (Fig 3 a) but disappeared in enzymatically hydrolysed sample (Fig 3b). In contrast, peaks at 1488.14 cm^{-1} (aromatic C=C compound), 1033.74 cm^{-1} (alkylhalide, ether and ester group), 3002.48 cm^{-1} (heteroaromatic compounds like pyridines, pyroles, furans and thiophenes having C-H stretching, aromatic compounds having N-H and C-H starching) were observed in enzymatically degraded sample which were absent in

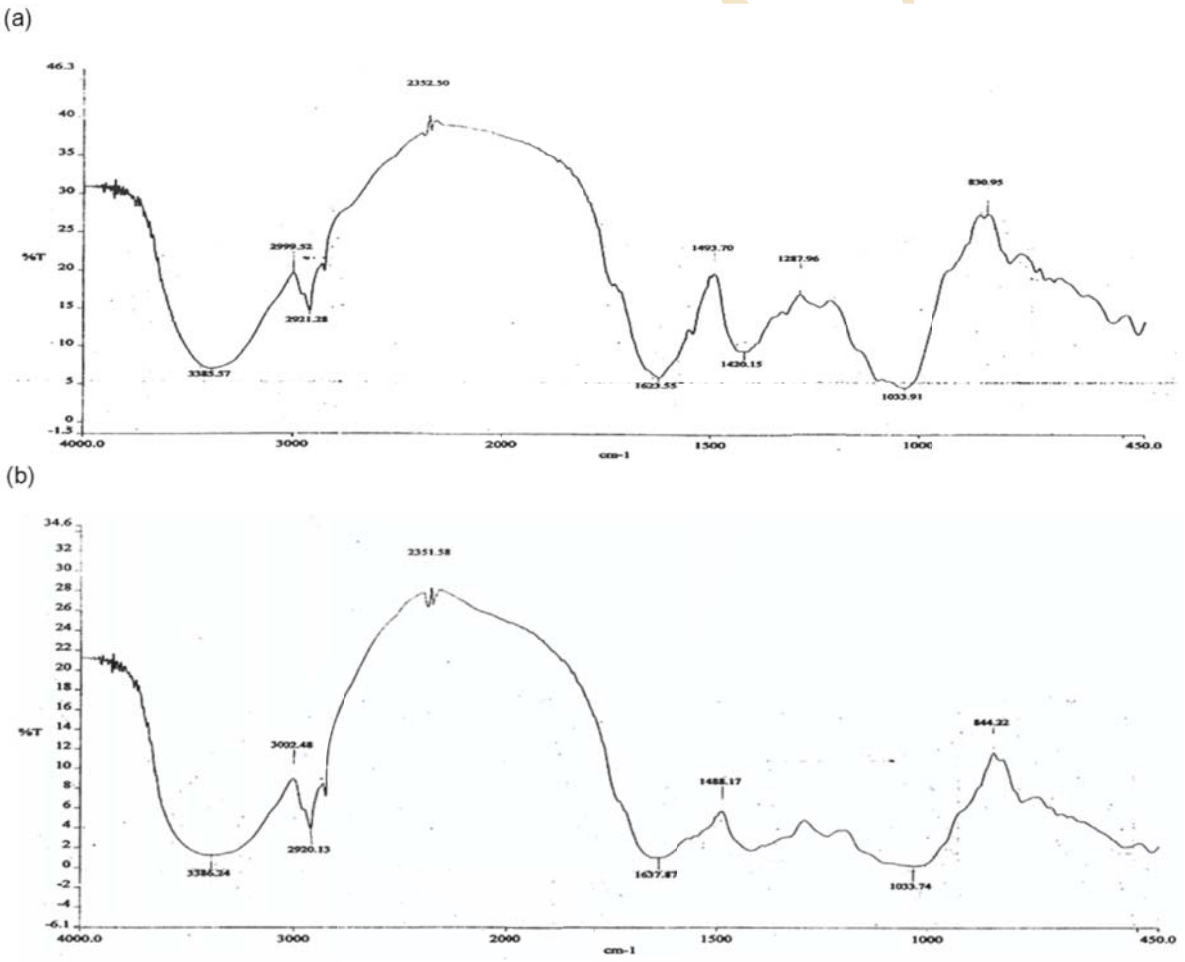

Fig. 3: Tobacco leaf dust before (a) and after (b) hydrolysis

undegraded sample. The intensity of FTIR peaks observed at 3385.57cm^{-1}, 2999.52 cm^{-1}, 2921.28 cm^{-1}, 1623.55 cm^{-1}, 1493.70 cm^{-1}, 1033.91 cm^{-1} and 830 cm^{-1} (corresponds to O-H bond in alcohols, alkane group, C=O banding in amine group, aromatic group, C=N bond in amines and C-H bond in alkene group respectively) decreased in tobacco leaf dust after enzymatic hydrolysis compared to earlier hydrolysis. Signal vibrations at 1420 cm^{-1} and 1287 cm^{-1} representing scissoring and bending of C-H bond in alkanes and group of carboxylic acids disappeared inenzymatically hydrolysed samples.

The results revealed that after degradation some compounds of tobacco leaf dust altered in its composition which confirmed its microbial degradation. Changes in functional groups of agricultural biomass during microbial degradation were also reported previously by Seneesrisakul *et al,* (2014) and Waghmare *et al*. (2014).

From the present study it can be concluded that the isolated bacterial strain of *Bacillus* sp. was capable of producing xylanase using *Nicotiana tabacum* leaf dust as substrate in submerged fermentation. Further, xylanase can saccharify lignocellulosic *Nicotiana tabacum* leaf dust and release xylose and glucose sugar. During bacterial action, the composition of *Nicotiana tabacum* leaf dust changed which confirmed microbial degradation.

Acknowledgment

Authors are grateful to the Head, Department of Microbiology, Sadara and Gujarat Vidyapith, Ahmedabad for providing necessary laboratory facilities to conduct the present experiment.

References

Aysegul, E. Y., I. S. Feride and H. Mehmet: Isolation of endophytic and xylanolytic *Bacillus pumilus* strains from *Zea mays*. *Brazil. Arch. Biol. Technol.,* **14**, 374-380 (2008).

Battan, B., S. Jitender, S. S. Dhiman and R. CKuhad: Enhanced production of cellulase-free thermostable xylanase by *Bacillus pumilus* ASH and its potential application in paper industry. *Enzyme. Microb. Tech.,* **41**, 733–739 (2007).

Bayer, E. A., Y. Shoham and R. Lamed.: Cellulose-decomposing bacteria and their enzyme systems. In: The prokaryotes: an evolving electronic resource of the microbiological community (Eds.: M. Dworkin, S. Falkow, E. Rosenberg, K.H. Schleifer and E. Stackebrandt). 3rd Edn.,Springer-Verlag, New York, (2000).

Beg, Q. K., M. Kapoor, L. Mahajan and G. S.Hoondal, Microbial xylanases and their industrial applications: A review, *Appl. Microbiol. Biotechnol.,* **56**, 326–338 (2001).

Bernfield, P.: In: Methods of Enzymol; (eds, Colowick, S. P. and Kalpan, N. O.) Academic Press, New York, 1, 149 (1955).

Camacho, N. A. and O. G. Aguilarm : Production, purification, and characterization of a low-molecular-mass xylanase from *Aspergillus* sp. and its application in baking. *Appl. Biochem. Biotechnol*., **104**, 159-72 (2003).

Ghose, T.: Measurement of cellulases. *Pure appl. Chem*., **59**, 257-268 (1987).

Irfan, M., M. Nadeem, Q. Syed and S. Baig, : Effect of Medium composition on Xylanase Production by *Bacillus subtilis* using various agricultural wastes. *American-Eurasian J. Agric. Environ. Sci*., **12**, 561-565 (2012).

Jacox, M. E.: Vibrational and electronic energy levels of polyatomic transient molecules. Supplement B. *J. Phy. Chem. Ref. Data,* **32** , 1(2003).

Khandeparkar, R. and N. Bhosle: Isolation, purification and characterization of the xylanase produced by *Arthrobacter* MTCC 5214 when grown in solid state fermentation. *Enz. Microbiol. Tech.*, **39**, 732–742 (2006).

Krishna, C.: Solid-state fermentation system - An overview. *Critic. Rev. Biotechnol.,***25**, 1-30 (2005).

Latif, F., M. Asgher, R. Saleem, A. Akrem and R. Legge : Purification and characterization of xylanase produced by *Cheatomium thermophile* NIBGE. *World J. Microbiol. Biotechnol.*, **22**, 45-50 (2006).

Lowry, O. H., N. J.Rosebrough, A. L. Farr and R. J.Randall : Protein measurement with the Folin-phenol reagent. *J. Biol. Chem.,* **193**, 265-275 (1951).

Mahadevan, A. and R. Sridhar : In Methods of Physiological Plant Pathology. 3rd edn., Sivakami publications, Chennai, 66-67 (1986).

Maria, L. G. S. and M. T. Samia : Optimization of xylanase biosynthesis by *Aspergillus japonicus* isolated from a "caatinga" area in the Brazillian state of Bahia. *Afr. J. Biotechnol*. **5**, 1135-1141 (2006).

Matsufuji, H., H. Nakamara, M. Chino and M.Takeda : Antioxidant activity of capsanthin and the fatty acid esters in Paprika (*Capsicum annuum*) *J. Aagricul. Food Chem.*, **46**, 3468-3472 (1998).

Miller, G. L.: Use of dinitrosalicylic acid reagent for determination of reducing sugars. *Anal. Chem.,* **31**, 426-428 (1959).

Murugan, S., D. Arnold, U. Pongiya and P. M. Narayanan, : Production of Xylanase from Arthrobacter sp. MTCC 6915 Using Saw Dust as Substrate under Solid State Fermentation. *Enz. Res*. 1-7 (2011).

Nagar, S., V. Gupta, D. Kumar, L. Kumar and R. Kuhud : Production and optimization of cellulose free, alkali stable xylanase by *Bacillus pumillus* SV85S in submerged fermentation. *J. Microbiol. Biotechnol*., **37**, 71-83 (2010).

Pandey, P. and A. K. Pandey, : Production of cellulose free thermostable xylanases by an isolated strain of *Aspergillus niger* PPI, utilizing various lignocellulosic wastes. *World J. Microbiol. Biotechnol*., **18**, 281-283 (2002).

Pason, P., K. L. Kyu and K. Ratanakhanokchai : *Paenibacillus curdlanolyticus* Strain B-6 Xylanolytic-cellulolytic enzyme system that degrades insoluble polysaccharides. *Appli. Environ. Microbiol*., **72**, 2483-2490 (2006).

Rehman, S., H. Aslam, A. Ahmad, S. A. Khan and M. Sohail: Production of plant cell wall degrading enzymes by monoculture and co-culture of *Aspergillus niger* and *Aspergillus terreus* under SSF of banana peels. *Braz. J. Microbiol.,* **45**, 1485-1492 (2014).

Sapre, M. P., H. Jha and M. B. Patil : Purification and characterization of a thermoalkalophilic xylanase from *Bacillus sp. World J. Microbiol. Biotechnol.*, **21**, 649-654 (2005).

Seneesrisakul, K., E. Gulari and S. Chavadej: Study on Microbial Pretreatment for Enhancing Enzymatic Hydrolysis of Corncob. *Int. J. Biol. Food Vet. Agril. Eng.*, **8**, 961-966 (2014).

Sepahy, A. A., S. Ghazi and M. A. Sepahy: Cost-effective production and optimization of alkaline xylanase by indigenous *Bacillus mojavensis* AG137 fermented on agricultural waste. *Enz. Res*., 593-624 (2011).

Shah, A., R. Shah and D. Madamwar: Improvement of the quality of whole wheat bread by supplementation of xylanase from *Aspergillus foetidus*. *Bioresource Technol.*, **97**, 2047-2053 (2005).

Sharma, P. K. and D. Chand: Production of cellulase free thermostable xylanase from *Pseudomonas* sp.XPB-6 *I. Res. J. Biological Sci.,* **1**, 31-41 (2012).

Smidt, E. and M. Schwanninger: Characterization of Waste Materials Using FT-IR Spectroscopy – Process Monitoring and Quality Assessment. *Spectro. Let.*, **38**, 247-270 (2005).

Subramaniyan, S. and P. Prema: Biotechnology of microbial xylanases: enzymology, molecular biology and application. *Crit. Rev. Biotechnol*., **22**, 33-64 (2002).

Verma, D. and T. Satyanarayana: Molecular approaches for ameliorating microbial xylanases. *Biores. Technol.* **17**, 360–367 (2012).

Waghmare, P. R., S. D. Kshirsagar, R. G. Saratale, S. P. Govindwar and G. D. Saratale: Production and characterization of cellulolytic enzymes by isolated *Klebsiella* sp. PRW-1 using agricultural waste biomass. *Emir. J. Food Agric*., **26**, 44-59 (2014).