Assessment and Characterization of Xylanolytic Bacteria Isolated from the Gut of Microtermes Obesi for Biomass Pretreatment

Iram Batool
University of Narowal

Falak Sher Khan
University of Sialkot

Muhammad Awais
National Center for Physics

Dawood Ahmed
University of Haripur

Sami Ullah Khan
COMSATS University Islamabad

M. Ijaz Khan (✉ mikhan@math.qau.edu.pk)
Riphah International University I-14

Research Article

Keywords: Termite, 16S rRNA, Bacillus, Xylanase, Sccharification

DOI: https://doi.org/10.21203/rs.3.rs-282399/v1

License: ☕️ 🌟 This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License
Abstract

The aim of current research was to examine the potential for the production of hemicelluloses degrading enzymes from bacteria harbor in termite gut. The research was also focused on the conversion of lignocellulosic biomass (Corn stover, rice straw and cotton stalk) into fermentable sugars by using enzymes from the bacterial isolates. The bacterial isolates from termite gut were screened for their ability to degrade xylan that is the major constituent of hemicelluloses. Two bacterial isolates were chosen and identified by 16S rRNA gene sequencing. Both isolates TGB9 and TGB10 belong to Bacillus geneus. The isolates have shown higher xylan degrading activity at 50°C and optimum pH was 6.0. Xylanases from isolate TGB9 and TGB10 were utilized for sccharification of agricultural substrates (stover, rice straw and cotton stalk). As a result higher contents of reducing sugars were observed from corn stover. Xylanases from isolate TGB9 produced higher yields of reducing sugar than isolate TGB10. A comparative study was also performed among chemical pretreatment and xylanases from bacterial isolates. For this purpose agricultural substrates were also treated with H2SO4 and NaOH. Xylanases produced by TGB9 and TGB10 released higher content of sugar from agricultural substrates than chemical pretreatments. So it is concluded that termite gut have bacteria that can hydrolyze hemicelluloses more efficiently than chemicals.

Introduction

In the whole world, biofuel production has been drastically elevated due to increase in gasoline prices and decline in the emission of greenhouse gases [1]. For this situation, lignocellulosic biomass, that have stored high amount of energy with low-cost resource, is the vanguard supply for biofuel manufacture [2]. For biofuel production degradation of cellulose and hemicelluloses into monomeric sugars is the most expensive and crucial step from lignocellulosic biomass. But production of sugars from lignocellulosic resources (cellulose and hemicelluloses) at high concentration is for more intricate as compared to sugars harvested from resources having sugar or starch [3]. The main noteworthy research is required in pre-treatment area of biomass. There is a need for the development of cheaper and resourceful enzymatic strategy to remove lignin and to degrade cellulose and hemicellulose into reducing sugars. The pre-treatment is the most expensive step during biofuel production (just about 20% of whole cost), this is a surrounding area in which significant opportunities for up gradation exist. So, biological based approaches are advantageous and alternate to chemical and high energy consuming temperature treatment for lignin removal and degradation of hemicellulose [4].

Xylan is a heteropolymer of uronic monosaccharides and β-1,4-D-xylanopyranosyl, a major part of the hemicelluloses. It can be degraded by xylanase and β-xylosidase [5–6]. Xylanase is produced by a number of diverse microorganisms; include fungi [7] and bacteria [8]. It is useful in different industries including fuel, feed, food, paper, detergents and textile industries for treatment of waste products [9].

Accordingly, the exploration of genes for cellulases and hemicellulases come into focus from diverse organisms that include fungi and bacteria because the organisms have high potential for the production
of enzymes. Consequently, termites are thought to acquire diverse sets of resourceful miniature lignocellulose hydrolyzing systems [10]. Termites can damage wood into dust and a reason for a damage of billions of dollars every year. There are more than 200 species of different microorganisms that are present in termite gut which have the ability to produce cellulases and hemicellulases [11]. So termites can hydrolyze a significant amount of hemicellulose and cellulose [12].

By the advert of meta genomic study different microbial species have been discovered, that have a various bacterial genes concerned for hydrolyzing hemicelluloses and cellulose. However, only a little information is accessible on the subject of their functional diversity [13]. During current years, remarkable researches have been prepared by different research groups to recognize termite lignocelluloses integration. Therefore, concentration was paid to discover the capability of termite for modification of agricultural waste products into reducing sugars.

### Materials And Methods

#### Sample collection

Wood degrading termites (*Microtermes obesi*) were obtained from decaying trees of *Acacia nilotica*. Agricultural substrates (cotton stalk, rice straw and corn stover) were obtained from the National Agricultural Research Center (NARC) Islamabad, Pakistan.

#### Isolation and screening of xylan degrading bacteria

Ten termites were sterilized by using 70% ethanol, followed by sterilization under UV light for 10 minutes. The heads were detached and bodies were grounded in presterilized mortar and pestle. Milli Q water was used to make a suspension. Diluted suspension was spread over the plates containing nutrient agar media with 1% xylan from beechwood (Sigma-Aldrich) [11]. At 30 °C, petri dishes were incubated for 20 hours.

Followed by isolation and purification of bacterial colonies, screening was carried out using nutrient agar media with 0.2% xylan, by congo red dye method [11]. From overnight culture of isolates 5 µl was placed on petri plates having media for screening. At 30 °C the petri plates were incubated for 48 hours. Xylan degrading ability of bacteria was established by considering clear zones of bacterial colonies [14]. *E.coli* and *B. subtilius* were considered as negative and positive control respectively.

#### 16S rRNA gene sequencing

Bacterial colonies were utilized for the amplification of 16S rRNA gene of bacterial isolates [15]. Universal primers for 16S rRNA gene, 27F(5'-AGAGTTTGATCCTGGCTCA-3') and 1492R(5'-ACGGCTACCTTGTTACGACTT-3') were used to amplify 1.5kb 16S rRNA fragment through PCR. Ethanol precipitation method was used to purify PCR products [16]. Purified PCR products were sent to “Keck Center for Comparative and Functional Genomics” University of Illinois at Urbana-Champaign, USA.
Sequences were submitted to NCBI. Gene sequences from bacterial isolates were analyzed by BLASTN program of GeneBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify the genera.

**Xylanase production**

Nutrient broth with 1% xylan, pH 6.8–7.2 [17] was inoculated with isolated bacteria (TGB9 and TGB10) and incubated at 30 °C for 48 hours at mild rotation. After centrifugation supernatant was utilized as source of crude xylanase enzyme.

**Xylanase activity assays**

Xylanase activities were tested for pH 5, 6, 7, 8 and 9, also at various temperatures that include 30, 40, 50 and 60 °C. Xylan (1%) was made in different buffers to study the pH parameter [18]. Reaction time was 30 min for each condition. Without the addition of crude enzyme substrates in buffer were used as control. By using p-hydroxybenzoic acid hydrazide (PAHBAH) method, reducing sugar content was determined [19]. One unit (U) of enzyme activity was defined as the amount of enzyme that released 1µmol of reducing sugars per minute during the reaction.

**Sccharification of agricultural substrates by xylanase from TGB 9 and TGB 10**

Agricultural wastes were utilized as 5% dry weight (w/v) for saccharification. For reaction mixture the ratio was 1:1 for xylanases from bacterial isolates and agricultural substrates. At 50 °C reaction mixture was incubated for 24 hours at mild rotation. Substrates in distilled water were studied as control.

**Sccharification of agricultural substrates by chemicals**

Sccharification of agricultural substrates was performed in 1, 2 and 3% of H$_2$SO$_4$ and NaOH. Cotton stalk, rice straw and corn stover were utilized as 5% (w/v) then pretreated at 121 °C for 20 min.

**Experimental Analysis**

To obtain accuracy in results the experiments were carried out in triplicates. The results obtained form chemical and enzymatic pre-treatment were analyzed by Analysis of Variance (ANOVA) applying MSTAT-C program. The results from pretreatment area were studied by GraphPad Prism 5.0 software for obtaining standard error and standard deviation.

**Results**

**Isolation and screening of xylan degrading bacteria**

Among the 53 bacterial isolates, two isolates were selected based on considerable clear zone formation during congo red screening (Fig. 1). The hydrolyzed clear zone diameters to colony diameter ratios showed that both isolates TGB9 and TGB10 have nearly same ratio to degrade xylan.
16S rRNA gene sequencing

The sequence data of 16S rRNA gene was examined in BLASTN to find out similarity index. Consequently it was exposed that isolates TGB9 and TGB10 fit in to Bacillus genus. The similarity searches demonstrate that TGB9 was 99% analogous to Bacillus pumilus and isolate TGB10 was 99% similar to Bacillus licheniform. The sequences were submitted to NCBI data base, accession numbers are KR902555 are KR902570 for isolates TGB9 and TGB10 respectively.

Temperature and pH optimization of xylanase

It was determined that at 50 °C, both of the isolates TGB9 and TGB10 showed highest xylanase activity (Fig. 2). Figure 3 illustrates that highest enzyme activity for isolates TGB9 and TGB10 was observed at pH 6. Isolate TGB9 have significantly high enzyme (xylanase) activity (270.37 ± 5.3208 U/ml) as compared to isolate TGB10 having 158.26 ± 1.9412 U/ml.

Sccharification of agricultural substrate

A comparative study showed that the xylanases isolated from TGB9 and TGB10 released significantly higher sugar content as compared to chemically pretreated biomass (corn stover). It was observed that there is a decrease in sugar yield as the concentration of H$_2$SO$_4$ increased (Fig. 4). Figure 5 revealed that sugar yield by xylanases from both isolates was more than the chemical pretreatment, while using cotton stalk as a substrate. But sugar concentration released from cotton stalk is less as compared to corn stover. Pretreatment of rice straw showed inconsistent results from cotton stalk and corn stover. Highest concentration of sugar content was obtained when rice straw was pretreated with 1% H$_2$SO$_4$ in comparison with bacterial xylanases (Fig. 6). From the above results it was observed that among the bacterial isolates, xylanases released by TGB9 hydrolyzed all the substrates more efficiently.

Discussion

This study was focused on the enzymes produced by bacteria, symbiotically living in termite gut. To find the role of bacteria playing in hemicelluloses digestion in this biologically assorted mini-biorefinery [20]. During the research facultative anaerobic and aerobic xylanolytic bacterial isolates were studied from termite gut. It was premeditated that the gut of termite has anaerobic surroundings [21], whereas anaerobic and aerobic bacteria were also isolated [22] from termite gut. After congo red screening two bacterial isolates were selected because they illustrate a significant xylanase activity with xylan.

From molecular characterization of the selected isolates it was found that both of them belong to Bacillus genus. Commonly, enormous of cellulolytic and xylanolytic microaerophilic and facultative anaerobic bacteria inhabit in termite gut. But species of Bacillus genera predominate with concentration up to 10$^7$/ml gut content [22]. Bacillus species are ubiquitous in environment and include symbiotically associated and free-living species. Majority among them are efficiently active and involved in a series of wood degrading enzymes like laccase, cellulase, amylase, xylanase, hemicellulase, ligninase [23]. A study
reported that *Bacillus* and *Acinetobacter* have dominant biomass degrading ability from other isolates belonged to *Microcerotermes diversus* [24]. *Bacillus species* were described to be the leading lignocelluloses devastating bacterial isolates collected from soil and animal waste samples, as well as from paper mills sludges [25].

Maximum xylanase activity was observed at 50 °C for the bacterial isolates. During a research it was studied that most favorable temperature for xylanase activity was 50 °C from a *Bacillus sp.* obtained from soil [26]. During experimentation it was observed that isolate TGB9 and TGB10 showed highest xylanase activity at pH 6.0. purified two xylanases from *Bacillus sp.* namely xylanases N and xylanase A. Xylanase N showed highest activity at pH 6.0 and 7.0, whereas xylanase A showed best results at pH 6.0–10.0. But a study reported that gram positive bacteria from termite gut showed maximum xylanase activity, pH 5.0 at 55 °C [27].

The xylanases produced by TGB9 and TGB10 released highest sugar content when corn stover was used as substrate as compared to cotton stalk and rice straw. Although, the carbohydrate content is high in rice straw (mannose, xylose, glucose, etc) as compared to the corn stover [28]. But it was also determined that minerals content are high in rice straw [29] which slowed degradation of rice straw in soil. A study showed that xylanase activity was improved by using reducing agents. But chelating agents, such as detergents and Cu$^{2+}$ decreased xylanase activity. High mineral content of hinder the activity of these enzymes, but do not hinder the chemicals (H$_2$SO$_4$ and NaOH) pretreatment [30]. Therefore, chemical pretreatment released nearly the same concentration of sugar content during pretreatment of rice straw. Least sugar content is released from cotton stalk compared to other agricultural substrates. It was studied that lignin content are high in cotton stalk therefore it is hard to hydrolyze [31]. It was also observed that when concentration of acid was increased the sugar yield was decreased. It was verified that at acidic conditions, as sugar released some of it is changed to inhibitors such as hydroxymethyl furfural and furfural [32]. So as the concentration of acid increase concentration of inhibitors elevate. Therefore absolutely decrease concentration of sugar content.

**Conclusion**

It is concluded that gut of termites symbiotically harbor some significant number of bacteria those are helpful for degradation of lignocellulosic biomass. It was also observed that xylanses not only hydrolyze pure substrate (xylan) but they can hydrolyze the complex substrate as well. So isolation purification and characterization of specific bacterial xylanses for further utilization in fermentation process is very important. Which additionally depend on continues availability of cheaper agriculture resides and its handling. And also these bacteria which can digest hemicelluloses might helpful to ferment these sugars into valuable end products.

**Declarations**

**Acknowledgement**
Higher Education Commission of Pakistan (HEC) provided the financial, indigenous scholarship without which this study was not possible.

Conflict of Interest

The authors declare no conflict of interest

References

1. Pourramezan, Z., Ghezelbash, G. R., Romanic, B., Ziaeid, S., Hedayatkhah, A., 2012. Screening and identification of newly isolated cellulose degrading bacteria from the gut of xylophagous termite *Microcerotermes diversus* (Silvestri). Microbiol., 81: 736-742.

2. Rogers, P. L., 2008. Current developments in bioethanol production. J. Microbiol. Aus., 29: 6-10.

3. Lynd, L. R., Laser, M. S., Bransby, D., Dale, B. E., Davison, B., Hamilton, R., Himmel, M., Keller, M., McMillan, J. D., Sheehan, J., Wyman, C. E., 2008. How biotech can transform biofuels. J. Nat. Biotech., 26: 169-172.

4. Galbe, M., Zacchi, G., 2007. Pretreatment of lignocellulosic materials for efficient bioethanol production. J. Adv. Biochem. Engin/Biotechnol., 108: 41-65.

5. Scharf, M. E., Boucias, D. G., 2010. Potential of termite-based biomass pre-treatment strategies for use in bioethanol production. J. Ins. Sci., 17: 166-174.

6. Mandal, A., Kar, S., Mohapatra, P. K. D., Maity, C., Pati, B. R., Mondal, K. C., 2011. Purification and characterization of an endoxylanase from the culture broth of *Bacillus cereus* BSA1. Appl. Biochem. Microbiol., 47: 250-255.

7. Faik, A., 2010. Xylan Biosynthesis: news from the grass. Plant Physiol., 153: 396-402.

8. Dhiman, S. S., Garg, G., Mahajan, R., Garg, N., Sharma, J., 2009. Single lay out and mixed lay out enzymatic processes for bio-bleaching of kraft pulp. Bioresour Technol., 100: 4736-4741.

9. Sanghi, A., Garg, N., Kuhar, K., Kuhad, R. C., Gupta, V. K., 2009. Enhanced production of cellulase free xylanase by alkalophilic *Bacillus subtilis* ASH and its application in biobleaching of kraft pulp. BioResour., 4: 1109-1129.

10. Pal, A., Khanum, F., 2010. Characterizing and improving the thermostability of purified xylanase from *Aspergillus niger* DFR-5 grown on solid-state-medium. J. Biochem. Technol., 2: 203-209.

11. Bignell, D. E., Eggleton, P., Nunes, L., Thomas, K. L., 1997. Termites as mediators of carbon fluxes in tropical forests: budgets for carbon dioxide and methane emissions, In: Watt A. D., Stork, N. E., Hunter, M. D (eds), Forests and Insects, pp. 109-134. Chapman and Hall, London.

12. Dheeran, P., Nandhagopal, N., Kumar, S., Jaiswal, Y. K., Adhikari, D. K., 2012. A novel thermostable xylanase of *Paenibacillus macerans* IIPSP3 isolated from the termite gut. J. Ind. Microbiol. Biotechnol., 20: 1-10.

13. Ohkuma, M., 2003. Termite symbiotic systems: Efficient bio-recycling of lignocellulose. Appl. Microbiol. Biotech., 61: 1-9.
14. Warnecke, F., Luginbuhl, P., Ivanova, N., Ghassemian, M., Richardson, T. H., J. Stege, T., Cayouette, M., McHardy, A. C., Djordjevic, G., Aboushadi, N., Sorek, R., Tringe, S. G., Podar, M., Martin, H. G., Kunin, V., Dalevi, D., Madejska, J., Kirton, E., Platt, D., Szeto, E., Salamov, A., Barry, K., Mikhailova, N., Kyrpides, N. C., Matson, E. G., Ottesen, E. A., Zhang, X., Hernandez, M., Murillo, C., Acosta, L. G., Rigoutsos, I., Tamayo, G., Green, B. D., Chang, C., Rubin, E. M., Mathur, E. J., Robertson, D. E., Hugenholtz, P., Leadbetter, J. R., 2007. Metagenomic and functional analysis of hindgut microbiota of a wood-feeding higher termite. Nature, 450: 560-565.

15. Liang, Y. L., Zheng, Z., Min, W., Yuan, W., Jia, X. F., 2014. Isolation, screening and identification of cellulolytic bacteria from natural reserves in the subtropical region of China and optimization of cellulase production by Paenibacillus terrae ME27-1. Biomed, Resour. Int., DOI:10.1155/2014/512497.

16. Batool I., Gulfraz, M., M. Asad, J., Kabir, F., Khadam, S., Ahmed, A. 2018. Cellulomonas sp. Isolated from Termite Gut for Saccharification and Fermentation of Agricultural Biomass. Bioresources, 13(1): 752-763.

17. Ji, Y., Hofstadler, S. A. 2003. A highly efficient and automated method of purifying and desalting PCR products for analysis by electrospray ionization mass spectrometry. Anal. Biochem., 316 (1): 50-57.

18. Bashir, Z., Kondapalli, V. K., Adlakha, N., Sharma, A., Bhatnagar, R. K., Chandel, G., Yazdani, S. S., 2013. Diversity and functional significance of cellulolytic microbes living in termite, pill-bug and stem-borer guts. Sci. Rep., 3: 1-11.

19. Rastogi. G., Muppidi, G. L., Gurram, R. N., Adhikari, A., Bischo, K. M., Hughes, S. R., Apel, W. A., Bang, S. S., Dixon, D. J., Sani, R. K., 2009. Isolation and characterization of cellulose-degrading bacteria from the deep subsurface of the Homestake gold mine, Lead, South Dakota, USA. J. Ind. Micro. Biotech., 36: 585-590.

20. Moretti, R., Thorson, J. S., 2008. A comparison of sugar indicators enables a universal high throughput sugar-1-phosphate nucleotidyltransferase assay. Anal. Biochem., 377: 251-258.

21. Breznak, J. A., Brune, A., 1994. Role of microorganisms in the digestion of lignocellulose by termites. Annu. Rev. Entomol., 39: 453-487.

22. Brune, A., Emerson, D., Breznak, J. A., 1995. The termite gut microflora as an oxygen sink: Microelectrode determination of oxygen and pH gradients in guts of lower and higher termites. Appl. Environ. Microbiol., 61: 2681-2687.

23. Wenzel, M., Schonig, I., Berchtold, M., Kampfer, P., Konig, H. 2002. Aerobic and facultatively anaerobic cellulolytic bacteria from the gut of the termite Zootermopsis angusticollis. J. App. Microb., 92: 32-40.

24. Konig, H., 2006. Bacillus species in the intestine of termites and other soil invertebrates. J. Appl. Microbiol., 101: 620-627.

25. Kim, Y. K., Lee, S. C., Cho, Y. Y., Oh, H. J., Ko, Y. H., 2012. Isolation of cellulolytic Bacillus subtilis strains from agricultural environments. Microbiol., DOI: 10.5402/2012/650563.
26. Kamble, R. D., Jadhav, R. A., 2012. Isolation, purification and characterization of xylanase produced by a new species of *Bacillus* in solid state fermentation. Int. Microbiol., 12: 1-8.

27. Matteotti, C., Bauwens, J., Brasseur, C., Tarayre, C., Thonart, P., Destain, J., Francis, F., Haubruge, E., Pauw, E. D., Portetelle, D., Vandenbol, M., 2012. Identification and characterization of a new xylanase from gram-positive bacteria isolated from termite gut (*Reticulitermes santonensis*). Protein Expres. Purif., 83: 117-127.

28. Karimi, K., Kheradmandinia, S., Taherzadeh, M. J., 2006. Conversion of rice straw to sugars by dilute acid hydrolysis. Biomass Bioenerg., 6: 247-253.

29. Vlasenko, E. Y., Ding, H., Labavitch, J. M., Shoemaker, S. P, 1997. Enzymatic hydrolysis of pretreated rice straw. Bioresour. Technol., 59: 109-119.

30. Matteotti, C., Haubruge, E., Thonart, P., Francis, F., Pauw, E. D., Portetelle, D., Vandenbo, M., 2011. Characterization of a new β-glucosidase, β-xylosidase from the gut microbiota of the termite (*Reticulitermes santonensis*). FEMS. Micro. Lett., 314: 147-157.

31. Silverstein, R. A., Chen, Y., Ratna, R., Shivappa, S., Boyette, M. D., Osborne, J., 2007. Comparison of chemical pretreatment methods for improving saccharification of cotton stalks. Bioresour. Technol., 98: 3000-3011.

32. Mosier, N., Hendrickson, R., Ho, N., Sedlak, M., Ladisch, M. R., 2005. Optimization of pH controlled liquid hot water pretreatment of corn stover. Bioresour. Technol., 96: 1986-1993.