

CHARACTERIZATION OF BACTERIA ISOLATED FROM THE GUT OF *MICROTERMES OBESI* FOR THE PRODUCTION OF ETHANOL FROM SECOND GENERATION BIOMASS

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ABSTRACT

The current research was also focused on the conversion of lignocellulosic biomass samples into fermentable sugars and ethanol by using enzymes from the bacterial isolates obtained from termites gut . Two bacterial isolates were chosen and identified by 16S rRNA gene sequencing. Both isolates TGB9 and TGB10 belong to *Bacillus* genus. 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). Result revealed higher contents of reducing sugars 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.

Key words: Termite; 16S rRNA; *Bacillus*; Xylanase; Sccharificatio

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 (Pourramezan et al., 2012) For this situation, lignocellulosic biomass, that have stored high amount of energy with low-cost resource, is the vanguard supply for biofuel manufacture (Roger, 2000) 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 (Lynd et al., 2008).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 (Galbe and Zacchi, 2000; Scharf and Boucias, 2010).

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 (Mandal et al., 2011; Faik, 2010). Xylanase is produced by a number of diverse microorganisms; include fungi and bacteria (Dhiman et al., 2009) . It is useful in different industries including fuel, feed, food, paper, detergents and textile industries for treatment of waste products (Pal and Khanum, 2010).

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 (Bignell et al., 1999) 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 (Dheeran et al., 2012). So termites can hydrolyze a significant amount of hemicellulose and cellulose (Ohkuma, 2003).

By the advent 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 (Wamecke et al., 2007). During current years, remarkable researches have been prepared by different research groups to recognize termite lignocelluloses integration. Therefore, attention was paid to discover the capability of termite for modification of agricultural waste products into reducing sugars. Keeping in view importance of bio fuels in current scenario ,present study was undertaken with aims and objectives (1). Chemical analysis of various biomass samples (2) Assessment of ethanol produced by fermentation process (3). Study of termites gut bacteria for fermentation process

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 beech wood (Sigma-Aldrich) (Dheeran et al.,2012). 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 (Dheeran et al.,2012) . 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 (Wamecke et al., 2007; Liang et al., 2014). *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 isolate (Batool et al., 2018) 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 (Jian and Hofstadler, 2003). 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 (Bashir et al., 2013). 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 (Rastogi et al., 2009).. 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 (Moretti and Thorson, 2008). One unit (U) of enzyme activity was defined as the amount of enzyme that released 1µmol of reducing sugars per minute during the reaction (Breznank and Brune, 1994).

Saccharification 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 (Brune et al., 1995).

Sscharification of agricultural substrates by chemicals

Sscharification of agricultural substrates was performed in 1, 2 and 3% of H₂SO₄ and NaOH. Cotton stalk, rice straw and corn stover were utilized as 5% (w/v) then pretreated at 121 °C for 20 min (Wenzel et al., 200; Konig, 2006; Kim et al., 2012).

Experimental Analysis

To obtain accuracy in results the experiments were carried out in triplicates. The results obtained from 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 AND DISCUSSION

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). Fig. 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.

Saccharification 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 H₂SO₄ increased (Fig. 4). Fig. 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% H₂SO₄ 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 (Kim et al., 2012).

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. 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. Whereas anaerobic and aerobic bacteria were also isolated from termite gut. After congo red screening two bacterial isolates were selected because they illustrate a significant xylanase activity with xylan (Wenzel et al., 2002; Koniq, 2006; Kim et al., 2012; Kamble and Jadhar, 2012).

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 10⁷/ml gut content (Matteotti, 2012). *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 (Karimi et al., 2006). A study reported that *Bacillus* and *Acinetobacter* have dominant biomass degrading ability from other isolates belonged to *Microcerotermes diversus* (Vlasenko et al., 1997). *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 (Matteotti et al., 2011).

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. 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 (Silverstein et al., 2007; Mosier et al., 2005).

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. But it was also determined that minerals content are high in rice straw (Karimi et al., 2006). 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_2SO_4 and NaOH) pretreatment. 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. 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 (Bashir et al., 2013; Batoool et al., 2018), as the concentration of acid increase concentration of inhibitors elevate. Therefore, absolutely decrease concentration of sugar contents were observed in many cases.

CONCLUSION

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

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Figure 1: Congo red screening, from left to right: Isolate TGB9 and isolate TGB10

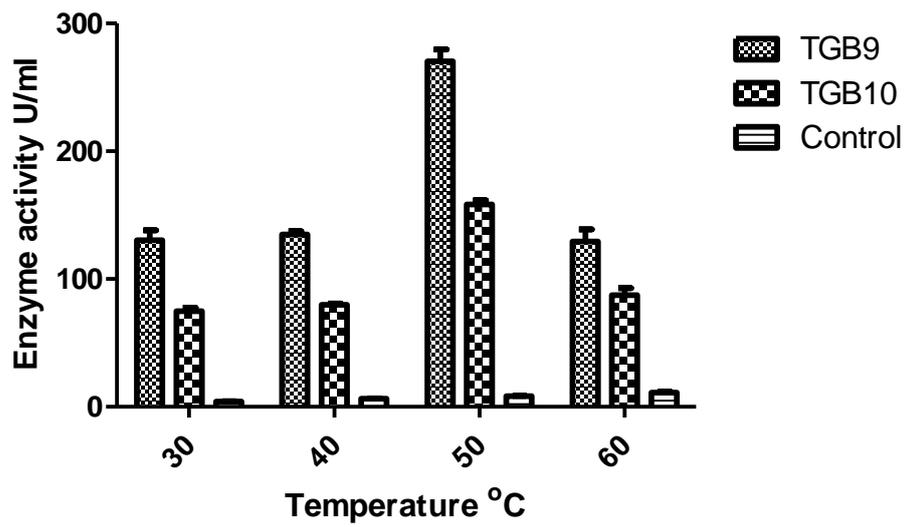


Figure 2: Temperature optimization of enzyme activity (U/ml) for isolates TGB9 and TGB10

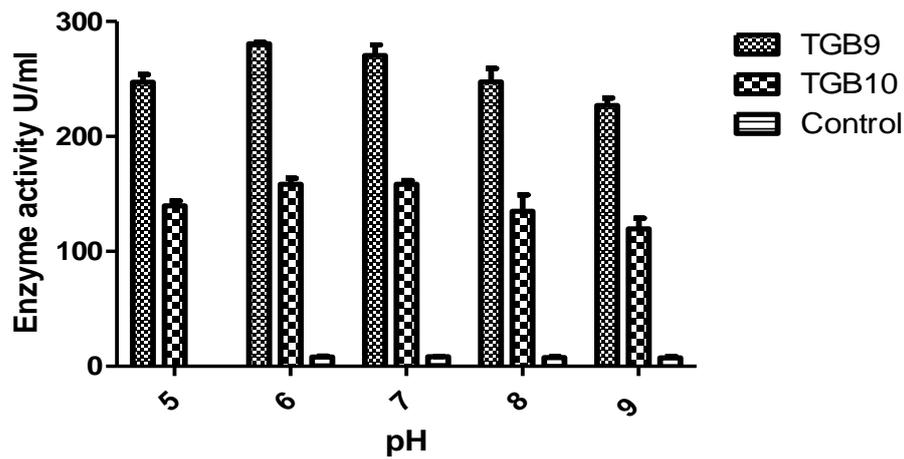


Figure 3: pH optimization of enzyme activity (U/ml) for isolates TGB9 and TGB10

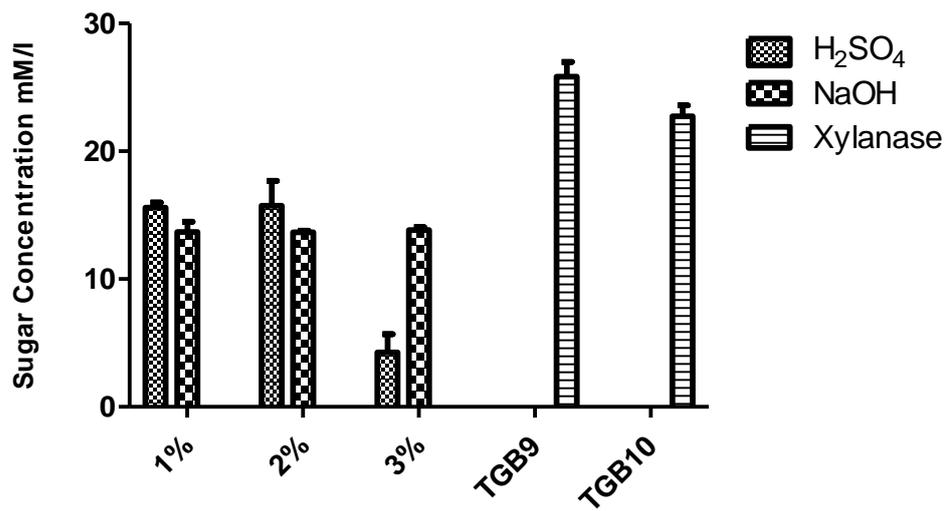


Figure 4: Comparative study of bacterial xylanases vs chemical pretreatment using corn stover

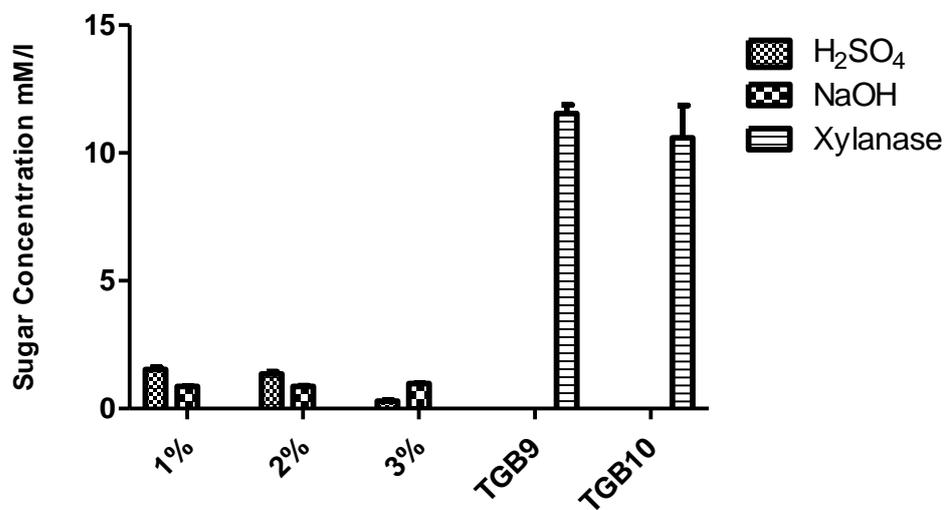


Figure 5: Comparative study of bacterial xylanases vs chemical pretreatment using cotton stalk

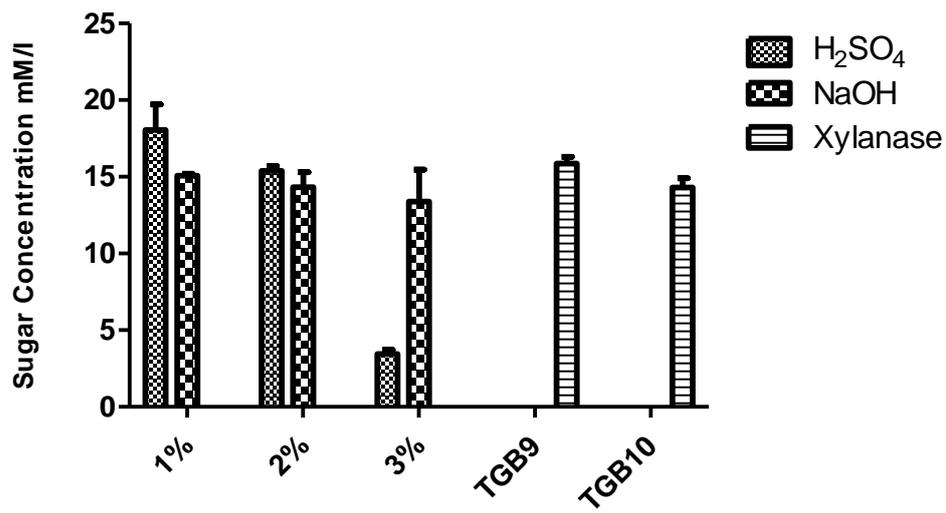


Figure 6: Comparative study of bacterial xylanases vs chemical pretreatment using rice straw