THE STUDY AIMED TO CHARACTERIZE THE CHARACTERISTICS OF BACTERIA ISOLATED FROM THE GUT OF MICROTERMES OBESI FOR THE PRODUCTION OF ETHANOL FROM SECOND-GENERATION BIOMASS

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ABSTRACT

Using enzymes from bacterial isolates extracted from termite guts, the present study also concentrated on converting lignocellulosic biomass samples into fermentable sugars and ethanol. Using 16S rRNA gene sequencing, two bacterial isolates were selected and identified. The Bacillus gene is shared by isolates TGB9 and TGB10. At 50oC and an ideal pH of 6.0, the isolates demonstrated increased xylan degradation activity. Stover, rice straw, and cotton stalk were among the agricultural substrates that were saccharified using xylanases from isolates TGB9 and TGB10. The maize stover produced results that showed increased levels of reducing sugars. Isolate TGB9's xylanases yielded greater yields of reducing sugar than isolate TGB10's. Additionally, xylanases from bacterial isolates and chemical pretreatment were compared. H2SO4 andNaOH were also used to agricultural substrates for this reason. In comparison to chemical pretreatments, the xylanases generated by TGB9 and TGB10 liberated a greater amount of sugar from agricultural substrates. Therefore, it may be said that bacteria found in termite guts are more effective than chemicals in hydrolyzing hemicelluloses.

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

Introduction

The global increase in gasoline costs and decrease in greenhouse gas emissions has resulted in a significant growth in the production of biofuels (Pourramezan et al., 2012). In this case, the most advanced source for producing biofuel is lignocellulosic biomass, which has a large energy storage capacity at a cheap cost (Roger, 2000). The most costly and important stage in the manufacture of biofuels from lignocellulosic biomass is the breakdown of cellulose and hemicelluloses into monomeric sugars. However, compared to sugars extracted from resources containing sugar or starch, the synthesis of sugars from lignocellulosic resources (cellulose and hemicelluloses) at high concentration is more complex (Lynd et al., 2008).The majority of significant research is needed in the biomass pre-treatment domain. It is necessary to create more inventive and more affordable enzymatic method to break down cellulose and hemicellulose into reducing sugars and extract lignin. Although the pre-treatment phase accounts for just 20% of the total cost of producing biofuel, it is the most costly and offers substantial room for improvement. For the elimination of lignin and the breakdown of hemicellulose, biologically based methods are therefore beneficial and a substitute for chemical and highly energy-consuming thermal treatment (Galbe and Zacchi, 2000; Scharf and Boucias, 2010).

Hemicelluloses, primarily β-1,4-D-xylanopyranosyl, and uronic monosaccharides form the heteropolymer known as xylan. According to Mandal et al. (2011) and Faik (2010), it can be broken down by xylanase and β-xylosidase. Several different microorganisms, such as bacteria and fungus, generate xylanase (Dhiman et al., 2009). The fuel, feed, food, paper, detergent, and textile sectors can all benefit from its application in the treatment of waste materials (Pal and Khanum, 2010).

Because fungi and bacteria have a significant potential for producing enzymes, they are among the various species from which cellulases and hemicellulases are explored genetically. According to Bignell et al. (1999), termites are believed to have obtained several sets of ingenious little lignocellulose hydrolyzing systems. Termites are responsible for billions of dollars' worth of damage annually and can reduce wood to dust. In the intestines of termites, there are around 200 species of various bacteria that can create cellulases and hemicellulases (Dheeran et al., 2012). Thus, hemicellulose and cellulose can be significantly hydrolyzed by termites (Ohkuma, 2003).

A variety of microbial species with distinct bacterial genes involved in the hydrolysis of cellulose and hemicelluloses have been found thanks to the advent of meta-genomic research. On the other hand, Wamecke et al. (2007) state that there is less evidence available on their functional variety. Several research groups have produced impressive studies in recent years to identify the incorporation of termite lignocelluloses. Thus, research was conducted to see whether termites might be used to modify agricultural waste products so that the amount of sugars they contained decreased. 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

Acacia nilotica dying trees provided the termites (Microtermes obesi) that degrade wood. From the National Agricultural Research Center (NARC) in Islamabad, Pakistan, agricultural substrates (cotton stalk, rice straw, and maize stover) were acquired.

Isolation and screening of xylan degrading bacteria

Following a 10-minute exposure to UV light, ten termites were sterilized using 70% ethanol. Using a pre-sterilized mortar and pestle, the heads were removed and the bodies were pounded. The suspension was made with Milli Q water. 1% xylan from beech wood (Sigma-Aldrich) was added to a diluted solution that was dispersed over plates containing nutrient agar media (Dheeran et al., 2012). Twenty hours were spent incubating petri dishes at 30 oC.

Using nutritional agar media with 0.2% xylan and the congo red dye technique, screening was done after bacterial colonies were isolated and purified (Dheeran et al., 2012). Using medium for screening, 5 µl of the isolates' overnight culture was put on petri plates. 48 hours were spent incubating the petri plates at 30 oC. By taking into account distinct zones within bacterial colonies, the capacity of bacteria to degrade xylan was determined (Wamecke et al., 2007; Liang et al., 2014). As positive and negative controls, respectively, E. Coli and B. subtilius were used.

16S rRNA gene sequencing

The 16S rRNA gene of a bacterial isolate was amplified using bacterial colonies (Batool et al., 2018). The 1.5 kb 16S rRNA fragment was amplified by PCR using universal primers for the 16S rRNA gene, 27F(5'-AGAGTTTGATCCTGGCTCA-3') and 1492R(5'-ACGGCTACCTTGTTACGACTT-3'). PCR products were purified usingthe ethanol precipitation technique (Jian and Hofstadler, 2003). The University of Illinois in Urbana-Champaign, USA's "Keck Center for Comparative and Functional Genomics" received the purified PCR products. NCBI received the sequences. Using the GeneBank BLASTN tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi), gene sequences from bacterial isolates were examined to determine the genera.

Xylanase production

Bashir et al. (2013) reported that a nutrient broth with 1% xylan had a pH of 6.8-7.2. was treated with a bacterial inoculum (TGB9 and TGB10) and incubated for 48 hours at a gentle rotation at 30 degrees Celsius. Using the supernatant from centrifugation, crude xylanase enzyme was obtained.

Xylanase activity assays

At a range of temperatures, including 30, 40, 50, and 60 oC, xylanase activities were examined for pH 5, 6, 7, 8, and 9. Rastogi et al. (2009) conducted a study on the pH parameter by preparing 1% xylan in various buffers. Every condition had a 30- minute reaction time. Substrates in buffer were utilized as a control group without any crude enzyme additions. The reducing sugar concentration was determined by employing the p-hydroxybenzoic acid hydrazide (PAHBAH) technique (Morietti and Thorson, 2008). Breznank and Brune (1994) defined an enzyme's activity as the quantity of enzyme that released 1µmol of reducing sugars per minute throughout the process. This was measured in units, or U.

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

Saccharification process using agricultural wastes at 5% dry weight (w/v). Xylanases from bacterial isolates and agricultural substrates were added to the reaction mixture at a ratio of 1:1. The mixture was subjected to a 24-hour gentle rotation incubation at 50 degrees Celsius. Brune et al. (1995) examined substrates in distilled water as a control.

Sccharification of agricultural substrates by chemicals

Using H2SO4 and NaOH at concentrations of 1, 2, and 3%, agricultural substrates were charred. Wenzel et al., 200; Konig, 2006; Kim et al., 2012) used cotton stalk, rice straw, and maize stover at a ratio of 5% (w/v) and processed them for 20 minutes at 121 °C.

Analytical Testing

Triplicates of the tests were run in order to ensure accuracy in the results. The MSTAT-C software was utilized to do an Analysis of Variance (ANOVA) on the outcomes derived from the chemical and enzymatic pre-treatment. To determine the standard error and standard deviation, the pretreatment area data were examined using GraphPadPrism 5.0 software.

RESULTS AND DISCUSSION

Isolation and screening of xylan degrading bacteria

A significant clear zone development during Congo Red screening led to the selection of two isolates from among the 53 bacterial isolates (Fig. 1). Both isolates TGB9 and TGB10 had roughly the same ratio to degrade xylan, according to the hydrolyzed clear zone diameters to colony diameter ratios.

Sequence of the 16S rRNA gene

A similarity index was determined by analyzing the 16S rRNA gene sequence data using BLASTN. The isolation of TGB9 and TGB10 was therefore shown to belong to the Bacillus genus. TGB9 was found to be 99% similar to Bacillus pumilus, whereas isolate TGB10 was found to be 99% similar to Bacillus licheniform after doing similarity searches. Accession numbers for isolates TGB9 and TGB10 are KR902555 and KR902570, respectively, for the sequences that were submitted to the NCBI..

Temperature and pH optimization of xylanase

It was discovered that the isolates TGB9 and TGB10 had the maximum xylanase activity at 50 oC (Fig. 2). The maximum enzyme activity for isolates TGB9 and TGB10 was seen at pH 6, as shown in Fig. 3. In comparison to isolation TGB10, which has 158.26 +1.9412 U/ml of enzyme (xylanase) activity, isolate TGB9 has substantially higher activity (270.37 +5.3208 U/ml).

Sccharification of agricultural substrate

When compared to chemically processed biomass (corn stover), a comparative research revealed that the xylanases isolated from TGB9 and TGB10 produced a much greater sugar content. Sugar production was shown to decrease when H2SO4 concentration rose (Fig. 4). When employing cotton stem as a substrate, Fig. 5 showed that the sugar yield produced by xylanases from both isolates was greater than the chemical pretreatement. However, compared to maize stover, cotton stalk releases less sugar. Pretreatment of rice straw had erratic outcomes when applied to maize stover and cotton stem. In comparison to bacterial xylanases, rice straw prepared with 1% H2SO4 yielded the highest concentration of sugar content (Fig. 6). According to Kim et al. (2012), the bacterial isolates with xylanases produced by TGB9 were able to hydrolyze all of the substrates with greater efficiency.

The enzymes generated by the bacteria that coexist symbiotically in the termite stomach were the main subject of this investigation. to determine the part that bacteria play in this biologically diverse mini-biorefinery's digestion of hemicelluloses. Termite gut isolates including facultative anaerobic and aerobic xylanolytic bacteria were investigated during the study. It was intended for the termite's stomach to have anaerobic conditions. On the other hand, both aerobic and anaerobic microorganisms were extracted from the termite stomach. Following Congo Red screening, two bacterialisolates were chosen due to their notable xylanase activity with xylan (Wenzel et al., 2002; Koniq, 2006; Kim et al., 2012; Kamble and Jadhar, 2012).

The two isolates that were chosen were discovered to belong to the same geneus, Bacillus, by molecular characterisation. Termites often harbor vast populations of facultative anaerobic, microaerophilic, celluloytic, and xylanolytic bacteria in their guts. According to Matteotti (2012), the majority of species in the gastrointestinal content are Bacillus species, with concentrations as high as 107/ml. Many free-living and symbiotically related Bacillus species may be found throughout the environment. A large proportion of them exhibit high levels of activity and participate in the production of several enzymes that break down wood, including ligninase, laccase, amylase, xylanase, and hemicellulase (Karmi et al., 2006). According to a research, among the isolates from Microcerotermes diversus, Bacillus and Acinetobacter were the most

effective in degrading biomass (Vlasenko et al., 1997). *As per Matteotti et al. (2011),* Bacillus species were identified as the predominant lignocellulose-destructive bacterial isolates obtained from soil, animal waste, and paper mill sludge samples.

In the case of the bacterial isolates, maximum xylanase activity was noted at 50 oC. In one study, it was shown that a Bacillus sp. isolated from soil responded best to a temperature of 50 oC for xylanase activity. The isolates TGB9 and TGB10 were found to have the maximum xylanase activity at pH 6.0 during the experiments. extracted and purified xylanases N and A from Bacillus sp. The optimal pH range for xylanase N was found to be between 6.0 and 7.0, while pH 6.0–10.0 was the optimal range for xylanase AHowever, gram positive bacteria from termite guts demonstrated maximal xylanase activity at pH 5.0 at 55 oC, according to a research (Silverstein et al., 2007; Mosier et al., 2005).

When maize stover was utilized as a substrate instead of cotton stalk or rice straw, the xylanases generated by TGB9 and TGB10 released the largest amount of sugar. However, in comparison to maize stover, rice straw has a higher carbohydrate content (mannose, xylose, glucose, etc.). However, Karimi et al. (2006) found that rice straw had a significant mineral concentration. It caused rice straw to decompose more slowly in the soil. According to a research, employing reducing agents increased xylanase activity. Xylanase activity was, however, reduced by chelating agents including detergents and Cu2+. The pretreatment chemicals (H2SO4 and NaOH) are not hindered by high mineral concentration, but the activity of these enzymes is. As a result, when rice straw was pretreated chemically, almost the same concentration of sugar was released. When comparing cotton stalk to other agricultural substrates, the least amount of sugar is liberated. Research revealed that cotton stalks had a significant lignin concentration, making hydrolyzation challenging. Furthermore, a drop in sugar output was noted in response to an increase in acid content. The release of sugar under acidic circumstances was confirmed to cause some of it to convert into inhibitors like hydroxymethyl furfural and furfural (Bashir et al., 2013; Batool et al., 2018), The concentration of inhibitors rises as the acid content climbs. As a result, a complete drop in the concentration of sugar contents was seen in several instances.

CONCLUSION

It is determined that a sizable population of bacteria that are beneficial for the breakdown of lignocellulosic biomass are symbiotically harbored in the stomach of termites. Furthermore, it was shown that xylanses are capable of hydrolyzing complex substrates in addition to their pure substrate, xylan. In order to use certain bacterial xylanses in the fermentation process, it is crucial to isolate, purify, and characterize them. They also rely on the continuation of less expensive agricultural resources and their management. Furthermore, the ability of these bacteria to break down hemicelluloses may aid in the fermentation of these sugars into useful final products.

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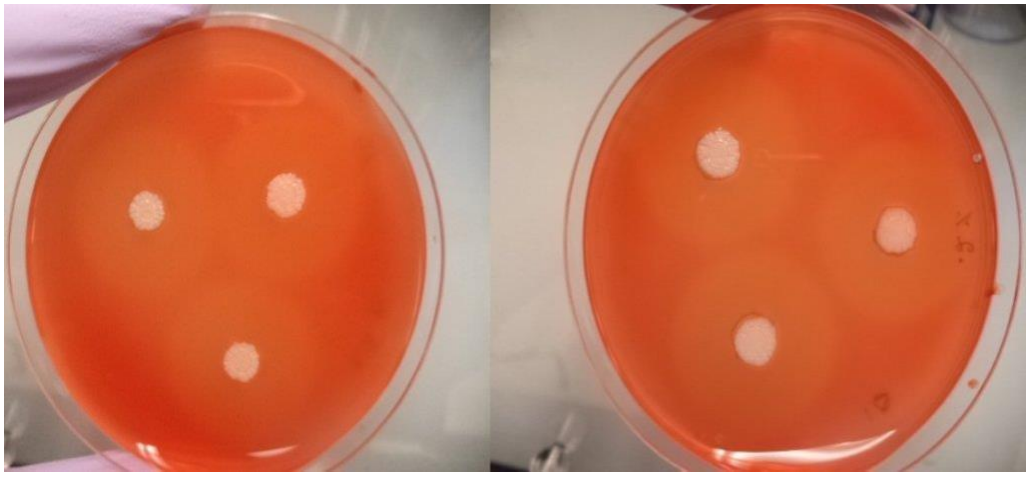

Figure 1: Congo red screening, from left to right: Isolate TGB9 and isolate TGB10

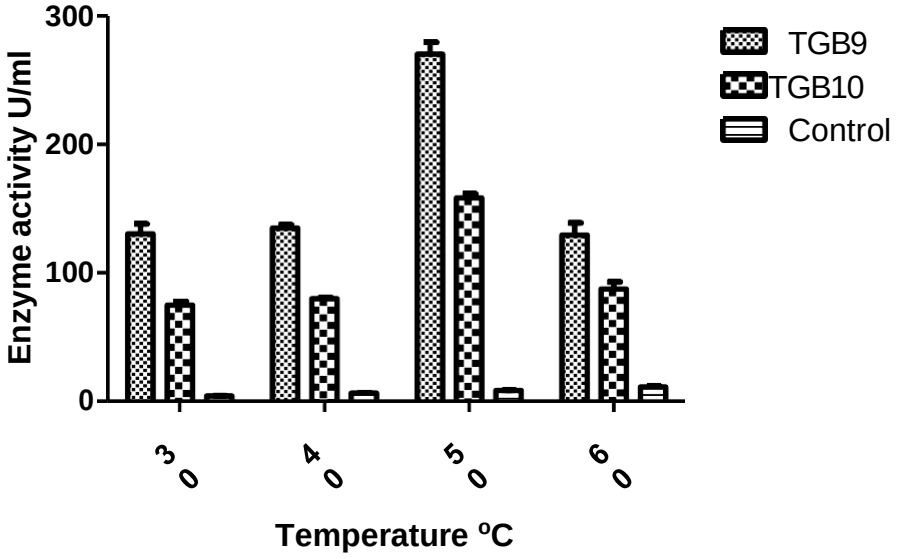

Figure 2: Temperature optimization of enzyme activity (U/ml) for isolates TGB9 and TGB10

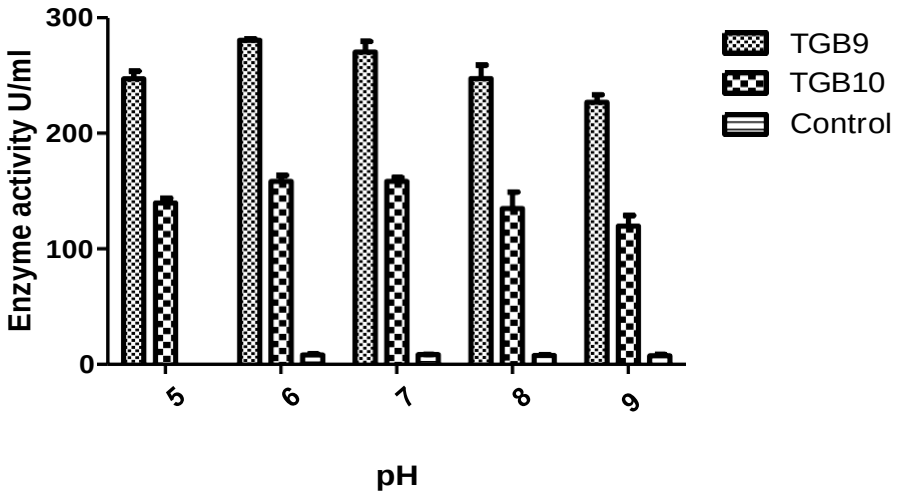

Figure 3: pH optimization of enzyme activity (U/ml) for isolates TGB9 and TGB10

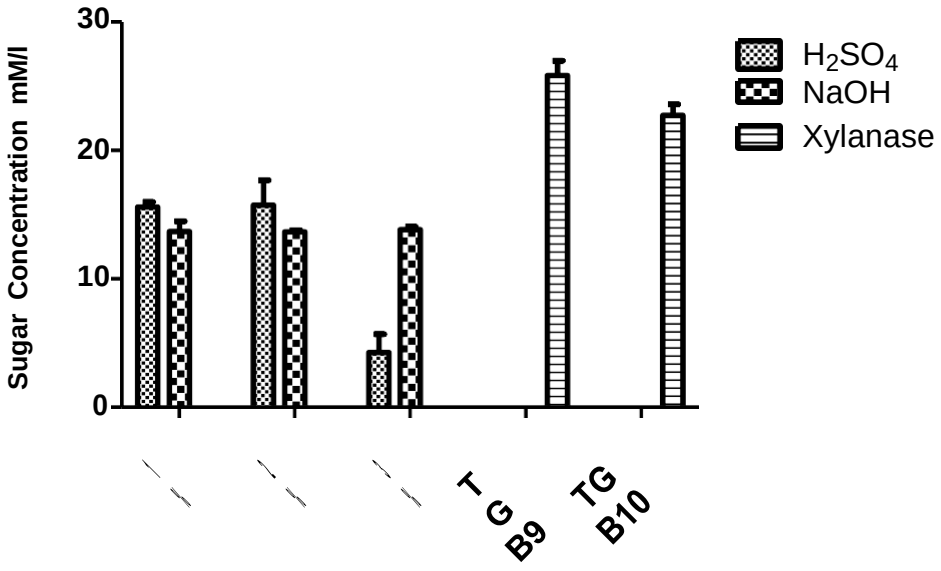

Figure 4: Comparative study of bacterial xylanases vs chemical pretreatment using corn stover

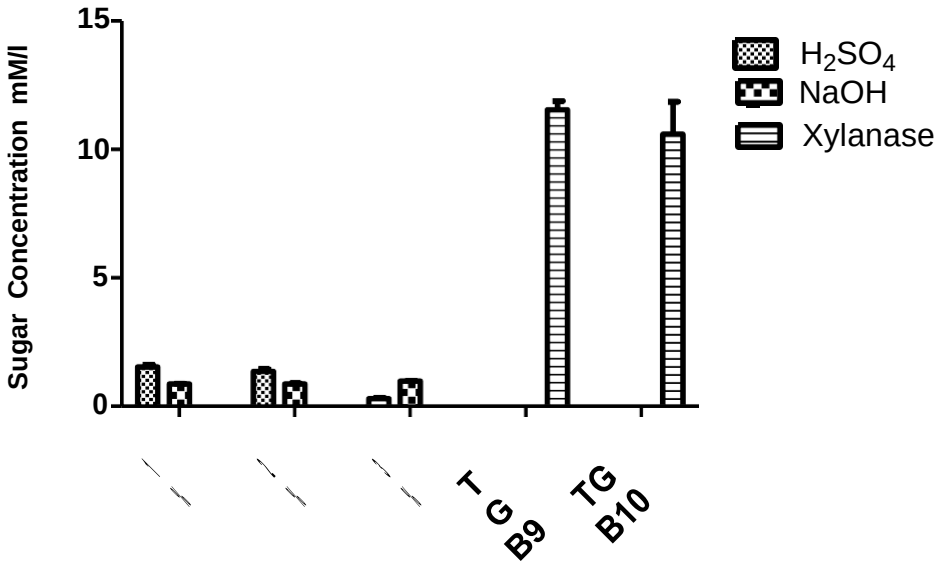

Figure 5: Comparative study of bacterial xylanases vs chemical pretreatment using cotton stalk

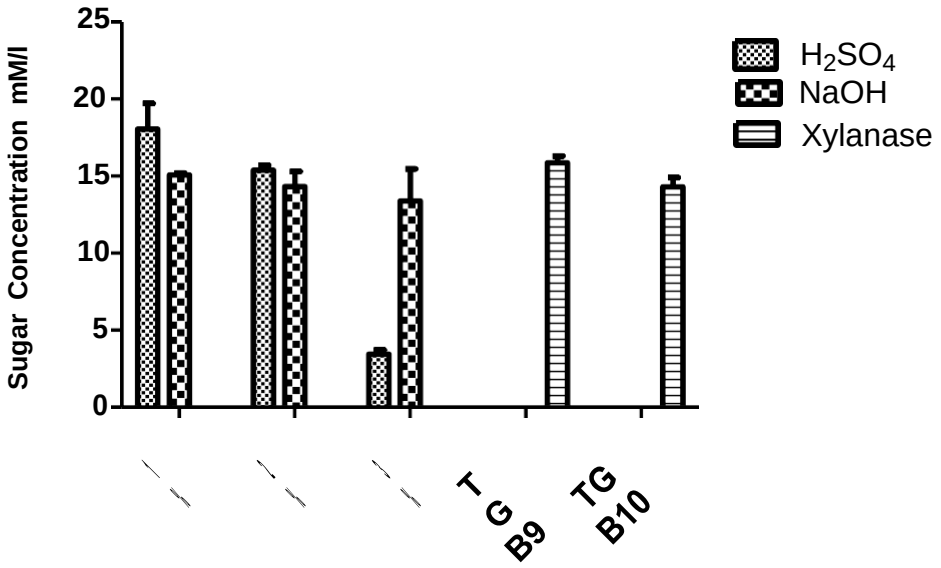

Figure 6: Comparative study of bacterial xylanases vs chemical pretreatment using rice straw