UTILIZATION OF TERMITE GUT BACTERIA FOR BIOFUEL PRODUCTION

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ABSTRACT

Researchers looked in the termite stomach for aerobic and facultative anaerobic xylanolytic and cellulolytic bacteria. on the Islamabad area, termites were found on trees that were decaying. Bacteria were tested for CMCase and xylanase activity using Congo red screening after being isolated from termite stomach. The two bacterial strains that were detected using the sequencing of the 16S rRNA gene. The results showed that the two samples are from the Bacillus family. Optimal conditions for the production of crude cellulase and xylanase enzymes were tested at 30, 40, 50, 60, and 70 ^{o}C using avicel, CMC, and xylan as substrates. Cellulase activity of isolates TGB9 and TGB10 is highest at 40 ^{o}C when using avicel as a substrate, and at 50 ^{o}C when using CMC and xylan. We tested the enzyme activity of the isolates at 5, 6, 7, 8, and 9 pH to find the optimal pH. When tested on avicel at pH 7 and CMC at pH 8, the cellulase activity of isolates TGB9 and TGB10 was shown to be greater. Enzyme activity is at its peak at pH 6 when xylan is used as a substrate isolate by TGB9 and TGB10. Isolate TGB9 has significant xylanase activity, whereas isolate TGB10 demonstrates high cellulase activity. The sccharification of maize stover, cotton stalk, and rice straw was carried out using crude enzymes (cellulase and xylanase) from isolate TGB9 and TGB10. Cotton stalks produced the least amount of sugar compared to maize

stover, which released the most reducing sugar. Isolate TGB9 xylanase yields reducing sugar at a high rate. Due to the relative difficulty of hydrolyzing cellulose and hemicelluloses, it was shown that xylanase from both isolates is more efficient than cellulase. Bacterial isolates TGB9 and TGB10 were used directly to sccharify and ferment the agricultural substrates (corn stover and rice straw) in place of chemical treatments and enzymatic hydrolysis. Using maize stover as a substrate resulted in the maximum bioethanol output for both isolates.

Key words: Termite, 16S rRNA, *Bacillus*, cellulase, xylanase, bioethanol

1. BACKGROUND

Pakistan has an enormous need for fossil fuel power generation, but the country can only meet 20% of that need using its own resources. The remainder of the world's oil and petroleum products are imported from various Arabian and other nations. Nowadays, almost 100 million barrels of oil are spent on gasoline annually (MPNR, 2014). The global concentration for bioethanol production has expanded dramatically because to the present high oil costs, the environmental advantages of decreasing GHG emissions, and the prospects for local growth (Rogers, 2008). Manufacturing bioethanol has the potential to significantly cut down on fuel costs. According to the Pakistan Defense Forum (2008), Pakistan has the potential to manufacture 500 million liters of ethanol today, which might assist reduce the need for petroleum goods.

To make biofuels, lignocellulosic resources are used primarily because they are abundant and inexpensive environmental energy storage options (Lynd et al., 2008). Approximately half of all biomass comes from lignocellulosic materials, and scientists predicted a 10–50 billion ton annual output of this material (Claassen et al., 1999). Various evaluations have shown that the agricultural area of Sanghar in Pakistan's Sindh province produces over 2.7 million tons of garbage

each year. This includes canola and wheat straw, rice straw and husks, sugarcane remnants, cotton stalks, and bagasse. The majority of these materials are burned off and around 75% to 85% of them are useless. Consequently, these resources might be put to use in producing electricity without compromising food supplies or other essential household items (UNEP, 2011).

Hydrolysis of hemicellulose and cellulose to monomeric reducing sugars is an important step in producing ethanol from lignocellulosic resources. The process of extracting sugars from plants that contain starch or sugar is simpler than the one that involves a high concentration of cellulose and hemicellulose (Galbe and Zacchi, 2007).

Improved and more cost-effective enzymatic methods for lignin removal and hydrolysis of cellulose and hemicellulose into their respective sugars rank high among the biomass pre-treatment area's most pressing research requirements. Pretreatment is where there is a lot of room for improvement as it is the most expensive part of bioethanol production (around 20% of overall expenditures). For delignification and hemicellulose hydrolysis, in particular, biologically based approaches are preferable to energy-intensive and chemically insensitive high-temperature treatments (Scharf and Boucias, 2010). For the simple reason that chemical pretreatments may also need some work. The process of extracting sugars from ionic liquids is quite difficult. Further complications arise from the fact that any residual ionic liquor in the pretreatment hydrolysate poses challenges for subsequent enzymatic hydrolysis and fermentation processes, in addition to the expense and chemical waste associated with losing portion of the ionic liquor (Sanderson, 2011).

Therefore, lignocellulase genes from other taxa, such as free-living fungus and bacteria, have been the main focus. Therefore, termites should have a variety of effective micro-scale

mechanisms for decomposing lignocelluloses (Bignell et al., 1997). Termites are well-known pests that may reduce wood to dust every year, a process that might cost billions of dollars. Dheeran et al. (2012) found that termites host over 200 kinds of microbes, some of which generate enzymes that degrade cellulose and hemicellulose. Research by Bretznak and Brune (1994), Watanabe et al. (1998), and Scharf and Tartar (2008) has shown that termites use endogenous and symbiont-based digestive enzymes to absorb lignocellulose.

Termites are known to consume a large amount of cellulose (75-99%) and hemicellulose (70-89%), according to Ohkuma (2003). Midgut of higher termites, namely Nasutitermes takasagoensis, often contains endo-β-1,4-glucanase enzymes, as shown by Tokuda et al. 1997. In 1998, Watanabe et al. sequenced the first termite cellulase gene.

In a bioreactor, a wide assortment of bacteria eat wood to transform the lignified cell-wall of plants into fermentation products that serve as an energy source. This process is similar to that of termites in their stomach. Termites rely on these bacteria for the digestion of lignocellulosic materials (Varma et al., 1994). While meta-genomic studies have uncovered hundreds of microbial species and a large collection of bacterial genes involved in hemicellulose and cellulose hydrolysis, very little is known about the functional diversity of these genes (Warnecke et al., 2007).

A number of studies have made significant strides in understanding the absorption of lignocelluloses by termites in recent years. The termites' ability to convert cellulosic material into sugar and ethanol was, however, the only focus. The current research set out to accomplish the following with the bioethanol's potential as a green alternative fuel in mind.

2. MATERIALS AND METHODS

2.1. Termite Collection

In Islamabad, subterranean termites (Microtermes obesi) were seen feeding on decaying *Acacia nilotica* trees found along roadside..

2.2. Isolation of Cellulose and Xylan Degrading Bacteria

We used 70% ethanol to surface-sterilize ten termites, and then we exposed them to UV light for 10 minutes to kill any remaining bacteria. In order to isolate bacteria, the heads were removed and the bodies were collected. After being sanitized beforehand, the corpses were crushed and suspended in Milli Q water using a pestle and mortar. According to previous studies (Dheeran et al., 2012; Pourramezan et al., 2012), the plates were made using nutritional agar media that included 1% carboxymethyl cellulose (CMC) and 1% xylan (Hemicellulose) independently, with the pH set to 7. Following a 24-hour incubation period at 30 oC, the plates were covered with the diluted solution. In order to purify the isolates, colonies that emerged after 24 hours were streaked on separate plates.

2.3. Screening of Cellulose and Xylan Degrading Bacteria

The congo red dye technique was used to screen bacterial isolates for their ability to degrade cellulose and xylan. One medium was made using CMC and referred to as CMC nutritional media; the other was made with xylan. The ingredients are as follows: 10 grams of beef extract, 10 grams of peptone, 5 grams of sodium chloride, and 18 grams of agar in 1 liter of distilled water. Separately, 0.2% CMC and xylan were added. The samples were incubated in nutritional broth over the night. Five microliters of the overnight culture was then added to the plates. Both mediums were used to test the cellulase and xylanse activities of each isolate. The plates were then kept at 30 °C for 48 hours to allow the bacteria to grow (Dheeran et al., 2012). For 20 minutes, the plates were submerged in a Congo red solution (0.2%) prepared with 5% ethanol. Extra dye color was removed by washing the plates with 1M NaCl 2-3 times. Bacterial colonies that had a clear

zone were identified as those that degraded cellulose and xylan (Tokuda and Watanabe, 2007). Ariffin et al. (2006) determined their hydrolysis capacity by dividing the diameter of the clean zone by the diameter of the colony.

2.4. 16S rRNA gene sequencing of CMC and Xylan degrading bacteria Isolates

The bacterial strains were incubated on nutrient agar for 24 hours at 30 °C. Matotti et al. (2011) immediately used the cultures for PCR amplification of the 16S rRNA gene of bacteria. Primers 27F(5'-AGAGTTTGATCCTGGCTCA-3') and 1492R(5'-ACGGCTACCTTGTTACGACTT-3') were used to amplify a full-length (1.5 kb) 16S rRNA fragment in order to conduct identification. Half a milliliter of PCR super mix, one milliliter of each primer, three milliliters of nano-pure water, and a little amount of colony served as the template for the 50 µl PCR reaction. There were 30 cycles of 94 °C for 30 seconds, 53 °C for 45 seconds, and 72 °C for 90 seconds in the thermal cycling process. After that, 72 °C for 7 minutes, and finally, a hold at 4 °C. The lid temperature was 105 °C. According to Shapiro (1981), the PCR products were isolated by using the ethanol precipitation procedure.

To the "Keck Center for Comparative and Functional Genomics" at Urbana-Champaign, USA, the University of Illinois at Urbana-Champaign received the purified DNA (PCR products). The Geneious R8 bioinformatic tool was used to construct consensus sequences after sequencing. The BLAST N tool from the National Center for Biotechnology (NCBI) was used to conduct the sequence similarity searches. Using CLUSTW, the sequences were matched up with their nearest homolog. Phylogenetic trees were constructed using MEGA6.06 and the neighbor joining technique with 1000 bootstrap values. Additionally, NCBI was notified of the sequence submission.

2.5. Characterization of Crude Cellulase and Xylanase

2.5.1. Enzymes Production

Overnight at 30 oC and 180 rpm, the bacterial isolates were grown in nutritional broth medium (ten grams per liter of beef extract, ten grams of peptone, and five milliliters of sodium chloride). Afterwards, enzyme production medium were injected with 1% of the culture. Enzyme manufacturing required two distinct media. One medium had 1% CMC as substrate, while the other contained 1% xylan. The other components of the two media were identical: 10 grams of beef extract, 10 grams of peptone, and 5 grams of sodium chloride in 1 liter of distilled water (Bashir et al., 2013; Dheeran et al., 2012). A pH range of 6.8-7.2 was achieved after inoculation, and the medium were cultivated for 48 hours at 30 °C and 180 rpm. Following the synthesis of enzymes, the cultures were centrifuged at 6,000 rpm for 20 minutes at 4 oC. The crude enzyme was extracted from the supernatant.

2.5.2. Enzymes Activity Assays

The activities of endoglucanase, exoglucanase, and xylanase were investigated using CMC, avicel, and xylan, respectively, serving as substrates. Per Ratogenet al. (2009), 0.025 mM of sodium phosphate (pH 6.0, 7.0, and 8.0) was used in the separate preparation of 1% avicel, CMC, and xylan substrates in 200 mM of sodium citrate (pH 5.0) and glycine-NaOH (pH 9.0) buffers. Sodium phosphate buffer in high concentrations is hazardous to reactions. A range of pH values (5.0, 6.0, 7.0, 8.0, and 9.0) and temperatures (30, 40, 50, and 60 oC) were used to evaluate the enzyme activity.

The enzymatic tests were carried out in a reaction volume of 0.2 ml, which included 0.1 ml of crude enzyme and 0.1 ml of buffered substrate. The control group consisted of substrates in buffer that had no enzyme added to them. Due of their hydrolysis resistance, CMC and avicel substrates required 60 minutes for the reaction, but xylan substrate only required 30 minutes.

According to Moretti and Thorson (2008), the sugar content was ascertained by means of the p-Hydroxybenzoic acid hydrazide (PAHBAH) technique. An enzyme was considered to be active if it emitted 1µmol of reducing sugars per minute during the process, which was measured in units of U.

2.5.3. Sccharification of Agricultural Substrates using Enzymes from Isolates of Termite Gut

Sccharification of maize stover, cotton stalk, and rice straw was carried out using enzymes from isolates TGB9 and TGB10.

To eliminate any impurities, 5% dry weight (w/v) of corn stover, cotton stalk, and rice straw were autoclaved. Substrate treatment continued with the addition of crude xylanase and cellulase enzymes from isolates 9x and 10, respectively. There was exactly one enzyme for every substrate. The reaction was allowed to sit at 50 degrees Celsius for a whole day. To determine the specific enzyme activity, cellulase and xylanase from isolate TGB9 and TGB10 were added to substrates independently. In order to determine the impact on substrates, the cellulase and xylanase from each isolate were also combined in a 1:1 ratio and evaluated together. As a control, we used maize stover, cotton stalk, and rice straw that had been treated with distilled water. Following substrate sccharification, the sugar content was assayed according to the protocol outlined by Moretti and Thorson (2008).

2.5.4. End Product Analysis of Isolates from Termite Gut

Bacterial isolates TGB9 and TGB10 were used directly to sccharify and ferment the agricultural substrates (corn stover, cotton stalk, and rice straw) in the absence of chemical treatment. The following water-soluble additives were added to the 5% dry weight (w/v) corn stover, cotton stalk, and rice straw: KH2PO4, MgSO4, NaCl, CaCl2, FeSO4.7H2O, NH4Cl, and

yeast extract, all in g/L. In order to eliminate any potential impurities, the substrates were autoclaved (Rastogi et al., 2009).

In nutrient broth medium, the bacterial isolates were pre-cultured overnight at 30 oC and 180 rpm. Inoculated with 1% of cultivated isolates, autoclaved maize stover, cotton stalk, and rice straw were incubated at 30 oC for 7 days at 100 rpm under microaerophilic conditions.

Protein estimation using the modified Bradford technique from 1976 was used daily to measure cell viability.

Centrifuged at 14,000 rpm for 20 minutes at 4 oC, the fermentative medium derived from maize stover and rice straw was strained to extract any leftover substrates and dead bacterial cells after six days. The filtrate was kept at -20 oC for HPLC analysis after being filtered through 0.22 µm membranes to eliminate any remaining contaminants from the collected supernatant. The analysis of cotton stalk end products was omitted due to the fact that the protein data demonstrated the absence of bacterial development.

A refractive index detector was used to inject the cell free filtrate into an HPX 87H column. The 5 mM sulfuric acid mobile phase was heated to 25 oC and flowed at 0.4 ml/min. The efficiency of the isolates for secondary metabolites synthesis was determined by testing them as end products with acetate, ethanol, formate, and lactate.

3. RESULTS AND DISCUSSION

3.1. ISOLATION AND SCREENING OF CELLULOSE AND XYLAN DEGRADING BACTERIA

Researchers looked in the termite stomach for aerobic and facultative anaerobic xylanolytic and cellulolytic bacteria. According to Brune et al. (1995), termite guts are anaerobic. Wenzel et

al. (2002) also found aerobic and anaerobic microorganisms in termite guts. The termite stomach contained 53 different types of microorganisms.

When testing for cellulose and xylan breakdown using Congo red, bacterial isolates that formed a clear zone around the colony were deemed positive. A positive control was chosen using B. subtilus, whereas a negative control was chosen using E. coli. Figure 1 shows that only 2 of 53 isolates were able to degrade both substrates. Table 1 displays the ratio of hydrolyzed clear zone diameters to colony diameters. Evidence showing isolation TGB9 is more capable of degrading xylan than isolate TGB10, despite the fact that their ratios for degrading CMC are same. Consequently, TGB9 and TGB10 were chosen for further investigation into enzyme activity using quantitative tests. The endoglucanase activity of cellulase enzyme is mostly catalyzed by CMC (Zhang and Lynd, 2004). Both isolates exhibit strong xylanase activity, since they are highly xylan specific compared to CMC.

3.2. MOLECULAR CHARACTERIZATION OF BACTERIAL ISOLATES

16S rRNA gene sequencing was used to characterize the bacterial isolates. Both isolates are members of the Bacillus genus, according to the results of a BLASTN similarity search conducted on the sequencing data. The sequence similarity between the two isolates and their respective genera is around 90%. The bacterial phylogenetic tree that breaks down xylan and cellulose is constructed. Isolate TGB10 is more closely related to B. licheniform, whereas all cellulose and xylan degrading bacteria TGB9 are closer to B. safensis, as shown in Figure 2. Isolate TGB9's accession number is KR902555, while isolate TGB10's is KR902570, both of which correspond to sequences that were submitted to the NCBI database. The capacity of Bacillus and Acinetobacter to degrade cellulose was shown to be primarily possessed by Microcerotermes diversus, according to a research conducted by Pourramezan et al., 2012. According to studies

conducted by Kim et al. (2012) and Maki et al. (2011), Bacillus emerged as the predominant cellulose-degrading bacterium in soil, animal waste, and paper mill sludge samples.

3.3. Characterization of Crude Cellulase and Xylanase

3.3.1. Temperature Optimization of Crude Cellulase

Figure 3 shows that when tested on avicel as a substrate, isolates TGB9 and TGB10 exhibited the highest cellulase activity at 40 oC. The enzyme activity of isolate TGB10 is much higher than that of isolate TGB9 (3.7486 +0.0309). Exoglucanase activity is strong in isolate TGB10. According to Zhang and Lynd (2004) and Sharrock (1988), the crude enzymes were tested for endoglucanase and exoglucanase activity, respectively, using CMC and avicel as substrates. Using CMC as a substrate, both isolates exhibited excellent enzyme activity at 50 oC (Figure 4). The cellulase activity of isolation TGB10 was the greatest at 5.4121+0.1541 U/ml, in comparison to isolate TGB9's 3.3068 +0.1245. The cellulase enzyme was also shown to be completely stable at temperatures of 40 and 50 oC by Pourramezan et al., 2012. The activity of both isolates is low when using avicel as a substrate, but it is high when using CMC. This is due to the fact that avicel is composed of microcrystalline cellulose, which is not readily biodegradable, in contrast to CMC, which is a soluble and hydrolyzable type of cellulose (Rastogi et al., 2009).

3.3.2. pH Optimization of Crude Cellulases

Enzyme activity is highest at 7 pH when avicel is used as a substrate for isolate TGB9 and TGB10 (Fig. 5). When comparing the enzyme activities of TGB9 and TGB10, the results demonstrate that TGB10 has a much higher level of activity (3.7486 +0.0309 U/ml) than TGB9. If you use CMC as a substrate for endoglucnase activity, as shown in Fig. 6, you may optimize the pH of bacterial isolates TGB9 and TGB10. The optimal pH for enzyme activity was found to be 8 for all isolates. The enzyme activity of isolate TGB10 is 5.6719 +0.2260 U/ml, which is higher

than that of isolate TGB9, which is 3.4962 +0.1657 U/ml. It is quite unusual for bacteria to exhibit their peak cellulase activity at pH levels below 6.0. On the other hand, Paenibacillus curdlanolyticus and Bacillus mycoides showed their highest activities at neutral or alkaline pH (Pason et al., 2006 and Balasubramanian et al., 2012).

3.3.3. Temperature Optimization of Crude Xylanase

Isolates TGB9 and TGB10 exhibited substantial levels of xylanase activity at 50 oC, as shown in Figure 7. In comparison to isolation TGB10, which has xylanase activity of 158.26 +1.9412 U/ml, isolate TGB9 demonstrates the greatest activity at 270.37 +5.3208 U/ml. Additionally, Kamble and Jadhav (2012) found that xylanase, which is made from a soil-isolated Bacillus sp., is most effective when heated to 50 oC.

3.3.4. pH Optimization of Crude Xylanase

The findings for optimizing the pH of the xylanase activity of bacterial isolates TGB9 and TGB10 are shown in Figure 8. Isolate TGB9 and TGB10 showed their maximum enzyme activity at pH 6. Isolate TGB9 had the greatest xylanase activity at 280.62 +0.6680 U/ml, whereas isolate TGB10 had the lowest at 158.37 +2.9687 U/ml. The xylanases N and A were isolated from Bacillus sp. and purified in 1985 by Honda et al. The optimal pH range for xylanase N is between 6.0 and 7.0, whereas the dynamic range for xylanase A is between 6.0 and 10.0.

Isolate TGB9 and TGB10 have a higher degree of substrate specificity for xylan than for CMC and avicel, according to data for all three substrates. This indicates that xylanase is more active than cellulase. The crystalline structure of cellulose makes hydrolysis more difficult than that of hemicelluloses (Cardona et al., 2009).

3.4. Sccharification of Agricultural Substrates using Enzymes from Isolates of Termite Gut

3.4.1. Sccharification of agricultural substrates by isolate TGB9

Utilizing maize stover as a substrate, it was shown that cellulase from isolate TGB9 produced 15.669 +0.5390 mM/l sugar, taking celluslase enzymes into account. Testing xylanase activity revealed that TGB9 isolate xylanase released 25.836 +0.8178 mM/l of sugar. In a comparison of cellulase and xylanase from isolate TGB9, the latter was shown to release a higher amount of reducing sugars. For optimal results, use xylanase on corn stover as a substrate. In Figure 9, the concentration of sugar released was found to be between that of sugar produced by cellulases and xylanases alone when a combination of the two was utilized. Xylanase (11.543 +0.2416 mM/l) and cellulases (2.7063 +0.1301 mM/l) isolated from TGB9 hydrolyzed cotton stalk, releasing sugar content, respectively. Following treatment of rice straw, the sugar content was 8.4758 +0.3717 mM/l for cellulase and 15.874 +0.2974 mM/l for xylanse, both of which were generated by isolate TGB9.

3.4.2. Sccharification of agricultural substrates by isolate TGB10

The sugar content released from maize stover utilizing the TGB10 cellulase enzyme was 12.900 +0.7063 mM/l, whereas the sugar content released using the xylanse enzyme was 22.732 +0.6134 mM/l. Using cotton stalk as a substrate, the cellulase and xylanase from isolate TGB10 yielded sugar concentrations of 3.0037 +0.0558 mM/l and 10.595 +0.8922 mM/l, respectively. Enzymes cellulase and xylanase digested rice straw to produce sugar at concentrations of 8.8290 +0.0186 mM/l and 14.294 +0.4275 mM/l, respectively. By combining cellulase with xylanase, the sugar concentration was found to be in the middle of what would have been achieved with either enzyme alone (Fig. 10).

3.5. END PRODUCT ANALYSIS OF ISOLATES FROM TERMITE GUT USING AGRICULTURAL SUBSTRATES

3.5.1. End product analysis of corn stover fermentation

The acetate concentrations for TGB9 and TGB10 were quite similar, measuring 1.1558 +0.0786 and 1.2748 +0.1234 mM/l, respectively. The results showed that either TGB9 and TGB10 isolates do not create formate as a fermentative product or the concentration of formate was so low that it was not detectable. Lactate concentrations of 3.4406 +0.3419 mM/l were achieved by isolate TGB10. The primary goal was to find out how well the bacterial isolates produced ethanol. In contrast to isolation TGB9's ethanol concentration of 5.7374 +0.2861 mM/l, isolate TGB10's ethanol production was 6.9896 +0.5830 mM/l (Table 2).

3.5.2. End product analysis of rice straw fermentation

Isolate TGB9 fermented rice straw to generate the highest quantity of acetate (3.0043 +0.6555 mM/l). Unlike when maize stover was used as a substrate, isolate TGB9 does not create formate. With rice straw, however, isolate 10 produces formate at a concentration of 1.2044 +0.1721 mM/l. So, it was concluded that formate is the end result of isolate TGB10. Formate concentration in maize stover, on the other hand, may be so low that it goes undetected. Isolate TGB10 produced the most lactate, at 6.1404 +0.5057 mM/l. When comparing the two isolates' ethanol production rates, it was found that TGB10 generated 5.9928 +0.2693 mM/l of ethanol, whereas TGB9 produced 3.3427 +0.4101 mM/l. However, when rice straw was used as a substrate instead of maize stover, the concentration of ethanol was found to be lower. In a study conducted by Fujimoto et al. (2011), it was shown that Bacillus licheniformis-related isolates R8 and R15 could degrade cellulose well in a microaerophilic environment; however, the quantity of ethanol produced during fermentation was minimal.

Table 1: D/d: Hydrolyzed zone diameter/colony diameter on agar media containing CMC or xylan as sole carbon source

Bacterial Isolates	Average	
	CMC D/d (mm)	Xylan D/d (mm)
Isolate 9x	3.3	4.12
Isolate 10	2.74	4.12

0.2% CMC

0.2% Xylan

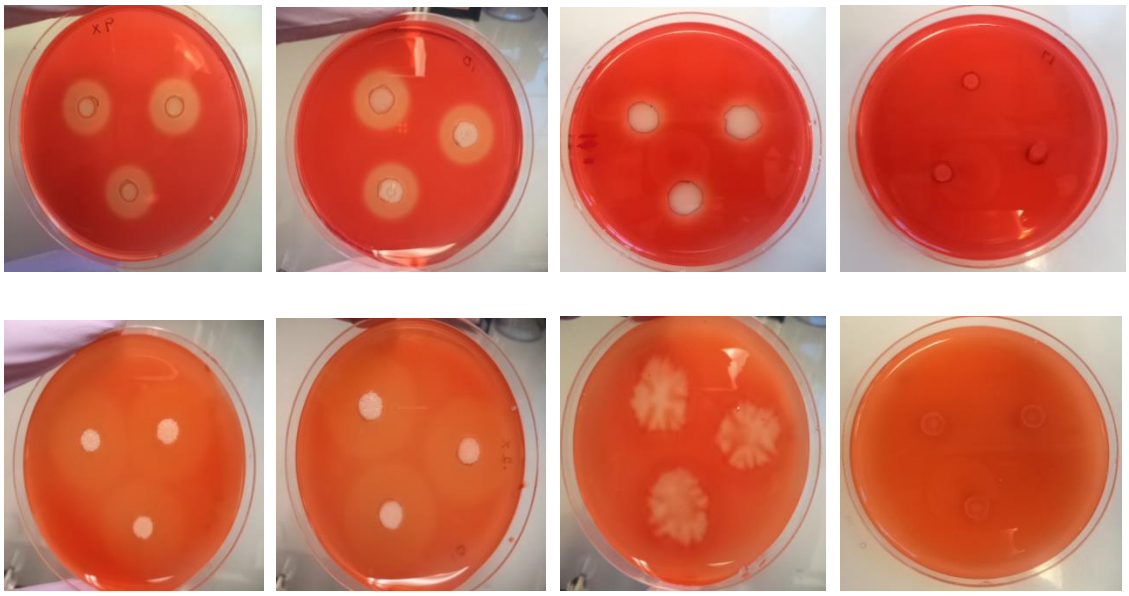

Figure 1: From left-right: Isolate TGB9, Isolate TGB10, *Bacillus subtillus*, *E. coli*

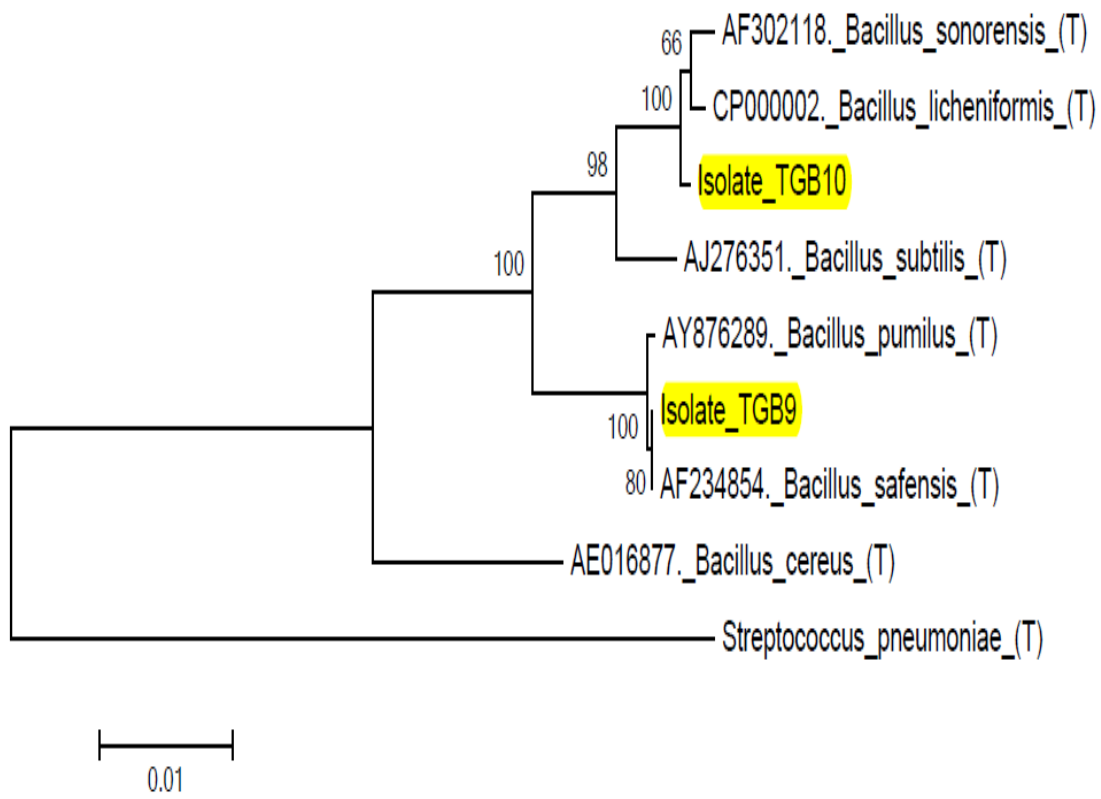

Figure 2: Phylogenetic tree for the cellulolytic and xylanolytic bacterial isolates from termite gut

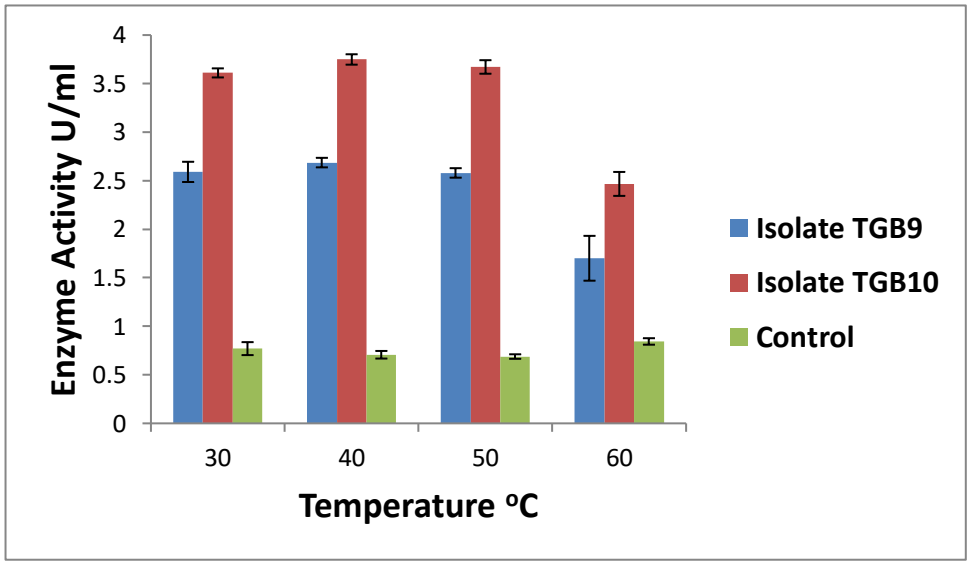

Figure 3: Temperature optimization of enzyme activity (U/ml) for isolate TGB9 and TGB10 using Avicel as substrate

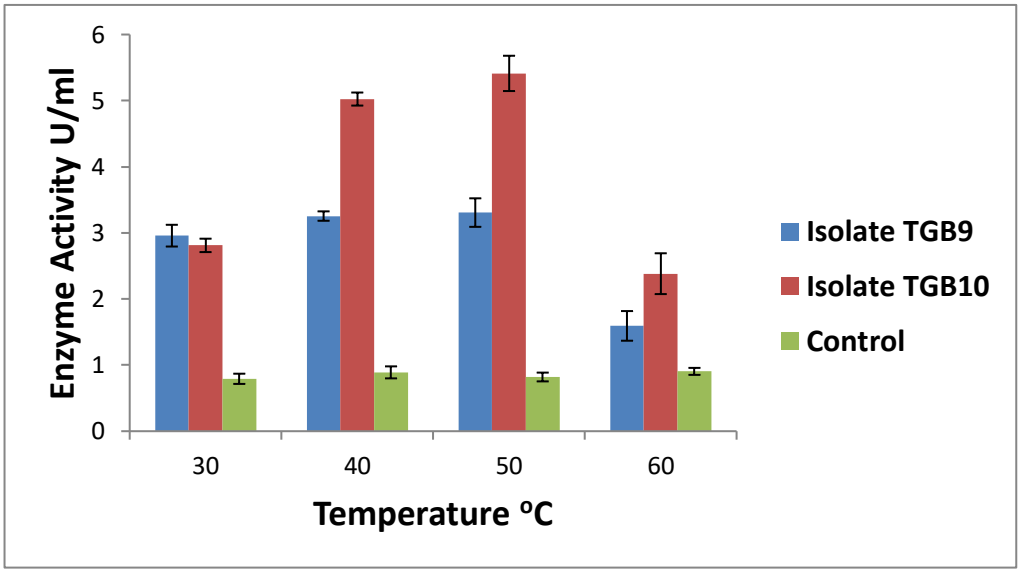

Figure 4: Temperature optimization of enzyme activity (U/ml) for isolate TGB9 and TGB10 using CMC as substrate

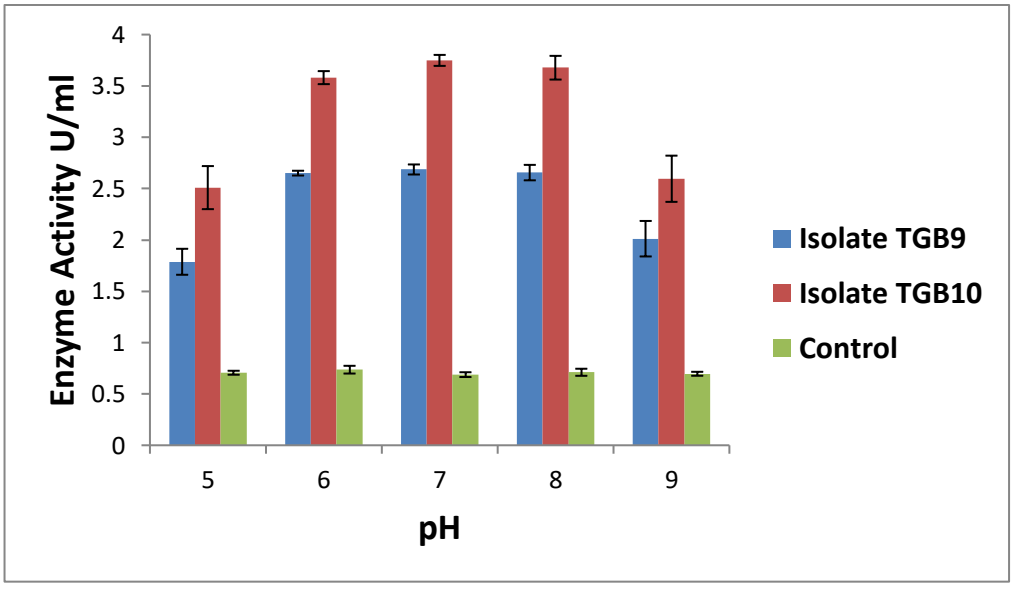

Figure 5: pH optimization of enzyme activity (U/ml) for isolate TGB9 and TGB10 using avicel as substrate

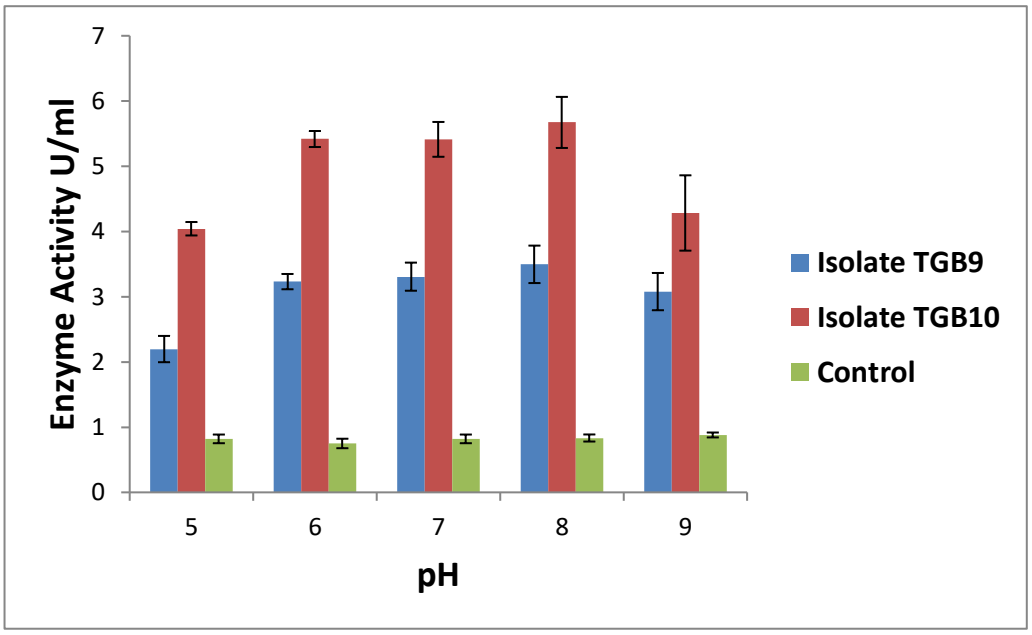

Figure 6: pH optimization of enzyme activity (U/ml) for isolate TGB9 and TGB10 using CMC as substrate

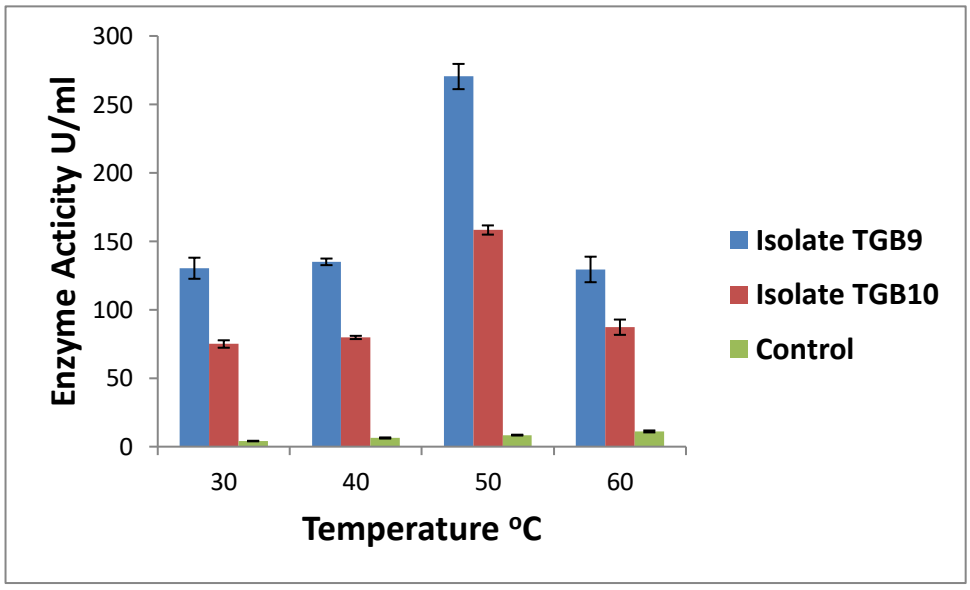

Figure 7: Temperature optimization of enzyme activity (U/ml) for isolate TGB9 and TGB10 using xylan as substrate

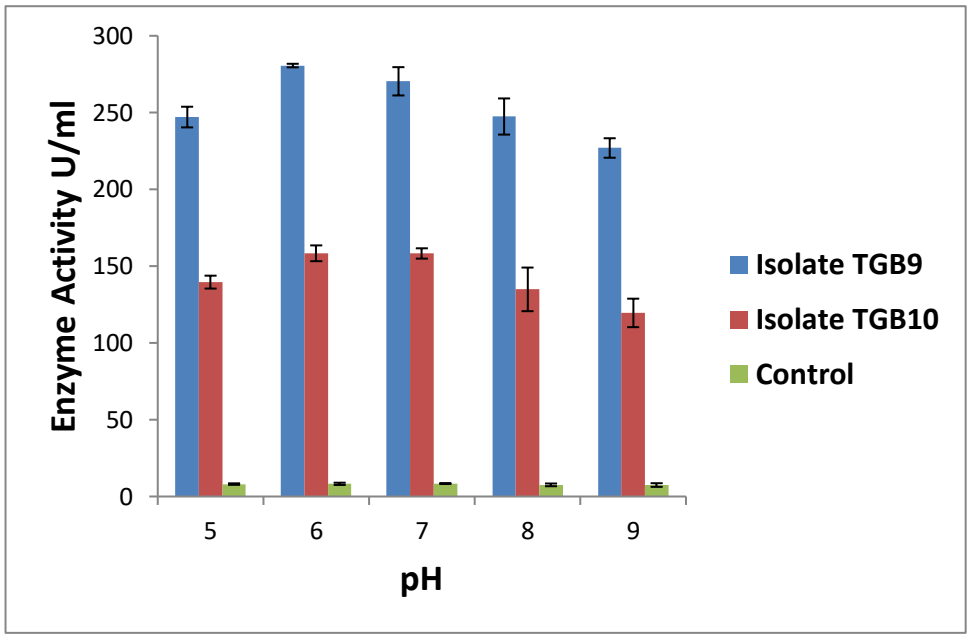

Figure 8: pH optimization of enzyme activity (U/ml) for isolate TGB9 and TGB10 using xylan as substrate

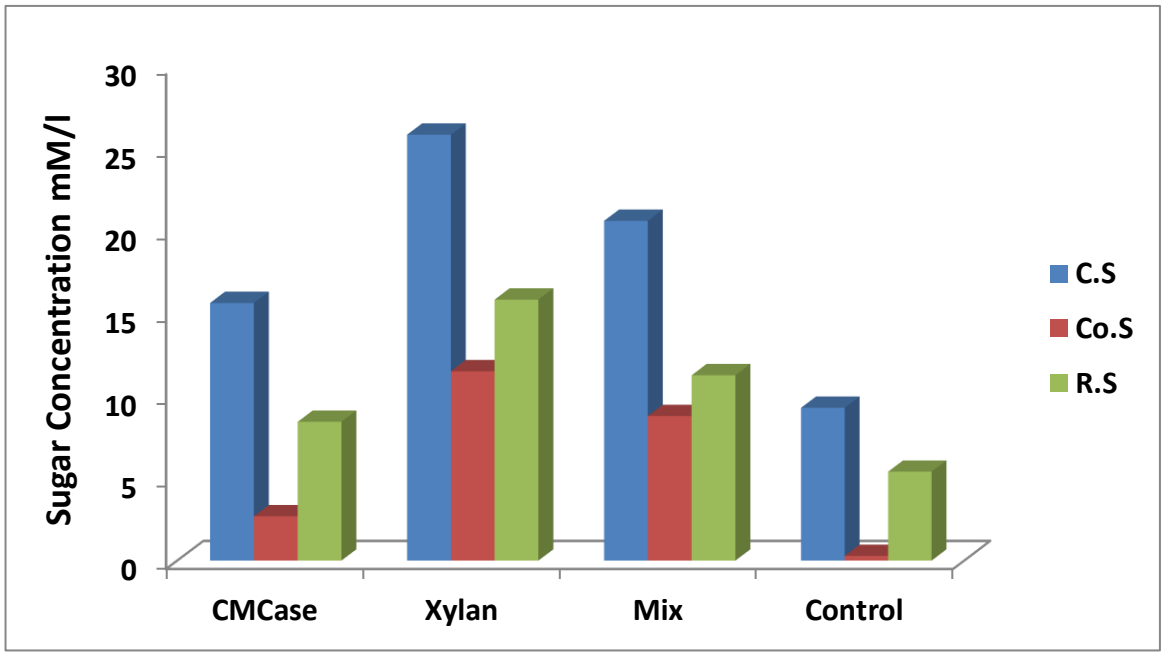

Figure 9: Comparison of cellulase and xylanase produced from isolate TGB9 using agricultural (C.S: corn stover, Co.S: cotton stalk, R.S: rice straw) substrates

Bacterial Isolates	Corn stover				Rice straw			
	Acetate	Formate	Lactate	Ethanol	Acetate	Formate	Lactate	Ethanol
Isolate TGB9	1.1558 ±0.0786	_	1.4068 ±0.1808	5.7374 ±0.2861	3.0043 ±0.6555	_	1.6159 ±0.3079	3.3427 ±0.4101
Isolate TGB10	1.2748 ±0.1234	_	3.4406 ±0.3419	6.9896 ±0.5830	1.5156 ±0.2088	1.2044 ±0.1721	6.1404 ±0.5057	5.9928 ±0.2693

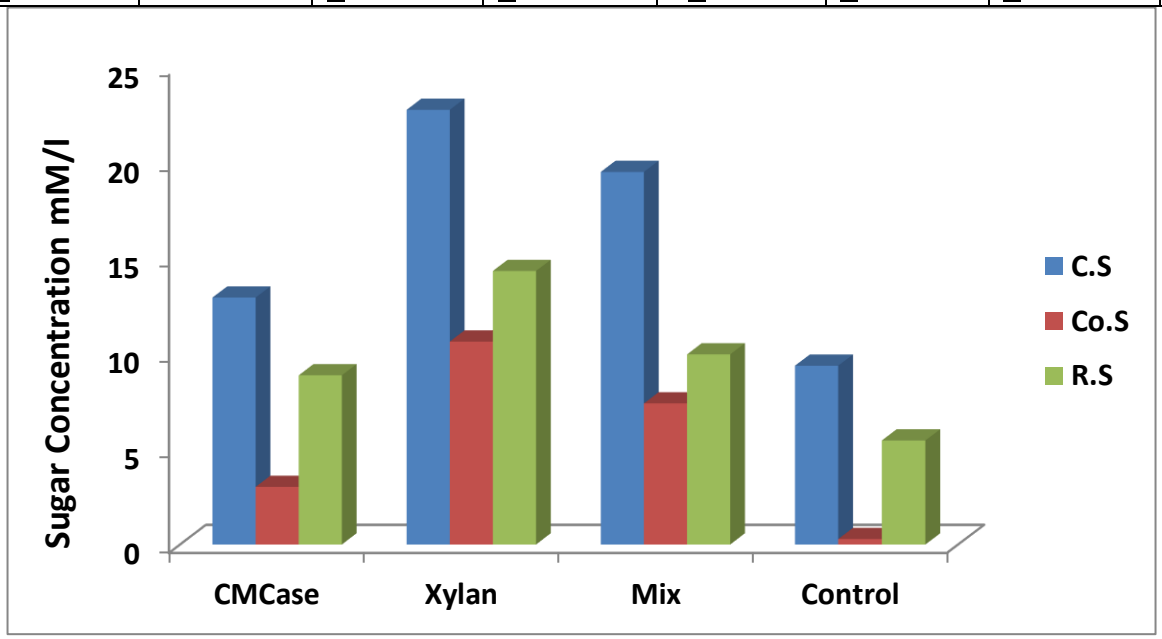

Figure 10: Comparison of cellulase and xylanase produced from isolate TGB10 using agricultural (C.S: corn stover, Co.S: cotton stalk, R.S: rice straw) substrates

References

Ariffin, H., N. Abdullah, M. S. Umi Kalsom, Y. Shirai and M. A. Hassan. 2006. Production and Characterisation of Cellulase by *Bacillus pumilus* EB3. Int. J. Eng. Technol., 3: 47-53.

Balasubramanian, N., D. Toubarro, M. Teixeira and N. Simos. 2012. Purification and biochemical characterization of a novel thermostable carboxymethyl cellulase from Azorean isolate *Bacillus mycoides* S122C. App. Biochem. Biotech., 168(8): 2191-2204.

Bashir, Z., V. K. Kondapalli, N. Adlakha, A. Sharma, R. K. Bhatnagar, G. Chandel and S. S. Yazdani. 2013. Diversity and functional significance of cellulolytic microbes living in termite, pill-bug and stem-borer guts. Sci. Rep., 3: DOI: 10.1038/srep02558

Bignell, D. E., P. Eggleton., L. Nunes and K. L. Thomas. 1997. Termites as mediators of carbon fluxes in tropical forests: budgets for carbon dioxide and methane emissions; A. D. Watt, N. E. Stork, and M. D. Hunter: 109-134.

Breznak, J. A. and A. Brune. 1994. Role of microorganisms in the digestion of lignocellulose by termites. Ann. Rev. of Ento., 39: 453-487.

Brune, A., D. Emerson and J. A. Breznak. 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.

Cardona, C. A., J. A. Quintero and I. C. Paz. 2009. Production of bioethanol from sugarcane bagasse: status and perspectives. Biores. Technol., 101(13): 4754-4766.

Claassen, P. A. M., J. B Van Lier., A. M. L. Contreras., E. W. J. Van Niel., L. Sijtsma., A. J. M Stams., S. S. D. Vries and R. A. Weusthuis. 1999. Utilisation of biomass for the supply of energy carriers. J. App. Microbiol. and Biotech., 52: 741-755.

Dheeran, P., N. Nandhagopal, S. Kumar, Y. K. Jaiswal and D. K. Adhikari. 2012. A novel thermostable xylanase of *Paenibacillus macerans* IIPSP3 isolated from the termite gut. J. Ind. Microbiol. Biotechnol., DOI 10.1007/s10295-012-1093-1.

Fujimoto, N., T. Kosaka, T. Naka and M. Yamada. 2011. *Bacillus licheniformis* bearing a high cellulose-degrading activity, which was isolated as a heat-resistant and micro-aerophilic microorganism from bovine rumen. Op. Biotech., 5: 7-13

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

Kim,Y. K., S. C. Lee, Y. Y. Cho, H. J. Oh and Y. H. Ko. 2012. Isolation of cellulolytic *Bacillus subtilis* strains from agricultural environments. Microbiol., 2012: ID650563.

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

Maki, M. L., M. Broere, K. T. Leung and W. Qin. 2011. Characterization of some efficient cellulase producing bacteria isolated from papermill sludges and organic fertilizers. Int. J. Biochem. Mole. Bio., 2(2): 146–154.

Moretti, R. and J. S. Thorson. 2008. A comparison of sugar indicators enables a universal high throughput sugar-1-phosphate nucleotidyltransferase assay. Anal Biochem., 377(2): 251-258.

MPNR (Ministry of Petroleum and Natural Resources) 2008. Government of Pakistan. www.mpnr.gov.pk

Pakistan defense forum, 2008. http://www.defence.pk/forums/economy-development/10237-bio-fuel-pakistan.html

Pason, P., K. L. Kyu and K. Ratanakhanokchai 2006. *Paenibacillus curdlanolyticus* strain B-6 xylanolytic-cellulolytic enzyme system that degrades insoluble polysaccharides. App. Enviro. Microbiol., 72(4): 2483-2490.

Pourramezan, Z., G. R. Ghezelbash, B. Romanic, S. Ziaeid and A. Hedayatkhah. 2012. Screening and identification of newly isolated cellulose degrading bacteria from the gut of xylophagous termite *Microcerotermes diversus* (Silvestri). Microbiol., 81(6): 736-742.

Rastogi. G., G. L. Muppidi, R. N. Gurram, A. Adhikari, K. M. Bischo, S. R. Hughes, W. A. Apel, S. S. Bang, D. J. Dixon, R. K. Sani. 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: 585-59.

Rogers, P. L. 2008. Current developments in bioethanol production. J. Microbiol. Aus., 29 (1): 6-10.

Sanderson, K. 2011. Lignocellulose: A chewy problem. Natu., 474: 12-14.

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

Scharf, M. E. and A. Tartar. 2008. Termite digestomes as sources for novel lignocellulases. Biofu. Bioprod. Biorefin., 2: 540-552.

Shapiro, D. J. 1981. Quantitative ethanol precipitation of nanogram quantities of DNA and RNA. Ana. Biochem., 110: 229-231 .

Sharrock, K. R. 1988. Cellulase assay methods: a review. J. Biochem. Biophy.l Met., 17: 81-106.

Tokuda, G. and H. Watanabe. 2007. Hidden cellulases in termites: Revision of an old hypothesis. Biol. Lett., 3: 336-339.

UNEP (United Nations Environment Programme). 2011. A project to make clean energy a reality for households in a rural region of Pakistan. http://www.unep.org/newscentre/

Varma, A., B. K. Kollia, J. Paula, S. Saxenaa and H. Konig. 1994. Lignocellulose degradation by microorganisms from termite hills and termite guts: a survey on the present state of art. F. E. M. S. Microbiol. Rev., 15: 9-28.

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

Watanabe, H., H. Noda, G. Tokuda and N. Lo. 1998. A cellulose gene of termite origin. Natu., 394: 330-331.

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

Zhang, Y.H.P. and L. R. Lynd. 2004. Toward an aggregated understanding of enzymatic hyrolysis of cellulose: noncomplexed cellulase systems. Biotech. Bioeng., 88: 797-824.