Cellulomonas sp. Isolated from Termite Gut for Saccharification and Fermentation of Agricultural Biomass

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Biofuel is an important alternative source of fuel, as many countries are looking to decrease their dependence on fossil fuels. One of the critical steps in biofuel production is the conversion of lignocelluloses to fermentable sugars, and there is need for cheaper and more efficient enzymatic strategies. Consequently, lignocellulase genes from various organisms have been explored. Termites possess varied sets of efficient micro-scale lignocellulose degrading systems. In this study, bacteria that degraded cellulose and xylan were isolated from termite gastrointestinal tract. The isolate was identified as *Cellulomonas sp*. by 16S rRNA gene sequencing. The bacterial enzymes cellulase and xylanase showed the highest activity at 50 °C and pH 8.0. The agricultural substrates were hydrolyzed by cellulases and xylanases, and more sugar was released from corn stover (18.903±0.65 mM/L) than from rice straw or cotton stalk. After direct hydrolysis and fermentation of agricultural substrates, ethanol (0.425±0.035 g/L) and lactate (0.772±0.075 g/L) were the major end products. Thus, termite gut bacteria can efficiently hydrolyze hemicellulose and cellulose, and these bacteria also have the potential to convert these fermentable sugars into valuable secondary metabolites.

Keywords: Cellulase; Xylanase; Termite; Saccharification; Cellulomonas

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INTRODUCTION

In the last few decades, there has been a global effort to reduce the reliance on non-renewable resources. Lignocellulosic biomass is a source of energy that is renewable and available in large quantities, but the process of bioethanol production from cellulosic biomass is more complicated than from sugars. Technologies for the cost-effective conversion of lignocellulosic material into biofuel are in development (Ohgren *et al.* 2007; Hendriks and Zeeman 2009).

There is a need for low-cost raw materials, effective enzymes, and pretreatment methods to decrease the expenditure for bioethanol production (Sanchez and Cardona 2008). Cellulosic biomass is a low-cost, renewable, and abundantly available material throughout the world. These materials include wood chips, residues of crops, grasses, *etc*. (Binod *et al.* 2010). In terms of quantity, sugarcane bagasse, rice straw, corn stover, and wheat straw are the most accessible agricultural wastes (Kim and Dale 2004).

During hydrolysis, monomeric sugars are generated *via* depolymerization of hemicelluloses and cellulose (Sarkar *et al.* 2012). The production of the cellulase enzyme

accounts for about 40% of the entire cost of bioethanol synthesis (Gray *et al.* 2006). To decrease the cost of cellulase, numerous efforts have been made to optimize the hydrolysis conditions (Sarkar *et al.* 2012). The hydrolysis of lignocellulose depends on the synergy of the enzymatic system, including β-glucosidase, β-1,4-exoglucanase, β-1,4-endoglucanase, (Alvira *et al.* 2010), and β-1,4-endoxylanase. This enzyme cocktail is needed to establish a cost-effective technology, in addition to the lower price of biomass (Arantes and Saddler 2011).

Currently, there is no enzyme system that can be efficiently employed on such a vast scale. However, some organisms utilize wood as food, and those systems could be explored and applied to industry (Sanderson 2011). Termites damage billions of dollars of wood each year. Molecular phylogenetic analysis has revealed that termites harbor more than 200 species of symbiotic microorganisms, which produce enzymes that degrade cellulose and hemicelluloses (Brune 2007; Matsui *et al.* 2009). A study by Warnecke *et al.* (2007) revealed the occurrence of a huge and varied set of bacterial genes that encode hydrolytic enzymes for degradation of xylan and cellulose. Termites consume 50 to 100% of the deceased biomass in humid ecosystems, and they degrade about 65 to 87% of hemicelluloses and 74 to 99% of cellulose in cellulosic biomass (Ohkuma 2003). The gut of wood eating termite is a bioreactor where a number of microbes utilize cellulose and hemicellulose content of lignified plant materials and convert them to fermentable products. Without these microorganisms, termites are unable to hydrolyze cellulose, which is their main food (Matsui *et al.* 2009)

In this study, *cellulomonas* sp. was isolated from termite gut. The isolate was screened for cellulolytic and xylanolytic activity and identified by 16S rRNA sequencing. The crude enzyme activity was checked at different temperature and pH. The agricultural substrates were hydrolyzed with the enzymes produced by the isolate. The substrates were directly hydrolyzed and fermented with the isolate to find the end products.

EXPERIMENTAL

Materials

Termites (*Microtermes obesi*) were collected from putrefying trees of *Acacia nilotica*. Corn stover, cotton stalk, and rice straw were obtained from the National Agricultural Research Center in Islamabad, Pakistan. The agricultural substrates were ground and sieved through 20- and 40-mesh sized sieves (0.420 mm and 0.841 mm, respectively) to produce equal size particles.

Isolation and screening of bacteria

Termites were sterilized with 70% ethanol and under UV light for 5 to 10 min. The bodies of termites were ground and serially diluted with Milli Q water. The dilute sample was spread over nutrient agar media with 1% carboxymethyl cellulose (CMC) and 1% beechwood xylan provided by M. S. Traders (Sigma, Lahore, Pakistan) (Dheeran *et al.* 2012; Pourramezan *et al.* 2012). The plates were incubated at 30 °C for 24 h.

The purified bacterial colonies were screened by the Congo red dye method using 0.2% CMC or 0.2% xylan separately (Dheeran *et al.* 2012). The bacteria were incubated at 30 °C for 48 h. Clear zones around the bacterial colonies established their ability to degrade cellulose and xylan (Liang *et al*. 2014).

16S rRNA gene sequencing

For bacterial identification, PCR amplification (PCR Super Mix (Invitrogen™) ThermoFisher Scientific, Waltham, MA, USA) was directly performed by using bacterial colonies (Matteotti *et al.* 2011). Full length (1.5 kb) 16S rRNA fragment was amplified. The universal 16S rRNA gene primers 27F(5'-AGAGTTTGATCCTGGCTCA-3') and 1492R(5'-ACGGCTACCTTGTTACGACTT-3') were used. PCR products were sequenced at the Keck Center for Comparative and Functional Genomics, University of Illinois at Urbana-Champaign, IL, USA, and the sequence was submitted to NCBI under accession number KR902590. The BLASTN program of GeneBank was used to analyze gene sequences.

Enzyme production and activity assays

Two enzyme production media containing nutrient broth with 1% CMC and 1% xylan, pH 6.8 to 7.2 (Dheeran *et al.* 2012; Bashir *et al.* 2013) were prepared. The media were inoculated with termite gut bacteria 31 (TGB31) and incubated at mild rotation for 48 h at 30 °C.

The enzyme activities of CMCase and xylanase of the TGB31 were studied by using CMC and xylan as substrates, respectively. The effect of a various range of temperatures, 30, 40, 50, and 60 °C, and also a pH at 5.0, 6.0, 7.0, 8.0, and 9.0 was assessed using crude enzymes. CMC (1%) and xylan (1%) were prepared in diverse buffers for different range of pH according to Rastogi *et al.* (2009). The reaction time for CMC was 60 min because it was slower to hydrolyze, and 30 min for xylan. Buffers with substrates only and no enzyme were used as controls. The p-hydroxybenzoic acid hydrazide (PAHBAH) method was used to determine the sugar content released during the reaction. Sodium citrate (100 mM) and 0.6 M NaOH was made and stored on ice. To determine sugar content 10 mg of p- hydroxyl benzahydride was added to 10 mL of the above solution. Then 150 µL of the working solution and 50 µL of the samples were mixed in 96-well microplates. The mixtures were boiled for 10 min and brought to room temperature. Absorbance was measured at 410 nm (Moretti and Thorson 2008). One unit (U) of enzyme activity was defined as the amount of enzyme that released 1 µmol of reducing sugars per min during the reaction.

Saccharification of corn stover, cotton stalk, and rice straw

First the contents of cellulose, hemicelluloses (Agblevor *et al.* 2003), and lignin (Anwar *et al.* 2012) were determined for corn stover, cotton stalk, and rice straw.

Then corn stover, cotton stalk, and rice straw were taken 5% by dry weight, which means 5 g in 100 mL of distilled water (w/v). The ratio of crude enzymes of TGB31 (CMCase and xylanase) to substrates was 1:1, means 100 mL of crude enzymes were added. With mild rotation, the reaction mixture was placed at 50 °C for 24 h. The combined effect of enzymes was also studied. Agricultural substrates treated with distilled water were used as controls.

End product analysis

The agricultural substrates were directly treated with bacterial isolates for saccharification and fermentation without any chemical treatment. Corn stover, cotton stalk, and rice straw were used at 5% dry weight (w/v) and supplemented (with, in g/L of

H_2O: KH_2PO_4 1.5, $MgSO_4$ 0.3, NaCl 0.01, $CaCl_2$ 0.1, $FeSO_4$ 7, H_2O 0.005, NH_4Cl 0.3, and yeast extract 0.05) (Rastogi *et al.* 2009). The agricultural substrates were inoculated with 1% of cultured isolates (1 mL of culture up to 100 mL of 5% substrate). The reaction mixture was incubated at 30 °C for 5 days at mild rotation and microaerophilic conditions. The cell viability was determined by protein estimation using the Bradford method.

The fermentative medium from corn stover and rice straw was centrifuged at 14,000 rpm for 20 min and 4 °C to remove the remaining substrates and dead bacterial cells. The supernatant was filtered through 0.22 µm membranes, and the filtrate was stored at -20 °C for high-performance liquid chromatography analysis (Protea, model: RID-10A, Shimadzu, Bucharest, Romania). The filtrate was later injected into HPX 87 H columns with a refractive index detector. The mobile phase was 5 mM sulfuric acid with a flow rate of 0.4 mL/min at 25 °C. Acetate, ethanol, formate, and lactate were tested as end products to find the efficiency of the isolates for secondary metabolites production.

Statistical analysis

The outcomes for enzymatic pretreatment and end products were analyzed by analysis of variance (ANOVA) using MSTAT-C software (Michigan State University, East Lansing, USA). GraphPad Prism 5.0 software (GraphPad Software, Inc., San Diego, USA) was used to determine the standard deviation.

RESULTS AND DISCUSSION

Isolation and Screening of Bacteria

In this study, the termite gut was explored to identify bacteria producing enzymes that degrade cellulose and hemicelluloses and to determine the role that these bacteria play in this small ecological niche. Termites harbor microbes that produce cellulases and hemicellulases, which hydrolyze lignocellulosic material (Scharf and Tartar 2008; Zhang *et al.* 2009). Approximately 53 colonies of bacteria were isolated from the termite gut. Only one bacterial isolate (TGB31) was considered for further study based on the screening method. The bacterial isolate degraded both CMC and xylan (Fig. 1), producing hydrolysis zones with diameters of 2 mm for CMC and 3 mm for xylan.

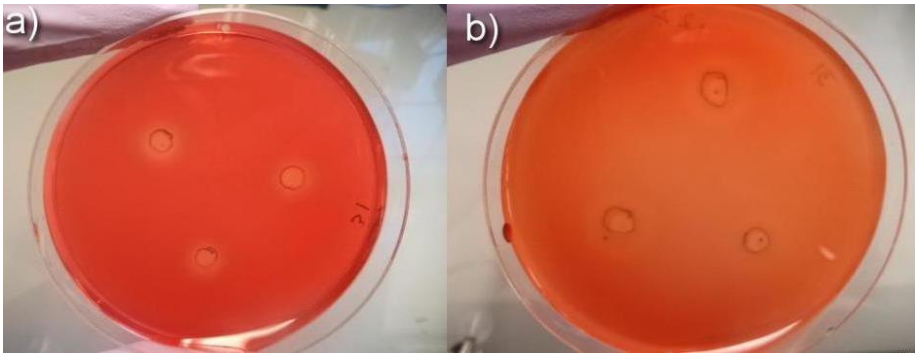

Fig. 1. Congo red screening method to show zone of clearance due to enzymatic hydrolysis produced by bacterial isolate TGB31 on (a) CMC media and (b) xylan media

16S rRNA gene sequencing

The bacterial isolate was identified by PCR amplification of the 16S rRNA gene. The BLASTN program was used to compare the sequence with GenBank data. The TGB31 isolate (GenBank accession no. KR902590) belongs to phyla Actinobacteria, and the genus was *Cellulomonas*. Based on the similarity analysis, isolate TGB31 is closely related to *Cellulomonas denverensis.* Fall *et al.* (2007) and Wenzel *et al.* (2002) also isolated and characterized *Cellulomonas* bacteria from the termite gut.

Enzyme activity assays

Figure 2 illustrates that TGB31 showed maximum endoglucanase activity at 50 °C and pH 8 (Fig. 3).

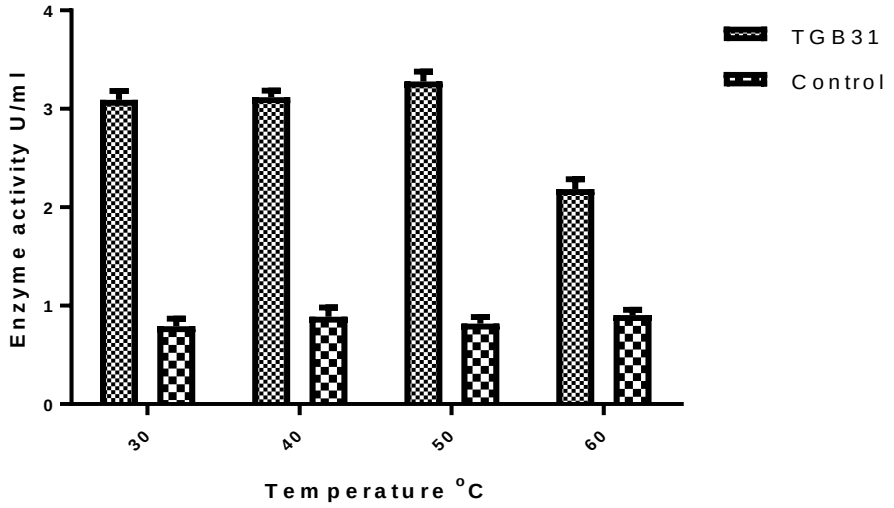

Fig. 2. Optimization of temperature for CMCase (cellulase) activity U/mL of TGB31

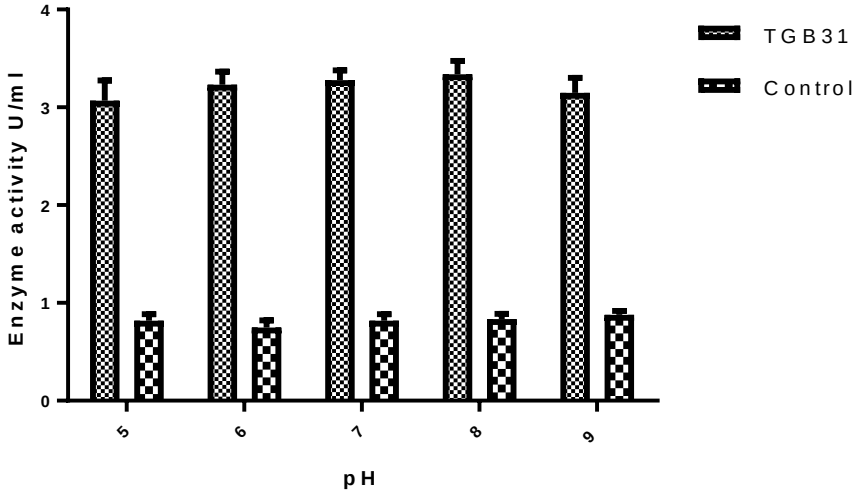

Fig. 3. Optimization of pH for CMCase (cellulase) activity U/mL of TGB31

Immanuel *et al*. (2006) observed that *Micrococcus*, *Bacillus*, and *Cellulomonas* species obtain maximum cellulase activity at neutral pH and 40 °C. Generally, cellulases isolated from microbes from mesophilic environments have an optimum pH of 4.0 to 8.0 and optimum temperature of 40 to 50 °C (Dutta *et al.* 2008).

Xylanase showed highest activity at 50 °C (Fig. 4). Lisov *et al.* (2017) found that the temperature optima for xylanases from *Cellulomonas flavigena* are 40 °C by CFXyl1 and 50 °C for CFXyl4, CFXyl3, and CFXyl2. Xylanases are stable below 60 °C and degrade rapidly at 65 to 70 °C (Amaya *et al.* 2010). Figure 5 shows that the best pH for xylanase was 8.0. The optimal pH of xylanase from *C. flavigena* was determined by Amaya *et al.* (2010) to be 6.5 and 5.7 in *C. fimi* by Chen *et al.* (2012). Xylanases show the highest stability at pH 8 to 10 (Lisov *et al.* 2017).

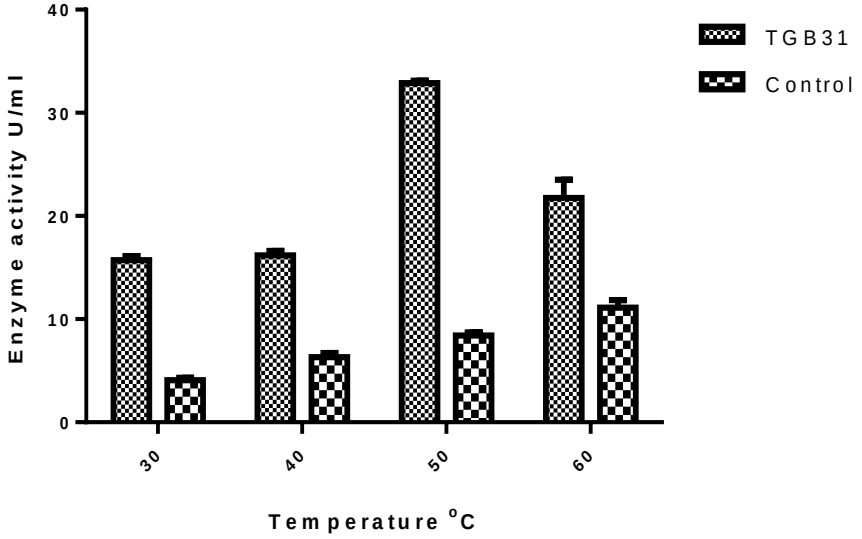

Fig. 4. Optimization of temperature for xylanase activity U/mL of TGB31

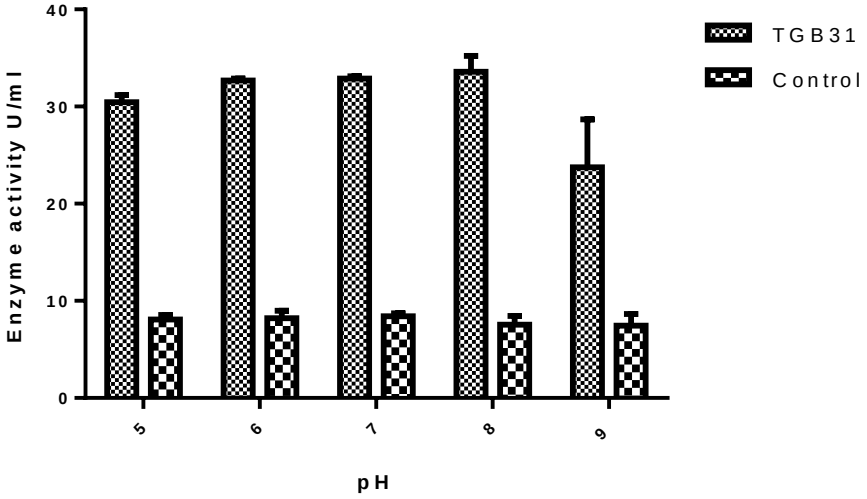

Fig. 5. Optimization of pH for xylanase activity U/mL of TGB31

Saccharification of corn stover, cotton stalk, and rice straw

Table 1 shows the content of cellulose hemicelluloses and lignin content in the agricultural substrates. Figure 6 demonstrates that both of the enzymes (xylanase and CMCase) hydrolyzed the agricultural substrates with diverse efficiency. Corn stover was a potential substrate for these enzymes. Saha and Cotta (2006) reported that the cellulose and hemicelluloses contents in corn stover were 42.6 and 21.3%, respectively. The contents of cellulose and hemicelluloses are high in corn stover as compared to rice straw and cotton stalk. Therefore the sugar contents produced by corn stover are higher than other substrates. The rice straw has contents of 32 and 19%, respectively, for cellulose and hemicelluloses (Karimi *et al.* 2006). The degradation of rice straw is very slow in soil, and also high mineral content is observed in rice straw (Vlasenko *et al.* 1997). It is possible that minerals interfere with enzyme activity. The least amount of sugar content was released from cotton stalk. The lignin content in cotton stalk is high enough, about 30%, and also the holocellulose (cellulose and hemicellulose) content is 41.8% (Silverstein *et al.* 2007).

Table 1. Percentage Composition of Cellulose Hemicelluloses and Lignin in Agricultural Substrates

Agricultural substrates	Amount in percentage (%)		
	Cellulose	Hemicellulose	Lignin
Corn stover	37	25	19
Rice Straw	33	21	18
Cotton stalk	29	14	24

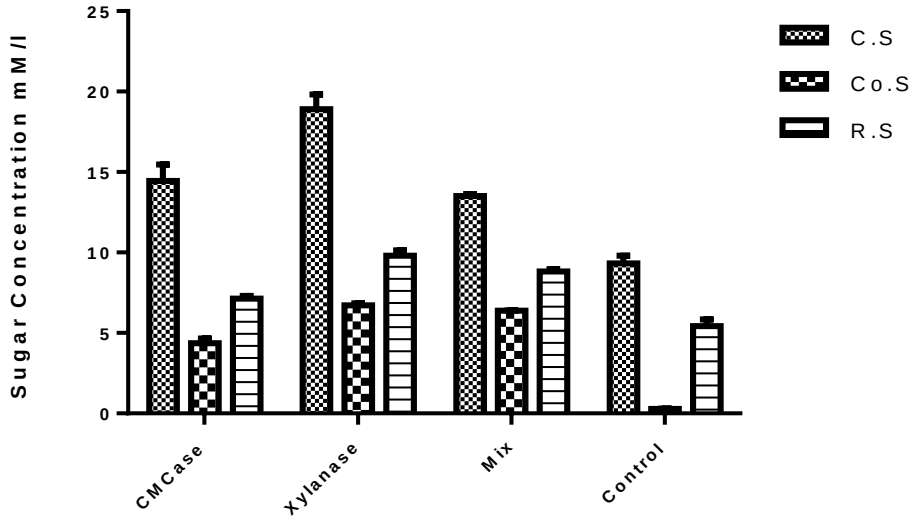

Fig. 6. Sugar concentration (mM/L) using CMCase (cellulase), xylanase, mix (CMCase, xylanase) to hydrolyze corn stover (C.S), cotton stalk (Co.S), and rice straw (R.S)

Xylanases released more sugar content from all substrates than the CMCases because the hemicellulose is easier to hydrolyze than cellulose (Cardona *et al*. 2009). The analysis of variance tested for both of the enzymes showed that the difference in the sugar yields among the agricultural substrates were highly significant (p=0.000). When the

mixture of both enzymes was used, the released sugar fell between the values released by both enzymes separately. Sugar content released from corn stover was 18.903±0.6506 mM/L when treated with crude xylanase as compared to sugar content released by treatment with CMCase (14.442±0.724 mM/L). Lisov *et al.* (2017) hydrolyzed rye, wheat, and oat with the xylanases isolated from *Cellulomonas flavigena* and obtained the highest yield from rye (approximately 1.3mM/L). *Bacillus* sp. and *Listeria* sp. isolated from leaf litter hydrolyzed pure cellulose, and the sugar concentration obtained was 0.0721 and 0.0772 mM/L, respectively (Gunathilake *et al.* 2013).

End product analysis

It was reported by Dermoun *et al.* (1988) that *Cellulomonas uda* ferments the hydrolyzed sugars into acetate, formate, ethanol, and lactic acids as primary end products. The genes for alcohol dehydrogenase (ADH) in *C. fimi* were observed to be more in number than the genes of ADHS of *C. thermocellum* and *Z. mobilis*, combined (Christopherson *et al.* 2013). So, it was designed to study different end products, specifically the ethanol. The end products from cotton stalk were not analyzed because there was no growth of bacterial isolates. Ethanol and lactate were the major end products of the experiment (Fig. 7). Poulsen *et al.* (2016) observed that formate was the dominant end product and lactate was the least product fermented by *C. uda*. Most *Cellulomonas* sp. does not encode pyruvate decarboxylase, which is necessary for homoethanol production (Christopherson *et al.* 2013). End products produced by isolate TGB 31 have shown statistically significant (p=0.000) results obtained from both of the agricultural substrates. Figure 7 also illustrates that more ethanol (0.425±0.035 g/L) was produced when corn stover was used as the substrate. This result confirmed that more sugar was released from corn stover. It was determined by Millati *et al.* 2005 that *S. cerevisiae* produced 0.42 g ethanol/g of glucose but cannot utilized xylose for fermentation. Metabolically engineered yeast, which was developed for xylose utilization, produced 0.24 and 0.28 g ethanol/g xylose (Fujitomi *et al.* 2012; Kato *et al.* 2013).

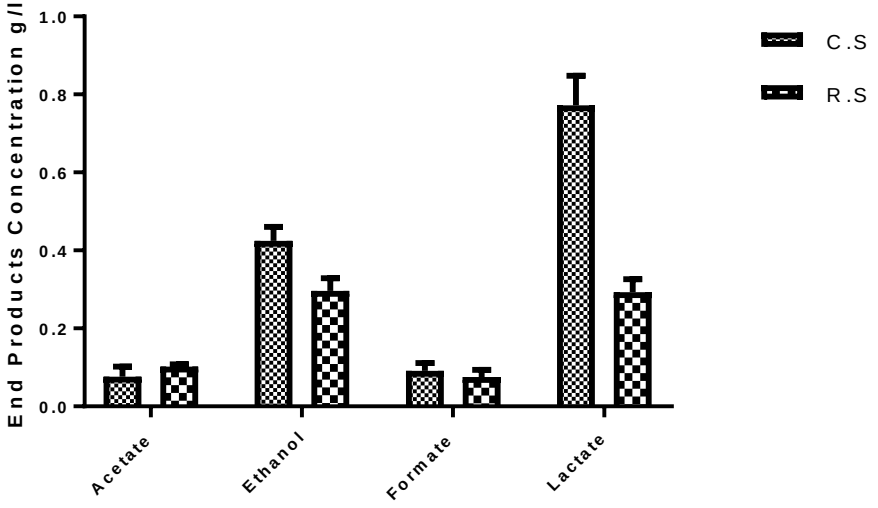

Fig. 7. Concentration of end products (g/L) of TGB31using corn stover (C.S) and rice straw (R.S)

Over all, the production of ethanol and other secondary metabolites is low. The production of sugars and end products can be increased by optimizing the different conditions to achieve the maximum potential of the bacterial isolate.

CONCLUSIONS

1. Termite gut bacteria were able to degrade hemicellulose and cellulose.
2. These enzymes have the potential to hydrolyze pure substrates and degrade agricultural substrates without any chemical pretreatment.
3. *Cellulomonas* sp. isolated from termite gut directly hydrolyzed agricultural substrates into valuable secondary metabolites.

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