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Ethanol production from agricultural residues by simultaneous saccharification and fermentation process (SSF) by using termites and *Saccharomyces cerevisiae*

[1]Iram batool, [1]Muhammad Javaid Asad, [1]M, Shareez Ahmad, [2]Raja Tahir Mahmood, [1]Hina gul, [5]Lubna Nisar , [3]Inam ul-Haq, [4]Imran Bodlah and [1]Muhammd Gulfraz

[1]Institute of biochemistry and Biotechnology PMAS Arid Agriculture University Rawalpindi
[2]Department of Biotechnology, Mirpur University of Science and Technology (MUST), Mirpur-10250, AJK, Pakistan
[3]Department of Plant pathology PMAS Arid Agriculture University Rawalpindi
[4]Department of Entomology PMAS Arid Agriculture University Rawalpindi
[5] Department of Biochemistry, FBS, Quaid-i-Azam University, Islamabad, Pakistan

Address For Correspondence:
Muhammd Gulfraz, Institute of biochemistry and Biotechnology PMAS Arid Agriculture University Rawalpindi
E-mail: gulfrazsatti@uaar.edu.pk

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ABSTRACT

Termites are considered as voracious insects and famous for damaging household materials made up by wood. As second generation biomass is a rich source of cellulose and termites can degrade this cellulose by using enzymes available in its foregut. During current study two strains of bacteria were isolated from the gut of termite in different media. The cellulases from the isolated bacteria were characterized for temperature, pH and time period. Cellulose fermentation ability and production of ethanol of the isolated bacterial strains was also analyzed. Results indicates that strains of termites gut bacteria 9 and 10 (TGB9 and TGB10) have shown significant cellulolytic activity on congo red assay and that these bacterial strains were belongs to *Bacillus* genera. Maximum cellulase activity was observed after 48 hrs at pH8 and 50 ^{o}C temperature. The agricultural substrates (Corn stover and rice straw) were directly treated with bacterial isolates TGB9 and TGB10 for sccharification and fermentation without any chemical treatment and enzymatic hydrolysis. It was observed that these isolates have produced ethanol from rice straw (7.52 ± 0.5 to 9.33 ± 0.4 g/L) followed by corn stove (6.35 ±0.6 to 6.95 ±0.5 g/L) having theoretical yields of ethanol 43.31 % (rice straw) and 39.62 % (corn stove).

KEYWORDS: Termite, *Bacillus*, cellulase, Cellulose, Biomass, Bioethanol

INTODUCTION

Global fossil fuel supplies are shrinking and atmospheric carbon concentrations rises, pressure is on to find practical alternatives to petroleum products like biofuels.. Ethanol (C_2 H_5 OH) from renewable resources has been of interest in recent decades as an alternative fuel to the current fossil fuels [19]. Bioethanol is made biologically by fermentation of sugars derived from a variety of sources and recognized as a unique transportation fuel with powerful economic, environmental and strategic attributes [14]. One of the greatest challenges of twenty-first century is to meet the growing demand of energy for transportation, heating and industrial processess and biofuels have emerged as an ideal option to meet these requirements in a sustainable manner [22].

Increasing concerns about climate change and energy security have motivated the search for alternative forms of energy. Since the transportation sector is responsible for a significant fraction of the greenhouse gases emissions, substitution of oil derived fuels by biofuels, like ethanol, could significantly decrease environmental impacts, besides providing gains on the socio-economic levels as well [16]. Lignocellulosic biomass like wood and agricultural crops are potential raw materials for producing several high-value products like fuel ethanol and biodiesel. lignocelluloses contains up to 80% from the polysaccharides [9]. These renewable raw materials are promising for replacing fossil hydrocarbon raw materials those are creating environmental problems ,where as bioethanol does not contribute to the greenhouse effect, being a CO_2 neutral resource.

Bioethanol is a high-octane, water-free alcohol produced from cellulosic biomass through fermentation. It is a colorless clear liquid with mild characteristic odor that boils at 78°C and freezes at -112°C. These are exclusively derived from plant origin. Bioethanol has all most all characteristics same as that of synthetic ethanol. Lignocellulosic biomass fall under this category are promising source for second generation bioethanol production [18].

Cellulase is an enzyme complex which breaks down cellulose to beta-glucose . It is produced mainly by symbiotic bacteria in the ruminating chambers of herbivores. Cellulase refers to a family of enzymes which convert cellulose into monosccharides like glucose and other similar sugar molecules [1,3].

Biomass contains varying amounts of cellulose, hemicellulose, lignin and small amounts of other organics besides inorganics. The relative proportion of the major organic components in biomass is particularly important in the development of processes for producing fuels and chemicals. The combination of cellulose, hemicelluloses, and lignin is called 'lignocellulose biomass and most abound on the earth [9]. The complex structure of lignocellulose therefore plays a huge role in inhibiting degradation of the hemicellulose and cellulose structure to monomeric sugars which is necessary to effectively convert biomass into ethanol. Processing of lignocellulose is therefore essential for the conversion of lignocellulosic biomass to biofuel such as bio-ethanol.

The rice straw is to be considered as the largest available biomass in the world which is about 7.31×10^{14} dry rice straw per year. Asia is the largest region in the world which is responsible for 90% of the annual global production. Maize is the best of the cereal stovers and very abundant livestock feed. It can be grazed off; otherwise, it is mostly burned in fields in the many areas of Pakistan before next crops to sow and all parts of maize are usable for different purposes ([17].

Termites are generally regarded as nuisance to human society, but they are in fact beneficial for the nature since termites decompose dead trees, and some 20 % of plant biomass in the nature is decomposed by termites [5]. The clarification of cellulose-decomposition process in termite guts will undoubtedly open the way to establish a new production technology for bioethanol, since this new technology can make it possible to dispense with traditional pretreatment of cellulose, and hence it will be much more energy-efficient and of low cost [10]. The present study was conducted based on objectives of isolation of cellulase producing bacteria from the termites gut. The characterization of cellulases to enhance their applicability for certain industrial processes. Further isolated bacterial strain used for fermentation of agricultural waste for the production of ethanol.

MATERIALS AND METHODS

Collection of Biomass samples:

The corn stover and rice straw samples were collected from different areas of Punjab province. The samples were sun dried followed by oven drying at 60 °C. The dried samples were ground into powder from (80 msh) and saved in fine plastic bags duly labeled till further uses**.**

Proximate and chemical analysis of rice straw and corn stove samples:

The Proximate and chemical analysis of rice straw and corn stover samples were carried out for volatile matter, fixed carbon, ash contents, crude fiber, ether extract, protein contents, cellulose, hemicelluloses and lignin contents by using method reported by AOAC (1980) and Hames *et al*. [15].

Termite Collection:

Wood feeding subterean termites (*Microtermes obesi*) were collected from decomposing trees (*Acacia nilotica*) from road side of Islamabad areas during month of January/ February Ordinary termites involved in wood degrading were selected as shown in figure 1.

Fig. 1: various termites used in this study as indicated by following scheme

Cellulosic Biomass→Pretreatment→Glucose→Fermentation→Distillation /separation → Bioethanol
Yeast
‖

Cellulosic Biomass----------Termites----------Glucose

Preparation of gut extract from termites:

Termites were surface sterilized in 70% ethanol for 2 minutes followed by three washes in sterile distilled water. The posterior paunch and colon regions were dissected from the termite abdomen and placed in separate reaction vessels containing 2 ml sterile phosphate buffer (10 mM $Na_2 HPO_4$. $2H_2O$,1.8 mM KH_2PO_4; pH 7.0). The contents were vortexed for 5 minutes and serial dilution was performed using sterile phosphate buffer as reported earlier by Tokuda and watanabe [28].

Isolation of cellulase producing bacteria from termite's gut extract:

The macerated gut of the collected organisms were inoculate in working media and individual colony was obtained by streaking the culture. Confirmation of cellulose-degrading ability of bacterial isolates was performed by streaking on the Carboxy methyl cellulose (CMC) media with the following composition: 15 g CMC, 3 g $NaNO_3$, 3 g $K2HPO_4$, 3 g KCl, 0.5 g $MgSO_4$, 0.5 g yeast extract, 1g glucose, 17g agar. Congo red (1mg/ 1 ml of distilled water) was used as staining solution and 1M NaCl as a destaining solution. The use of Congo-Red as an indicator for cellulose degradation in an agar medium provides the basis for a rapid and sensitive screening test for cellulolytic bacteria as reported by [29].

Cellulase Extraction:

The selected isolates were cultured at 37 ^oC at 150 rpm in media (1g $NaNO_3$, 1g $KH2PO_4$, 1g KCl,0.5 $MgSO_4$, 0.5g yeast extract, 1g glucose at pH 6.8–7.2). Broth culture after three days of incubation was subjected to centrifugation at 5000 rpm for 15 minutes and Supernatant was collected and stored as crude enzyme at 4 ^oC for enzyme assays [11].

Cellulase Assay:

The activity of isolates as cellulase enzymes was assessed by measuring the amount of reducing sugar from amorphous cellulose. In these tests, reducing sugars were estimated spectrophotometrically with3, 5-dinitrosalicylic acid using glucose as standards as earlier reported by Ghosh and Ghose [13] Then enzymatic activities of total endoglucanase were defined in units. (One unit of enzymatic activity is defined as the amount of enzyme that releases one micromole reducing sugars (glucose) per minute).

Effect of time period on cellulase activity:

Different incubation times (12, 24, 36, 48, 54, and 72 hours) were employed to study their effect on the cellulase activity. The culture filtrates were collected at respective time interval and assayed for enzyme activity.

Effect of temperature on cellulase activity:

The effects of various range of temperature (30 ^{o}C, 40 ^{o}C,50 ^{o}C, and 60 ^{o}C) was assessed on crude cellulase activity as reported earlier by Daheeran *et al.* [10].

Effect of pH on cellulase activity:

The pH of the test solution was adjusted to different pH range of 4.5, 5.5, 6.5, 7.0, 7.5, 8.5, and 8.8 with phosphate buffer (10 mM $Na2HPO_4.2H_2O$, 1.8 mM KH_2PO_4). The production was carried out to study their effect on enzyme activity [7]

Saccharification:

The bacterial isolates TGB9 and TGB10 isolated from termite gut were directly treated with biomass samples for process of Saccharification and fermentation. Total 100 geach of rice straw and corn stover samples was mixed with 10 ml of crude enzymes (isolates) as reported earlier by Arillo *et al*., [2] and Binod *et al*., [6] The reaction mixture was incubated fro 72 hours and after that this mixture was heated up to 90°C cooled and centrifuged at 5000 rpm for 10 minutes. The supernatant obtained was subjected to fermentation process [30]

Fermentation:

Saccharomyces cerevisiae grown in glucose yeast extract broth medium for 48 hours and 10% inoculum was inoculated into 50 mL fermentation medium containing previously saccharified solution and kept for 3 days at room temperature [4,26]

Analytical procedures:

The amount of glucose, cellobiose, hydroxymethylfurfural, furfural and other organic acids obtained during different stages of fermentation were analyzed by using HPLC method (Shimadzu, Japan at 65°C. 5 mM sulfuric acid in MilliQ water as eluent, a flow rate of 0.5mL/min and refraction index detector. Whereas, fermentation products like ethanol, glucose and ethanol yields were also determined by using method reported by Mozier *et al.* [21].

Statistical analysis:

Data obtained were statistically analyzed by using one way ANOVA

RESULTS AND DISCUSSION

Analysis of moisture, volatile matters, fixed carbons, fiber contents, ash contents, ether extracts and crude proteins are important parameters to determined the quality of any biomass samples for its use as feed or for extraction of bio based products. Whereas chemical components of biomass samples like cellulose hemicelluloses and lignin indicates its suitability for conversion cellulose into monomeric sugars like glucose and its further conversion into ethanol and others alcoholic products [8]. The results of all of these parameters obtained in current study are given in the following sections.

Table 1: Proximate and chemical analysis (%) of rice straw and corn stove samples

Parameters	Rice straw	Corn stove
Moisture contents	6.0	7.0
Volatile Matter	79.0	75.0
Fixed Carbon	10.5	19.5
Ash contents	4.5	6.0
Crude Fiber	30.0	32.0
Ether extract	1.5	2.5
Crude Protein	4.5	3.8
Cellulose	35.5	29.6
Hemicellulose	28.5	32.5
Lignin	17.5	18.5

Triplicate analysis (n=3).

Table 2: Analysis of rice straw and corn stove samples with HPLC

Components	Retention time (min)	Concentration (mg/ml) Rice straw	Concentration (mg/ml) Corn stove
Glucose	8.6	21.52	22.3
Cellobiose	7.1	1.02	1.05
Xylose	11.6	2.51	2.32
Succinic acid	12.0	5.45	6.32
L-Lactic acid	13.2	8.45	7.38
Acetic acid	15.5	8.62	7.85
Furfural	42.5	3.42	2.65

HMF	28	3.21	2.84

Triplicate analysis (n=3)

Isolation and Screening of Cellulose producing Bacteria:

Cellulose producing bacteria were enriched and isolated by inoculating termite gut extract on TSA medium and by restreaked on CMC agar medium [1,3]. A total of two bacterial isolates found to be positive on screening media (CMC agar) producing clear zone with Congo red stain as shown in Figure 1 and 2 and table 3.

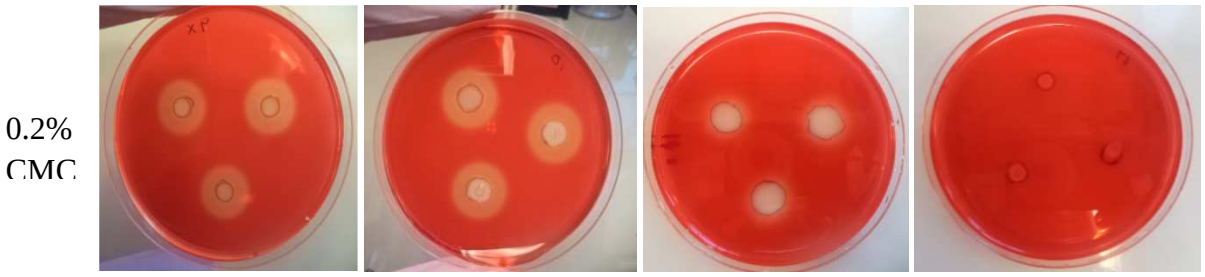

Fig. 1: Zone of clearance on CMC agar plates for isolate cellulose producing bacteria after 48 hrs of incubation. The formation of clearing zone around the colonies confirms the secretion of extracellular cellulase.

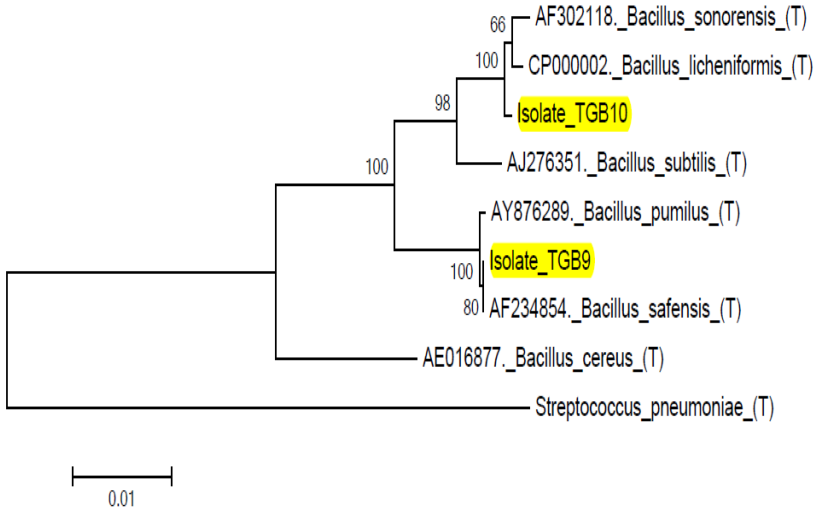

Fig. 2: Phylogenetic tree for the cellulolytic and xylanolytic bacterial isolates from termite gut

Table 3: 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

Diameter (mm) of Bacterial isolates.

Effect of time period on cellulase activity:

Enzyme activity recorded at different time period revealed that cellulase production was maximum at 48 hours of incubation (Table 4). The time period was found to influence extracellular enzyme secretion, possibly by changing the physical properties of the cell membrane.

Effect of Temperature on enzyme activity:

The crude enzymes produced during this experiment were assessed for activity using CMC and avicel as substrates as reported earlier by Zhang and Lynd [30]. It was observed that isolate TGB9 and TGB10 have shown higher cellulase activity at 40 ^{o}C when avicel was used as substrate (Figure 3). The Isolate TGB10 shows higher exoglucanse activity (3.74 $\pm$0.03 U/ml) as compared to isolate TGB9 (2.68$\pm$0.02 U/ml). Both isolates have also shown higher enzyme activity at 50 ^{o}C when CMC was used as substrate (Figure 4). The isolate TGB10 shows higher cellulase activity (5.4121$\pm$0.1541 U/ml) as compared to isolate TGB9 (3.30 $\pm$0.12).

Which indicates that TGB10 have higher enzyme activity for both substrates (avivel and CMC) at both temperatures 40 ^{o}C and 50 ^{o}C (Fig.3 and 4). Pourramezan *et al.* [24] reported that cellulase is thermal stable at 40 ^{o}C and 50 Furthermore CMC is a soluble form of cellulose which can be efficiently hydrolyzed and avicel is microcrystalline cellulose which cannot be easily degradable [25,23].

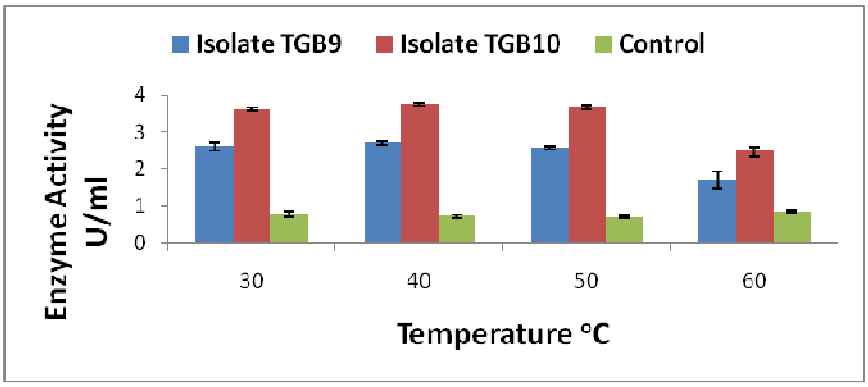

Fig. 3: Temperature optimization of enzyme activity (U/ml) for isolate TGB9 and TGB10 using Avicel as substrate

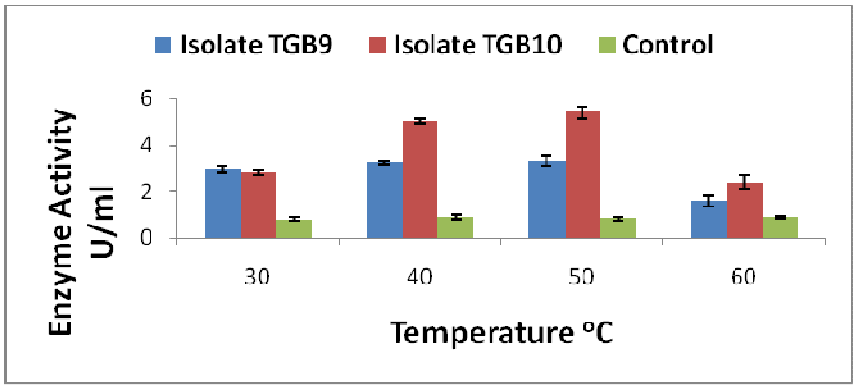

Fig. 4: Temperature optimization of enzyme activity (U/ml) for isolate TGB9 and TGB10 using CMC as substrate.

pH Optimization of Crude Cellulases:

As pH play important role in enzyme activity during conversion of cellulose to monomeric sugars (glucose). It was observed that that activity of isolate TGB10 (3.74 $\pm$0.03 U/ml) was higher as compared to isolate TGB9 (2.68 $\pm$0.02 U/ml) at pH 7 when avicel was used as substrate (Figure 5). The results recording pH optimization of bacterial isolates (TGB9 and TGB10) when CMC was used substrate are shown in Figure 6. The enzyme activity at pH 8 for TGB10 (5.67 $\pm$0.2 U/ml) as compared to TGB9 (3.49 $\pm$0.16 U/ml) was found. Higher cellulase activity of bacteria at pH lower than 6.0 was rarely observed and highest activities of *Paenibacillus curdlanolyticus* and *Bacillus mycoides* were observed at neutral or alkaline pH. Similar results were also reported earlier by Pason *et al.* [23], Balasubramanian *et al.* [3] and Kim *et al*. [18].

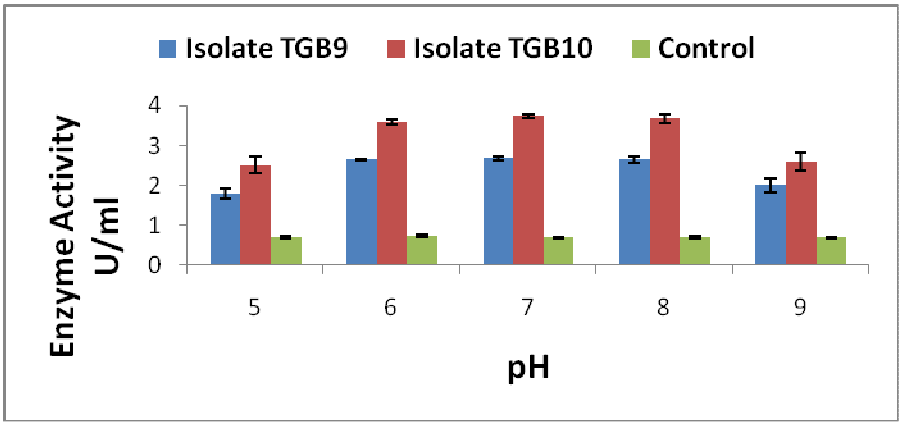

Fig. 5: pH optimization of enzyme activity (U/ml) for isolate TGB9 and TGB10 using avicel as substrate.

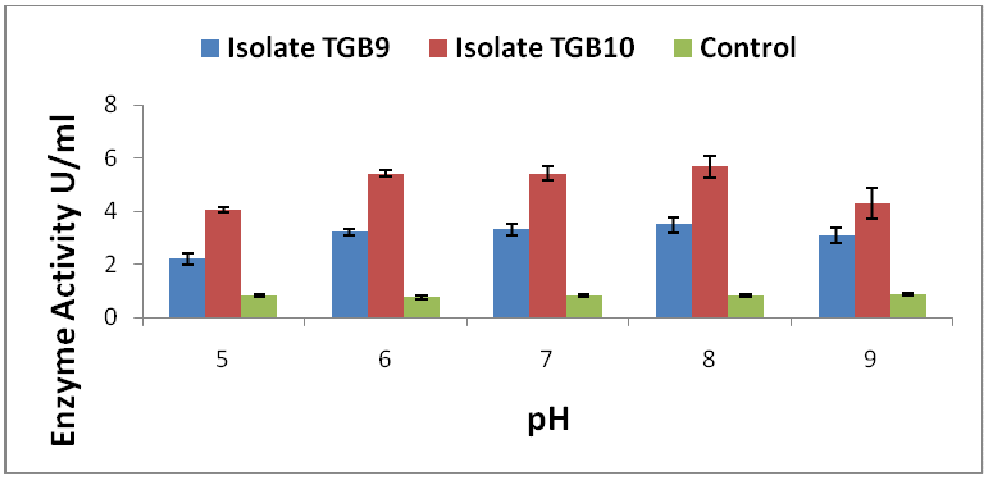

Fig. 6: pH optimization of enzyme activity (U/ml) for isolate TGB9 and TGB10 using CMC as substrate.

Table 4: Analysis of fermentation products by using optimized conditions

Isolates TGB9	Substrate	Time duration (hours	Temperature °C	pH	Glucose (g/g)	Ethanol (g/L)	Ethanol yield (%)	Acetate	Formate
	Rice straw (100 g	48	50	8	65.5 ±0.3	7.52 ±0.5	33.17	2.5 ±0.2	Nil
	Corn stove (100)	48	50	8	62.3 ±1.5	6.35±0.2	31.33	2.1±0.7	Nil
Isolates TGB10	Rice straw (100 g)	48	50	8	85.5±2.3	9.35±0.5	43.31	2.8 ±0.7	1.2 ±0.2
	Corn stove (100 g)	48	50	8	78.5 ±1.9	6.94 ±0.6	39.62	1.7 ±0.4	3.1 ±0.7

Triplicate analysis (n=3)

End product analysis of corn stover and rice straw substrates after fermentation:

Results regarding analysis of acetate, formate, glucose and ethanol produced from corn stove and rice straw by using bacterial Isolates (TGB9 and TGB10) and finally fermentation by *S.Cerecives* are given in table (Table 4). It was observed that isolate TGB9 completely failed to produced fromate from corn stover and rice straw samples during fermentation , or might be their concentration was below detection limits. However, isolates TGB10 have produced some amount of formate from both substrates (Table 4). The level of acetate produced in this experiment was also low but comparable results reported earlier by research workers [12]. The amount of glucose obtained from rice straw (85.5 ±2.3 g/g) was higher as compared amount of glucose obtained from corn stover (78.5 ± 1.9 g/g) when saccharification was carried out by Isolates TGB10. Whereas Isolates TGB9 has provided lower amount of glucose from both substrates. It was observed that isolate TGB10 produced higher amount of ethanol from both corn stove and rice straw as compared to isolate TGB9 (Table 4). The higher concentration of ethanol (9.35 ±0.5 g/L) was obtained from rice with Isolate TGB10 as compared to isolate TGB9 that have produced 6.94 ±0.6 g/L of ethanol from rice straw (Table 4). Whereas yields of ethanol (39.62 to 43.31 %) were obtained by TGB10 from corn stover and rice straw respectively as compared to 31.33 to 33.17 % obtained by TGB9 from corn stover and rice straw. It was reported by Fujimoto *et al.* [11] that isolates obtained from termites gut were closely related to *Bacillus licheniformis* and have a strong cellulose-degrading [31, 32] ability under a micro-aerophilic condition but very low amount of ethanol was observed as fermentative product**,** that might be effected by some unknown factors during fermentation and handling process [27,20].

Bioethanol production:

The saccharification process was conducted by using bacterial isolates (TGB 9 and TGB 10) extracted from termites gut and was mixed with broth containing *Saccharomyces cerevisiae* and ethanol produced was quantified by using HPLC. It was found that rice straw have produced ethanol (9.35 ±0.5 g/L) equally to ethanol yields of 43.31 % as compared to 6.94 ±0.6 g/L obtained from corn stover and yields were 39.62 %. It was assumed that lower amount of ethanol obtained in current study was due to influences of different factors those effects on final concentration of ethanol as well as ethanol yields. These factors might be concentration of cellulase enzymes, level of lignin and hemicellulose in biomass samples, yeast growth as well as optimal conditions like temperature, pH and time duration [33, 34, 24].

Conclusion:

It is concluded that bacteria isolates (TGB9 and TGB10) produced responsible cellulase enzyme activity after 48 hours at 50 ^{o}C and pH 8. Activity of Cellulase enzyme produced by bacteria isolated from termite gut was found to be increased by the addition of 5 mM MnSO4. By using these isolates commercially enzymes (cellulase) as well as acidic for alkaline treatments of biomass samples are not required. That can reduce the overall cost of ethanol, when it is being use as different types fuels. However, if effects of different factors, like level of lignin and hemicllulose as well as enzyme concentration, temperature, pH and time duration well managed then amount of ethanol per gram of biomass samples could be increased.

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