ISOLATION AND SCREENING OF CELLULOSE AND HEMICELLULOSE DEGRADING BACTERIA

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ABSTRACT

As the demand of the world for energy is increasing day by day there is a need of alternate resource of energy. Pakistan is a country using huge budget for the import of petroleum goods due to limited energy resources. There is huge amount of lignocellulosic biomass is present because Pakistan is an agricultural country. This lignocellulosic material can be utilized for the production of alternate energy resource. There are many challenges while using lignocellulosic biomass for energy production. One among of those is the hydrolysis of biomass by enzymes. These enzymes are expensive, so there is a need to produce enzymes from different sources that can efficiently hydrolyze biomass. Termite guts are micro environments and also called as mini biorefineries. Due to their proficiency to utilize lignocellulosic biomass. Therefore, a study was planned to isolate bacteria in termite gut that have the ability to degrade hemicellulose and cellulose into respective reducing sugars. Five bacterial isolates were selected on the basis of screening by Congo red dye method. All these bacterial isolates have the ability to hydrolyze cellulose and hemicelluloses. Biochemical and morphological characterization of the selected bacterial isolates were performed. These bacteria may have the enzymes that can efficiently hydrolyze biomass that will be helpful for production of biofuel and also for biological waste management.

Key words: cellulose, hemicelluloses, termites, enzyme, lignocellulosic

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INTRODUCTION

Pakistan has huge demand to yield power by fossil fuel, only 20% can be accomplished by domestic resources. 80% of petroleum goods and fuel are trade in from diverse countries of world. Energy is most important source for the growth of a country (MPNR, 2008).The current extraordinary charges of oil,environmental assistances of reduction in greenhouse gas discharge and potential for the local development,all around the world there is considerably enlarged concentration for the production bioethanol (Rogers, 2008). In this context, lignocellulosic assets, that is a rich and cost efficient resource

of deposited energy present in environment. These biological materials are the lead source for biofuel production (Lynd *et al*., 2008). Among different steps for bioethanol production one critical step is the conversion of cellulose and hemicelluloses present in lignocellulosic material, into monomeric sugars (Galbe and Zacchi, 2007). The major research is required in the step of pre-treatment of biomass, to find efficient and cheaper enzymatic systems for lignin exclusion and to degrade hemicelluloses and cellulose into individual reducing sugars. Pretreatment signifies the costliest division of bioethanol production (~ 20% of entire costs) (Scharf and Boucias, 2010). Termites assimilate and utilize lignocellulosic material by different digestive enzymes among which some are endogenous and some are symbiont based (Breznak and Brune, 1994; Watanabe *et al.*, 1998; Scharf & Tartar, 2008). Keeping in opinion the significance of bioethanol as alternative and renewable fuel, current research was accompanied to isolate and screen out the hemicellulose and cellulose degrading bacteria from the termite gut.

MATERIAL AND METHODS

Sample Collection

Wood assimilating termites were collected from the road side, Islamabad from decaying trees.

Isolation and Screening Of Hemicellulose And Cellulose Degrading Bacteria
. Sample preparation

Sterilized petriplates were retained in laminar flow hood. It was cleaned up with 70% ethanol and also sterilized using UV light for approximately 10 minutes. To remove surface contamination, the termites were placed for 10 minutes in 70% ethanol after sterilization the termites were washed using sterilized distal water. The termites were cut into two parts, head and body. The bodies were selected for isolation of symbiotic bacteria present in termite gut. By using pre-sterilized mortar and pestle the bodies of termites were grounded and a suspension was made in pre-sterilized distal water.

Culturing and purification of bacteria

The petri plates were organized and prepared by using nutrient agar, some of them have 1% carboxymethyl cellulose (CMC) and others have 1% xylan, 7 pH was maintained (Dheeran *et al.,* 2012, Pourramezan *et al.,* 2012). The suspension made was serially diluted and spread over media petri plates. The petri plates were incubated for 24 hours at 30 ^{o}C. After incubation period colonies appeared that were streaked on new petri plates for purification. To purify the bacterial cultures, the bacterial colonies were re-cultured several times.

Screening of hemicellulose and Cellulose Degrading Bacterial isolates

Congo red dye method was used to confirm hemicelluloses and cellulose degrading capacity of bacterial isolates. Two different nutrient agar media were prepared, one was with CMC, and other xylan for hemicelluloses screening bacteria. The media have composition: peptone 10g; beef extract 10g; agar 18g and NaCl 5g per L distilled water. CMC media was supplemented with 0.2% CMC and xylan media was supplemented with 0.2% xylan. To get the actively growing bacterial isolates, they were cultured overnight in broth media. The actively growing bacterial culture (5ul)

was poured on petri plates having screening media. The petri plates were incubated for 48 hours at 30 ^{o}C (Dheeran *et al.*, 2012).

Congo red dye solution (0.2%) was prepared in 5% ethanol. The petri plates were flooded with the above mentioned solution for 20 minutes after incubation. Extra dye was removed by washing petri plates, 2-3 times with 1M sodium chloride. The plates having clear zones around colonies were selected for specific bactertial isolates having xylan and cellulose degrading ability (Tokuda and Watanabe, 2007). The hydrolyzing ability of bacterial isolates was measured by taking the ratio of the diameter of both clear zone and bacterial colony (Ariffin *et al.*, 2006).

Biochemical and Morphological Categorization of Selected Bacterial Isolates

Biochemical characterization of bacterial isolates

Biochemical Characterization of selected bacterial isolates was conducted by using different tests. Catalase test was conducted according to Reiner, 2010, oxidase test was performed according to Shields and Cathcart, 2010, Triple sugar iron (TSI) medium is used to determine the ability of isolates for sugar fermentation and H_2S production (Lehman, 2005), Simon citrate (Williams, 2009) and SIM (Sulfide- indole motility medium) was conducted to check the mobility of bacterial isolates by using ASM, 2011 method.

Colony morphology

Various characteristics of bacterial colonies were considered that include: form of colony, margin, colony color and elevation of isolate TGB1, TGB2, TGB3, TGB4 and TGB5.

Gram staining

Sigma-Aldrich kit was used for Gram Staining. After staining the slides were examined under microscope.

RESULTS AND DISCUSSION

Isolation and Purification of Bacteria

During the study facultative anaerobic and aerobic bacteria isolated from termite gut were examined having xylanolytic and cellulolytic ability. Brune *et al.*, 1995 considered that only anaerobic environment exists in the termite gut. Whereas anaerobic and aerobic bacteria were likewise deliberated by Wenzel *et al.*, 2002. Termites collected from decaying trees were selected from Islamabad, Pakistan. The bacterial isolates from termite gut were cultured on media having xylan and CMC as carbon source on nutrient agar (Fig. 1). Pure bacterial cultures were obtained by streaking them again and again on various petri plates.

Figure 1. **Bacterial colonies from termite gut**

Screening of Xylan and Cellulose Degrading Bacteria

For screening of bacterial isolates to validate hemicelluloses and cellulose degrading ability congo red dye method was used. This method for screening is sensitive and rapid to determine cellulolytic potential of bacteria due to high non-covalent affinity for cellulose (Yoon *et al.*, 2007). Bacterial isolates having clear zone on nutrient media having xylan and CMC were selected as positive for xylan and cellulose degradation. During experiment positive control was *B. subtilus* and negative controlwas *E. coli*. Purified bacterial isolates were 53, among which 5 exhibited degradation capacity for both, xylan and cellulose substrates. The diameter ratio of clear zone and colony of all 5 bacterial isolates is listed in (Table 1). For enzymatic activities isolates TGB1, TGB3 and TGB5 were selected because they have high capacity to hydrolyze xylan and cellulose according to screening results when compared with isolate TGB2 and TGB4. For isolate TGB1, TGB2 and TGB3 the diameter ratio of clear zone to colony for CMC was calculated as 3.4, 2.84 and 2.3 mm respectively presenting that isolate TGB1 has high capacity to hydrolyze CMC when compared to TGB3 and TGB5 (Table 1). Generally, CMC is considered as a substrate for endoglucanase activity and Avicel as exoglucanase activity for cellulase (Zhang and Lynd, 2004) this means isolate TGB1 has high endoglucanase activity.

When xylan is considered, diameter ratio of clear zone to colony, isolates TGB1, TGB3 and TGB5 is 4.32 mm for both isolate TGB1 and TGB3 and 3.3 mm for TGB5 (Table 1). All the three isolates indicate high enzymatic activity for xylan when matched with CMC, means having high xylanase activity and hydrolyze hemicellulose more efficiently than cellulose. When results from both substrates were compared it was observed that TGB1 has high xylanase and endoglucanase activity when compared to TGB3 and TGB5.

Table 1. Screening of bacteria by Congo Red Dye Method.

Bacterial Isolates	Average	
	CMC D/d* (mm)	Xylan D/d* (mm)
TGB1	±3.4	±4.32
TGB2	±1.39	±1.24
TGB3	±2.84	±4.32
TGB4	±1.15	±1.2
TGB5	±2.3	±3.3

*D/d: Hydrolyzed zone diameter/colony diameter

Biochemical and Morphological Categorization of Selected Bacterial Isolates

Morphological and Biochemical characterization of TGB1, TGB2, TGB3, TGB4 and TGB5 was conducted that have ability to hydrolyze xylan and cellulose.

Biochemical Characterization

Different biochemical tests were performed to characterize bacterial isolate. For current study Simon citrate, SIM (Sulfide- indole motility medium), oxidase, catalase and TSI (Triple sugar iron) tests were done to biochemically characterize isolates TGB1, TGB2, TGB3, TGB4 and TGB5.

It was observed that isolate TGB3, TGB4 and TGB5 utilize citrate while isolateTGB1 and TGB2 do not use citrate as sole carbon source (Table 2). Simon citrate test is utilized to determine that bacterial isolates have the ability to use citrate as carbon source and ammonium salt ($NH_4H_2PO_4$) present in the media is a source of nitrogen (Williams, 2009).

SIM is a semisolid agar media used to determine motility of bacteria, hydrogen sulfide production and indole formation. All of bacterial isolates did not produce hydrogen sulfide and indole but it was observed that all isolates have the motility (Table 2). The SIM media inoculated with the isolates was matched with un-inoculated one to discriminate a slight motility.

Bacterial isolate TGB1, TGB3 and TGB5 did not show oxidase activity but isolate TGB2 and TGB 4 were found to be oxidase positive (Table 2). Oxidase test is utilized to find bacteria that have the ability to produce cytochrome c oxidase. Oxidase is an electron transport chain enzyme that can utilize oxygen during respiration as terminal electron acceptor (Acharya, 2012). Results showed that TGB1, TGB3 and TGB5 do not have the ability to produce cytochrome c oxidase.

Catalase test is performed to discriminate between obligate anaerobes, facultative anaerobes and aerobes. The obligate anaerobes generally do not produce catalase enzyme (Mahon *et al*., 2011). All the bacterial isolate TGB1, TGB2, TGB3, TGB4 and TGB5 were found to have catalase activity (Table 2). Therefore, results tell that above mentioned isolates were facultative anaerobes

or aerobes.

It was observed that TGB1, TGB2, TGB3, TGB4 and TGB5 do not produce any kind of gas while performing TSI test. Only glucose was consumed by TGB1 and TGB2 but they do not utilize lactose and sucrose (Table 2). Isolate TGB3, TGB4 and TGB5 have the ability to consume glucose, sucrose and lactose (Table 2). The media utilized during experiment showed acidic slant and acidic butt in the test tube which indicates that all sugars were consumed (Lehman, 2005).

Table 2. Biochemical categorization of bacterial isolates

Tests	TGB1	TGB2	TGB3	TGB4	TGB5
Gas production	–	–	–	–	–
H_2S production	–	–	–	–	–
Gram stain	+	+	+	+	+
Shape	Rod	Rod	Rod	Rod	Rod
Catalase	+	+	+	+	+
Oxidase	–	+	–	+	–
TSI	Alk/Acid	Alk/Acid	Acid/Acid	Acid/Acid	Acid/Acid
Indole	–	–	–	–	–
Citrate utilization	–	–	+	+	+
Motility	+	+	+	+	+

Morphological Characterization of Bacterial Isolates

Colony morphology

Various characters of the colony morphology were considered that include; form of colony margin, colony color and elevation for TGB1, TGB2, TGB3, TGB4 and TGB5 that are given (Table 3) and Figure 2 shows all isolates cultured on nutrient agar plate.

Gram staining

It is a technique used to distribute the bacterial isolates into two huge groups, founded on the various constituents of cell wall (Bergey *et al.*, 1994). All the bacterial isolates TGB1, TGB2, TGB3, TGB4 and TGB5 were observed to be gram positive and rod shaped. Gram positive bacteria have a thick layer of peptidoglycan (50-90% of cell wall) on the other hand gram negative bacteria have a very thin layer of peptidoglycan (10% of cell wall). Regardless of thick layer of gram-positive bacteria are reachable to antibiotics as compared to gram negative bacteria, due to absence of outer membrane (Bergey *et al.*, 1994).

Table 3. Colony morphology of bacterial isolates

Bacterial Isolates	**Morphology of colony**			
	Elevation	**Margin**	**Form**	**Color**
TGB1	Convex	Undulate	Irregular	White
TGB2	Flat	Entire	Circular	Foggy white
TGB3	Raised	Filamentous	Filamentous	White
TGB4	Convex	Entire	Circular	Foggy white
TGB5	Raised	Entire	Punctiform	Pale yellow

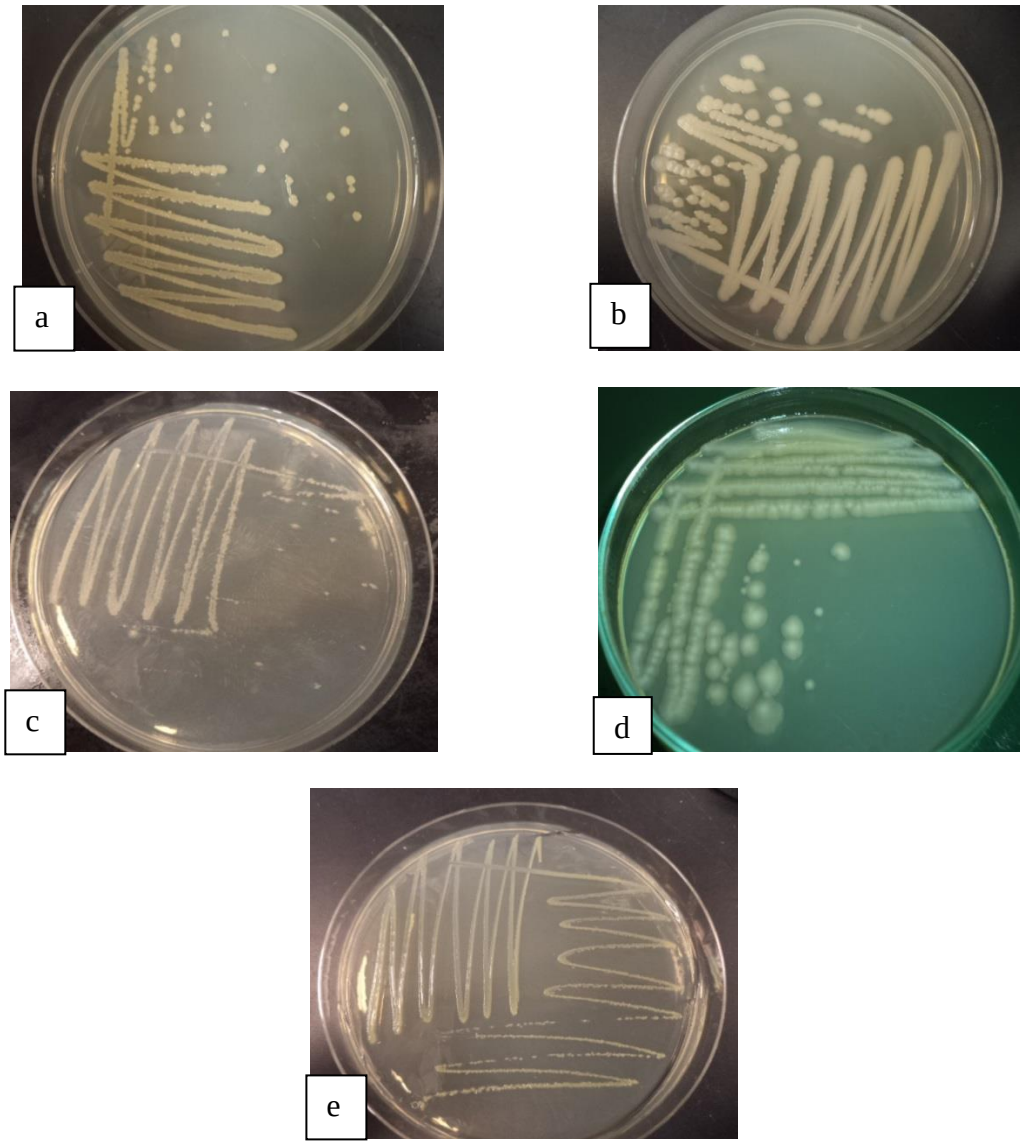

Figure 2: (a) TGB1 (b) TGB2 (c) TGB3 (d) TGB4 (e) TGB5

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