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16s rRNA Identification of Lignocellulose Degrading Bacteria from Cow Dung and Termite GUT Revealed Pathogenic Bacteria Strains

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Abstract: Lignocellulose is the most abundant renewable resource available from plants in this case from coconut husk. Bioconversion products of lignocelluloses have biomedical significance. This study was to identify previously isolated lignocellulosic bacteria strains which have the ability to degrade lignin and cellulose. The techniques used in this study confirms bacteria identity by sequence analyses of 16s rRNA, Gram staining, motility test, crystal formation test and penicillin sensitivity test (10 μg of Penicillin G-P-10). Gram staining was performed on six bacteria strains to distinguish between Gram negative and Gram positive cells. Extraction of genomic DNA for 16s rRNA sequence analyses were also conducted on the six bacteria strains to differentiate between species. Four out of six bacteria were identified as *Bacillus thuringiensis* (T10), *Enterobacter aerogenes* (T19), *Bacillus pumilus* (T22) and *Bacillus vireti* (T24) using sequence analysis of 16s rRNA compared to NCBI database. Two bacteria strains, C19 and C37 belong to the Genus *Bacillus* which had more than one possible species results which were distinguished by using biochemical test. Four bacteria strains were Gram positive (T10, T22, C19 and C37) and two Gram negative (T19 and T24). Motility test was negative for C19 and positive in C37 isolates. Isolate C19 had a diameter of 32 mm which showed susceptibility to Penicillin whereas for isolate C37 had a diameter of 19 mm which showed resistance to Penicillin. Isolate C37 was confirmed as *B. thuringiensis* after four days incubation by using crystal formation test. This study suggests that the isolates T10, T19, T22, T24, C19 and C37 were identified as *B. thuringiensis*, *E. aerogenes*, *B. pumilus*, *B. vireti*, *B. anthracis* and *B. thuringiensis*, respectively. Future studies using metabolic engineering techniques can optimise their lignocellulose degradation potential in suitable vectors.

Key words: Bacillus, 16s rRNA, gram stain, biochemical characterisation, pathogenic, lignocellulose, bioconversion

INTRODUCTION

Lignocellulose is a major constituent in plant biomass which consists of cellulose, lignin and hemicellulose. Naturally, lignin is not susceptible to degradation due to its strong chemical bonds although the composition of lignin varies in different species. In previous studies since the mid-1980s, white-rot fungi and brown-rot fungi are known lignin degraders, especially basidiomycetous fungi were the most effective lignin degraders (Chen *et al*., 2012). However, bacterial strains have rarely been cited to significantly degrade lignocellulosic materials. Isolating bacterial strains from cow dung and termite guts with the potential to degrade or bioconvert lignin and or cellulose was an idea born from the capability of these organisms to digest cellulose and lignin by the latter. It is supported by the fact that these organisms host symbiotic bacteria expressing necessary enzymes for the breakdown of cellulose in the gastrointestinal tract.

We have isolated bacterial from the guts of termites and cow dung (pers. comm.) and in this study their identity was elucidated by molecular methods complementing biochemical characterization where necessary. In retrospect, phenotypic method includes the identification through microscopic and macroscopic observation and biochemical testing while genotypic methods are also known as molecular techniques which include PCR, electrophoresis and DNA sequencing of concensus sequences. In addition to that phenotypic identification is known as traditional bacterial identification with disadvantages because highly related species cannot be distinguished. Molecular methods have been proven to be more efficient in overcoming the limitations of phenotypic identification where the 16s rRNA gene sequence with <97% similarity is known as a different species (Elwary *et al*., 2008; Das *et al*., 2014).

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Lignin-degrading bacteria can usually be isolated from soil and guts of wood-eating insects which mainly belong to three classes, Actinomycetes, α-proteobacteria and α-proteobacteria (Huang *et al*., 2013, 2012). *Pseudomonas* and *Bacillus* sp. were previously identified as potential lignin degraders in which *Sphingomonas paucimobilis* SYK-6 was one of the best characterized lignin degraders (Bandounas *et al*., 2011). Other genera of lignin degraders including Klebsiella and Citrobacter (Chen *et al*., 2012). Cellulose-degrading bacteria containing cellulolytic anaerobes include the bacterial genus Fibrobacter and lineages of Clostridium (McDonald *et al*., 2012). This study's aim is to identify the genera and species of bacteria compared to known bacteria isolated from termites gut and cow dung which are suggestively lignocellulosic bacteria in order to elucidate their metabolic pathway for lignin and cellulose degradation or conversion.

MATERIALS AND METHODS

The previously isolated six bacteria strains (pers.comm.) were sub-cultured in nutrient agar and trypsin soy agar and incubated for 12h at 37°C to proceed with Gram staining, biochemical tests and 16s rRNA sequence analysis. Glycerol was prepared in 40% concentration for long term storage of the bacteria. One volume of bacterial culture at OD 0.6 was added with one volume of 40% glycerol. The stock was then kept at -80°C to maintain the cells viability.

Molecular techniques used in this study was 16s rRNA sequence analysis by using consensus sequence comparison of the small ribosomal subunit of the RNA. Genomic DNA of all six bacteria strains were extracted and subjected to PCR for amplification of the gene of interest. There were three main stages in PCR include denaturing, annealing and elongation in a cycle. In denaturing stage, the cells were separated from double stranded to single strand at 94°C for one minute. Followed by annealing stage in which the primers were bind to the gene of interest at 50°C for 30 sec. Then, the cells entered the elongation stage to allow the synthesis of new DNA strand by taq polymerase at 72°C for 5 min. This cycle was repeated 25 times and then stored at 4°C until further use. After the amplification of gene interest, the PCR product was then subjected to ligation into PGEM-T Easy vector followed by transformation of ligated product into *E. coli* JM109 competent cells. Blue-white screening was used as insertion screening. The randomly selected white colonies were picked for plasmid extraction and sent for sequencing. The completed sequencing results were then identified based on NCBI database.

Gram staining was done for morphological characteristics studies. Biochemical tests were carried out for further confirmation of bacteria strains that were unable to be identified to its species. Biochemical tests included motility test, penicillin sensitivity test and crystal formation test. Motility test and penicillin sensitivity tests were used to differentiate between *B. anthracis* from *B. cereus* species. Crystal formation test was used to differentiate among *B. thuringiensis* and *B. cereus* species.

RESULTS

Six selected isolated strains, T10, T19, T22, T24, C19 and C37 were sent for sequencing following 16s rRNA sequence analysis which were then compared to NCBI database. One of the sequence result, T10 (*Bacillus thuringiensis*) obtained was shown in Fig. 1. The sequence result from the forward reading and reverse reading were combined together to obtain a full sequence before compared to NCBI database. The four isolates, T10, T19, T22 and T24 were then identified as *Bacillus thuringiensis*, *Enterobacter aerogenes*, *Bacillus pumilus* and *Bacillus vireti* accordingly. However, bacteria isolate C19 and C37 have more than one possible result as their species overlapped according to the sequencing results which may be *Bacillus thuringiensis*, *Bacillus cereus* or *Bacillus anthracis*. Thus, both the isolates, C19 and C37 were further confirmed using biochemical testing.

Gram staining was done showing four isolates as Gram positive and two isolates as Gram negative. Gram staining of two isolates (C19 and C37) is shown in Fig. 2. All *Bacillus* sp. are motile except *B. anthracis*. This test was used to differentiate *B. anthracis* from *B. cereus*. Motility results were compared before and after incubation. The C19 was non-motile a known characteristic of *B. anthracis* and it was futher confirmed using penicillin sensitivity test. The C37 shows motility after the incubation suggesting a possibility of either *B. cereus* or *B. thuringiensis*. Thus, C37 was further confirmed by using crystal formation test and penicillin test.

Biochemical test were carried out systematically to distinguish between bacteria of the same genus, these tests were chosen by its distinguishing factors between the two bacilli. The following test results, i.e., motility test, penicillin sensitivity test and crystal formation test can be summarized in Table 1.

All Bacillus are resistant to penicillin except *B. anthracis*. Penicillin (P-10) sensitivity test was used to differentiate *B. anthracis* from *B. cereus* group. In this test isolate C19 showed a clearing zone with a diameter of

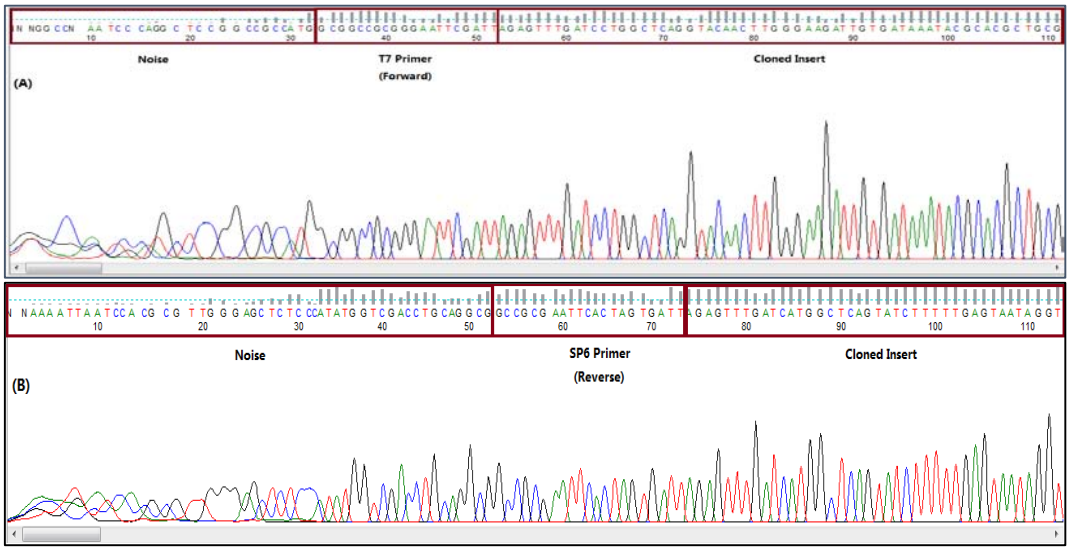
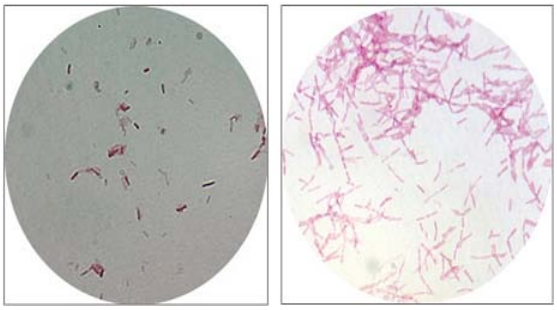


Fig. 1: Intensity spectra for T10 (*Bacillus thuringiensis*) shows good quality sequencing results where both forward and reverse reading results were combined to obtain a full sequence result: A) Forward reading sequence result for sample T10 by using primer T7 and 760 bases was taken to combine with reverse reading and B) Reverse reading sequence result for sample T10 by using primer SP6. 760 bases were taken to proceed with reverse complement before combining with forward reading sequences



B. anthracis
Purple, rod-Gram positive

B. thuringiensis
Purple, rod-Gram positive

Fig. 2: Gram staining results for C19 (*B. anthracis*) and C37 (*B. thuringiensis*)

Table1: Summarization of motility test, penicillin sensitivity test and crystal formation test for two isolates (C19 and C37)

Motility test		
Isolates	Result	Species
C19	Non-motile	*B. anthracis*
C37	Motile	Possibility of either *B. thuringiensis* or *B. cereus*
Penicillin sensitivity test		
C19	Susceptible	*B. anthracis*
C37	Resistant	Possibility of either *B. thuringiensis* or *B. cereus*
Crystal formation test		
C19	Negative	*B. anthracis*
C37	Positive (Cuboid-shaped)	*B. thuringiensis*

34 mm which suggests susceptible to penicillin and C37 showed a clearing zone with a diameter of 18 mm which

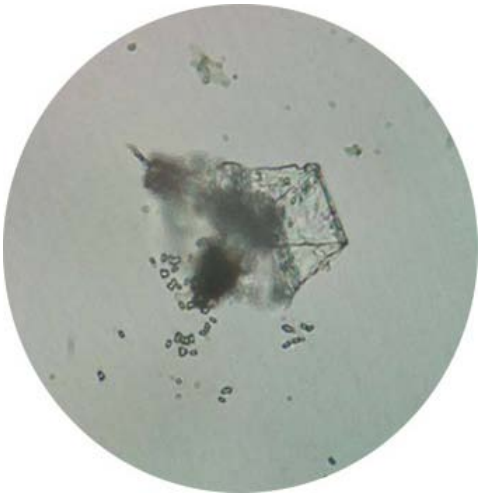


Fig. 3: A cuboid shaped crystal was observed in C37 (*B. thuringiensis*) after 4 days incubation under 100x magnification x

suggests resistant to penicillin. Thus, isolate C19 can be confirmed to be *B. anthracis*. Crystal formation test was used to differentiate between *B. thuringiensis* and *B. cereus* in which *B. thuringiensis* will form crystal in its life cycle under appropriate condition as a defense mechanism. A cuboid shaped crystal was observed in isolate C37 after four days of incubation as shown in Fig. 3. This indicated that C37 is *B. thuringiensis*.

DISCUSSION

The bacteria from *Bacillus* genus are known to be used in biomedical applications and as a bio-insecticide in agriculture (Tan *et al*., 2012; Sansinenea, 2012; Priyadarshini *et al*., 2013). It is also a biological control agent developed against mosquitoes to avoid ecological pollution which may be caused by wide use of chemicals as pesticides (Huguette and Donald, 2012). *Bacillus thuringiensis* is an insect pathogen which can be found in soil. Insecticidal pore forming proteins, possibly the cause of the crystal formation in *B. thuringiensis* are able to kill their insect larva host (Bravo *et al*., 2011). It is interesting to note that *B. thuringiensis* emerged as one of the isolates capable in bioconverting lignocellulosic material into reducing sugars isolated from termite gut and cow dung. It would suggest that not only that this bacteria was used as a biological control agent around the vicinity it was isolated from, its capability in forming endospores enabled it to survive the harsh digestive tracts of cows and termites and into their guts possibility assisting with enzymes used to digest lignocellulosic materials in wood and grass.

The next isolate found in termite gut is *Bacillus pumilus* which can be naturally found in lake sediments as well as earthworm guts (Jayakumar *et al*., 2012; Shankar and Isaiarasu, 2011). The protease enzyme from *B. pumilus* shows effective result as a dehairing agent and detergent (Jayakumar *et al*., 2012). Besides that, *B. pumilus* can also be used as a biological control agent to control *Rhizoctonia solani* Q1 in cucumbers (Huang *et al*., 2012).

Bacillus anthracis was isolated from local cow dung is the causative agent for anthrax. It is interesting to note that this infamous pathogen used in bioterrorism attacks typically causing three types of infections at cutaneous, gastrointestinal and inhalation levels (Hicks *et al*., 2012) can also degrade lignocellulose into reducing sugars as shown in unpublished results (pers. comm.). Anthrax infection rarely occurs in developed countries, however, workers in contact with herbivores and its products, heroin use or clustering of patients with similar respiratory symptoms are at high risk of antrax infection (Sweeney *et al*., 2011).

Enterobater aerogenes isolated from termite gut is a nosocomial and pathogenic bacterium that causes opportunistic infections especially in immuno compromised patients. A strain of *E. aerogenes* was reported to be resistant to all current antibiotics except gentamicin which is commonly used to treat Enterobacterial infections (Diene *et al*., 2013). According to Panda and Sarkar (2012), *E. aerogenes* is useful in bioremediation of chromium as it is able to survive in actual heavy metal environmental conditions. Besides that *E. aerogenes* was widely used in bioengineering for fermentative hydrogen production to enhance in anaerobic cultivation (Zhang *et al*., 2011). Its presence in termite guts and its capability to degrade lignocelluloses may point to further exploitation of this bacterium.

CONCLUSION

All six bacteria isolated from local termite gut and cow dung were successfully identified using 16s rRNA methods complemented by biochemical characterisation. Five isolates were *Bacillus* sp. and one *Enterobacter aerogenes*. Although, these identified bacteria are commonly known pathogens, various applications of these bacteria outweigh their patogenecity. It is interesting to note that they are found ubiquitously in herbivores and insects which can digest lignin and cellulose for energy. Their capability to degrade carboxy methyl cellulose on solid minimal media without other carbon sources as reported in unpublished work indicated that these bacteria can thrive solely on complex carbohydrate molecules. This preliminary work supports our aim to utilize coconut husk as the sole carbon source. Further optimization studies on agitation, temperature, pH and carbon sources are underway to select the best conditions for these bacteria to degrade lignocelluloses. Sequential identification of the metabolic processes involved in degradation or bioconversion of lignocelluloses to smaller molecules or larger but high value metabolites paves the way for molecular engineering techniques in order to optimise their lignocellulose degradation potential in suitable vectors for in situ bioconversion.

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