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Diversity of cellulose hydrolyzing bacteria from the gut of *Coptotermes heimi* (Rhinotermitidae)

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Abstract

This study was aimed to investigate the cellulolytic bacteria of termite gut symbionts of worker *Coptotermes heimi* (Wasmann) and to identify bacteria with the ability to degrade the cellulosic biomass to glucose monomers. These bacterial isolates were checked for cellulolytic activity on CMC agar by staining with Gram's Iodine. The bacteria were identified on the basis of their morphology like shape and colour of the colony, Gram's staining and KOH test. The Biochemical analysis was done by performing different tests (Citrate Utilization, Cellulase activity, Starch hydrolysis, EMB, MacConkey, Mannitol Salt agar, TSI, MR, Nitrate test etc.) and molecular characterization was done by using bacterial 16S primers to amplify the 16SrRNA gene. The culturable isolates obtained from the termite guts were: *Bacillus atrophaeus, B. pumilus, B. licheniformis, B. amyloliquefaciens, B. coagulans, B. megaterium* and *B. cereus*. All these isolates were rod shaped Bacilli, Gram positive, KOH negative. *B.pumilus* showed maximum cellulolytic index (0.69). *B.cereus* was cellulase negative. The study suggested that the termite guts are an important source for the study of bacteria and bacterial enzymes benefit for biotechnology. These guts can be utilized for microbiological, genetic, agricultural and industrial applications.

Key words : Termite ; *Coptotermes heimi* ; KOH test; Bacilli ; cellulase ; 16SrRNA

INTRODUCTION

Termites (Isoptera) are roughly divided into higher and lower termites. Lower termites harbor a dense population of prokaryotes and protists (single celled Eukaryotes) in their gut while higher termites lack protists [1]. These termites feed on variety of food material mainly contains cellulose, hemicellulose and lignocellulose. The association between gut cellulolytic protists and lower termite is a well- known example of mutual symbiosis. It is a serious pest because of their ability to destroy all materials containing cellulose. The protists endocytose wood or cellulose particles to produce acetate, which is then absorbed by termites as energy and carbon source [1]Cellulose is a linear polysachharide which is constructed from monomer of glucose bound together with β 1-4 glucosidal linkage [2]. Cellulose is a most abundant renewable polymer on the earth. Bacteria have a higher growth rate than fungi leading to greater production of cellulase and is often more effective catalysts [3].Cellulases are a group of enzymes catalyzing the hydrolysis of cellulose. In nature, complete cellulose hydrolysis that breakdowns of cellulose and subsequent biological conversion to glucose as end product is facilitated by a synergistic activity of three major types of cellulases: endoglucanases (EC 3.2.1.4), exoglucanases, including cellobiohydrolases (CBHs) (EC 3.2.1.91), and β-glucosidase (BG) (EC 3.2.1.21). All of these enzymes hydrolyse the β- 1,4- linkage in cellulose chain [4]. Cellulases play an extremely important role with abundant industrial applications like, textile industry, in detergents, pulp and paper industry, enhancing digestibility of animal feeds and food industry [5].

Several bacteria have been isolated from the pure culture from the termite gut [6],[7,8,9];in which some bacteria were reported as cellulolytic organisms or at least participating in cellulose decomposition. This reveals the potential of investigating cellulose degrading bacteria from termite gut [10].The aim of the current work is to isolate and characterize the bacterial strain of termite gut with cellulolytic activity.

MATERIALAND METHOD

Collection of Termites

Termites were collected from Kurukshetra University Campus. Kurukshetra is bounded by the region at latitudes of $29^0$53'00" N and $30^0$15'02" N and longitudes of $76^0$26'27"E to $77^0$7'57" E. They were brought to the laboratory for gut extraction and for further analysis.

Isolation of Cellulose Hydrolysing Bacteria

Termite species captured from the site was surface sterilized with 70% alcohol to remove contaminations and then rinsed with distilled water. The gut of approximately 50 termites was removed in Phosphate Saline Buffer (pH 7). These extracted guts were preserved in PBS and refrigerated at -20°C for future use. The guts were macerated in a test tube and homogenized guts were spreaded over nutrient agar plates for bacterial isolation. The isolated colonies were streaked on and incubated at 37^0 C for 24-48 hrs.

Now these isolated colonies were spotted on Carboxy Methyl Cellulose agar medium for the isolation of cellulose hydrolysing bacteria. These inoculated CMC agar plates were incubated at 37^0 C for 24-48 hrs. At the end of incubation the plates were flooded with Gram's Iodine stain (9) and after 10 minutes they were washed with distilled water to observe the clear zone around the colony. The diameter of clear zone shows the cellulose digesting activity of bacteria. Hence, isolates showing discoloration of Gram's Iodine were taken as positive isolates. These cellulose hydrolysing bacteria isolates showing a clear zone around the colonies were picked from the master plates (Nutrient agar plates) and streaked onto nutrient agar slants. They were served as stock culture.

Morphological Characterization of Cellulose-Hydrolysing Bacteria

Gram's Staining kit (11) was used to identify the Gram Positive and Gram Negative Bacteria. These isolates were also confirmed by KOH test [12].

Biochemical Characterization

Bacterial isolates were analysed by biochemical tests measuring carbon source utilization, enzymatic activities by performing different Biochemical tests like Cellulase, amylase, protease, Mannitol agar, Skim Milk Agar, TSI, EMB, MacConkey, Simmon Citrate, MR, Nitrate Reduction etc.

Molecular Characterization

Molecular Characterization of Cellulose- Hydrolysing Bacterial isolates was performed by BioAxis DNA Research Centre, Hyderabad, India.

Genomic DNA Extraction and PCR Amplification of 16S rDNA

Bacterial sample from slants was collected and DNA was isolated from all the seven samples by using Biopure™ kits (BioAxis DNA Research Centre, Hyderabad) for Bacteria Genomic DNA isolation. 16S rRNA gene was amplified by PCR from the above isolated DNA. Bacterial 16S primers were used to amplify the 16S rRNA gene.

F AGA GTT TGA TCM TGG CTC AG

R CGG TTA CCT TGT TAC GAC TT

Polymerase Chain Reaction was performed in a thermocycler. The amplification was performed as follows:

Initial denaturation for 5 minutes at 94^{0}C, 35 cycles each of denaturation for 60 sec. at 94^{0}C, annealing for 45 sec at 55^{0}C,, primer extension for 90 sec. at 70^{0}C, a final extension for 10 min at 70^{0}C. Amplified PCR product was subjected to electrophoresis using agrose gel 1% in TAE buffer and visualized by staining with ethidium bromide. The PCR product was purified by washing with sodium acetate and 70 % alcohol and eluted from the gel. Forward and Reverse sequencing reaction of PCR amplicons were carried out on ABI 3730XL sequencer to obtain the sequence. The assembled DNA sequence was used to carry out BLAST with the nr database of NCBI.

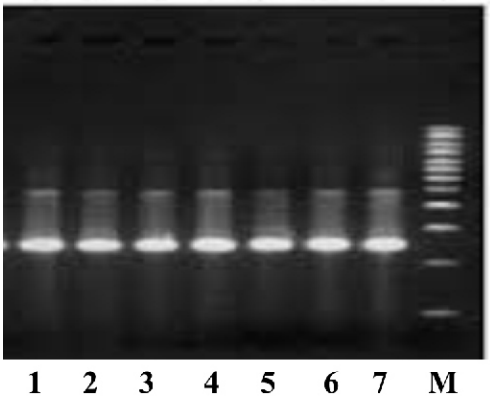

Fig 1: Gel Picture of the amplicons

RESULTS

Termite species collected from Kurukshetra University campus was identified by using identification keys "The fauna of India and The adjacent Countriues" (13). The species identified was *Coptotermes heimi* (Wasmann) found in furniture and dead wood. It belongs to family Rhinotermitidae and SubFamily Coptotermitinae.

Morphological Characterization of cellulolytic Bacteria

Seven bacteria were isolated from the gut of worker termite on nutrient agar. All the bacteria were subjected to Gram's staining and all found Gram positive as all the samples were showing colouration even after washing with absolute alcohol or decolorizer. These isolates were also confirmed on the basis of KOH test (Table 1). Morphologically all the isolates were rod shaped. Colonies of these isolates were smooth mucoid, transluscent,opaque, white, off white, irregular, rough and wrinkled.

Cellulolytic Activity of Bacterial Isolates

Cellulolytic activity of bacterial isolates as cellulose degrading bacteria were checked on CMC Agar and identified on the basis of clear zone formation around the colony. The colony with clear zone formation was considered as cellulase positive

Table 1: Cellulolytic Index Gram's Stain and KOH Test of cellulolytic gut bacteria

Bacterial Isolate	Gram Stain	KOH Test	Cellulolytic Index
Bacillus atrophaeus	+	-	0.4
Bacillus pumilus	+	-	0.69
Bacillus licheniformis	+	-	0.65
Bacillus amyloliquefaciens	+	-	0.44
Bacillus coagulans	+	-	0.39
Bacillus megaterium	+	-	0.67
Bacillus cereus	+	-	0

Table 2: Biochemical and Enzymatic properties of bacterial isolates obtained from the gut of *Coptotermesheimi* collected from Kurukshetra

Bacterial Isolate	Closest Taxonomic affiliation	Cellulase	Starch Hydrolysis	Catalase	EMB	Citrate Utilization	Mac Conkey	Indol Test	Nitrate Test	Skim Milk Agar	MR	Mannitol Salt Agar	TSI (But/Slant)
1	*B. atrophaeus*	+	+	+	-	-	-	+	+	-	-/V	-	R/R
2	*B. pumilus*	+	-	+	-	-	-	-	+	-	-/V	+	Y/Y
3	*B. licheniformis*	+	+	+	-	+	-	-	+	-	-	-	Y/R
4	*B. amyloliquefaciens*	+	+	+	-	+	-	-	+	-	-	+	Y/R
5	*B. coagulans*	+	+	+	-	+	-	-	+	-	-	+	Y/R
6	*B. megaterium*	+	-	+	-	-	-	-	+	-	-	+	Y/Y
7	*B. cereus*	-	+	+	-	-	-	+	+	-	-	+	Y/Y

and the colony not forming the clear zone was considered as cellulase negative. A total of 6 isolates showed clear of varying degree around the colony. Cellulolytic activity was measured by calculating the Cellulolytic index (Table 1)[14]using formula as follow:

Cellulolytic Index = Diameter of Zone Diameter of Bacterial Colony

Diameter of Bacterial colony

Biochemical characterization of Bacterial Isolates

A number of Biochemical tests were performed to know the biochemical activity of these isolates. Biochemical tests revealed that all the 7 isolates were catalase positive, Nitrate reduction positive, MR positive, Mac Conkey and EMB negative. Approximately 85.7% of the isolates showed cellulase positive activity to utilize cellulose as a source of carbon. Approximately 71.4% of the bacterial isolates showed amylase positive, Mannitol salt agar and Skim Milk Agar activity (Table 2).

Molecular Characterization of Bacterial isolates

16S rDNA sequence analysis of the cellulose- hydrolyzing bacterial isolates using bacterial 16S F and R primers. BLAST (Basic Local Alignment Search Tool) was carried out with the database of NCBI (National Centre for Biotechnology Information). The sequence obtained of isolate 1 showed 95% identity to the partial sequence of 16s rRNA of *Bacillus atrophaeus* strain M14. Isolate 2 showed 87% identity to the partial sequence of 16S rRNA of *Bacillus pumilus* strain CBS; isolate 3 showed 99% identity to the partial sequence of 16S rRNA of *Bacillus licheniformis* strain SY23; isolate 4 showed 96% identity to the partial sequence of 16S rRNA of *Bacillus amyloliquefaciens* strain KU19; isolate 5 showed 97% identity to the partial sequence of 16S rRNA of *Bacillus coagulans*; isolate 6 showed 100% identity to the partial sequence of 16S rRNA of *Bacillus megaterium* strain KLM14; isolate 7 showed 97% identity to the partial sequence of 16S rRNA of *Bacillus cereus* strain ARI.

DISCUSSION

Termites were collected and identified for a variety of cellulase secreting bacteria inside their gut. The digestive tract of soil-feeding termite provides a favourable environment for microorganisms and harbours dense and varied gut microbiota [15]. Soil- feeding termites are able to overcome the recalcitrance and significantly promote their mineralization [16]. All the bacteria obtained were rod shaped and Gram Positive showing KOH test negative. They were showing diverse colony colour, margin and texture. Through biochemical analysis some of the isolates showed their ability to secrete wide variety of enzymes such as amylase, protease and cellulose[17]Some of them were catalase, EMB, Simmon Citrate, Indol, Nitrate, MR, Mannitol Salt and TSI positive and some were negative. The ability to degrade starch, an indication of the role the amylases play in extracting organic matter from the soil in the gut, a process that is favoured by the alkaline conditions in the gut [18]. Their starch degrading property can be used for bioremediation process of waste products that contain amylose compound [19]The cellulase enzyme is very useful for industrial processes such as detergent and textile industries and reducing environmental pollution. Carboxymethyl Cellulose was used to screen for cellulolytic bacteria [20], [21] At present cellulase is preferred as the third enzyme for the industrial demand in worldwide due to the expansion of cellulase applications in many industries and it is expected to become the biggest amount of industrial enzyme [22], [23]. This cellulase enzyme can break down the complex organic compound into simpler

inorganic substances. This degradation can be checked by staining the colony or CMC agar plates with different stains [9]. The Gram's Stain was found to be the best stain. After staining with Gram's Iodine the cellulose degrading bacterial colonies showed a clear zone around the colony, which determines the hydrolysis capacity of a particular strain [24,25,26]. The termite species showed a number of bacteria with cellulolytic activity [25,26, 27]The differences in the hydrolysis capacity of these bacteria are because of production of variable amount of enzymes which depends on optimum pH and temperature [28]

The isolated strains of bacteria were *Bacilli* that showed varying degree of cellulolytic activity. Literature reviews also report the presence of diverse cellulolytic *Bacillus* species isolated from termite gut. *Bacillus megaterium* was also reported as a cellulose hydrolyzing bacteria, non- pathogenic host for the biotechnological production of several substances, including vitamin B_{12}, Penicillin acylase and amylase [29],[3],[14]. *Bacillus cereus* has identified as good cellulose degrading potential. It is able to produce Bacteriocin- like inhibitory substances (BLIS) such as Trochicin. *B. licheniformis* strains exhibit antimicrobial activity, *B. coagulans* produces a protease- sensitive peptide a bacteriocins such as coagulin [30]. *B.pumilis* has potential role as a bloodstream pathogen during infancy [31].

CONCLUSION

There is a great diversity of termites in Kurukshetra. They are diverse in terms of their morphology, habitat and microfauna inhabited in their gut. Their bacterial diversity showed various morphology and biochemical characterization. Bacteria obtained from *Coptotermes heimi* (Wasmann) gut were Gram positive and all were *Bacilli*. They showed various degree of cellulolytic activity. It has been observed that the termite gut bacteria are the rich source of many enzymes like cellulase, amylase, protease etc. their potential to hydrolyse cellulose can be used for various purposes in detergent industry, food industry and pharmaceutical industries [24]. The thermostability feature of proteases used in detergent industry, enzyme mediated synthesis, wheat dough rheology and leather dehairing [32,33].The cellulolytic potential of these bacteria can also be used in fermentation, ethanol production and the bio- inoculation of these bacteria may increase the soil fertility by decomposing the organic material. Thus they can reduce the environmental pollution.

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REFERENCES

1. Ohkuma, M. 2003. Termite symbiotic systems: Efficient bio -recycling of lignocellulose. *Appl. Microbiol. Biotechnol.,*61: 1 9.

2. Ghio et al 2012. Isolation of Paenibacillus sp. And Variovorax sp. Strains from decaying woods and characterization of their potential for cellulose deconstruction. *International Journal of Biochemistry and Molecular Biology*3(4): 352-364.

3. Tarun, L.J.R. 2016. Molecular Characterization of cellulose- hydrolysing bacteria isolates from the gut of Philippine Milk termite (*Coptotermes gestroi*). *Asia Pacific Journal of Research*1(37): 136-143.

4. Phuong et al 2015. Isolation of cellulose degrading bacteria from the gut of the termite *Coptotermes gestroi* Asian *Journal of Microbiol. Biotech. Env. Sc.*1(4): 931- 936.

5. Kumar et al 2008. Bioconversion of lignocellulosic biomass: biochemical and molecular perspectives *Journal of Industrial Microbiology and Biotechnology*35: 377-391.

6. Wenzel et al 2002. Aerobic and facultatively anaerobic cellulolytic bacteria from the gut of the termite *Zootermopsis angusticollis* Journal of Applied Microbiology*92*(1): 32-40.

7. Hatami et al 2008. Investigation on Aerobic Cellulolytic Bacteria in some of North Forest and Farming soil American-Eurasian Journal of Agricultural And Environmental Sciences3(5): 713-716.

8. Kakkar et al 2016a. *"Studies on Cellulolytic Activity and Structure of Symbiotic Bacterial Community in OdontotermesparvidensGuts" International Journal of Current Microbiology and Applied Sciences*4(10): pp. 310-315.

9. Kakkaret al 2016 b. Comparison of different stains in determination of extracellular cellulase activity of *Odontotermes obesus* gut bacteria. International Journal of Entomology Research 1(5): 54-56.

10. (Varma et al 1994).

11. Mitchell,P., and Moyle, J. 1954. The Gram Reaction And Cell Composition: Nucleic Acids And Other Phosphate Fractions *J. Gen. Microbiol.*10: 533-540.

12. Carlone et al 1983. Methods for Distinguishing Gram-Positive from Gram- Negative Bacteria Journal of Clinical Microbiology 16(6):1157-1159

13. Roonwal, M.L., and Chhotani, O.B. 1989. Thae Fauna of India and Adjacent Countries Vol I pp. 449-457.

14. Ferbiyanto et al 2015. Characterization and Identification of cellulolytic Bacteria from gut of worker Macrotermes gilvus. HAYATI *Journal of BIOSciences*22: 197-200.

15. Brauman et al 2000. Soil- feeding Termites: Biology, Microbial association and Digestive mechanism *Termites:* Evolution, Sociality, Symbioses, Ecology pp. 233-259.

16. Ji et al 2000. Transformation and Mineralization of synthetic ^{14}C- labeled humic model compounds by soil- feeding, Termites Soil Biology and Biochemistry32(8): 1281-1291.

17. Ji R., and Brune, A. 2001. Transformation and mineralization of ^{14}C- labeled cellulose, peptidoglycans and protein by the soil feeding termite *Cubitermes orthognathus* Biology and Fertility of Soils33: 166-174.

18. Egert et al 2003. Microbial community structure in Midgut and Hindgut of the Humus feeding Larva of *Pachnoda Ephippiata* (Coleoptera: Scarabaeidae) Applied and Environmental Microbiology 9(11): 6659-6668.

19. Hastuti et al 2015. Biodiversity and Enzyme activity of indigenous cellulolytic and amylolytic bacteria in decayed Mangrove stem waste product at Waai SeaShore, Ambon Island *Journal of Agricultural Science and Technology*B5: 252-256.

20. Gohel et al 2014. "A comparative study of various staining techniques for determination of extra cellular cellulase activity on Carboxy Methyl Cellulose (CMC) agar plates" *International*

*Journal of Current Microbiology and Applied Science*3(5):261-266.

21. Sakolvaree, J., and Deevong, P., 2016. Isolation and Characterization of cellulase producingbacteria from the gut of a Higher Termite, *Termes propinquus*. The 5th Burapha University International Conference *"Harmonization of Knowledge towards the Betterment of Society"* 193- 203.

22. Cherry J.R., and Fidantsef A.L. 2003. Direct evolution of Industrial enzymes: An update, *Current Opinion in Biotechnology*14: 438-443.

23. Nandimath et al 2016. Optimization of cellulase production for *Bacillus* sp. And *Pseudomonas* sp. Soil isolates African Journal of Microbiology Research 10(13): 410-419.

24. Liang et al 2014. Isolation, Screening and Identification of Cellulolytic Bacteria from Matural Reserves in the Subtropical Region of China and Optimization of cellulose production by paenibacillus terrae ME27-1. Biomed. Res. Int. Article ID 512497 1-13.

25. Sharma et al 2016. Isolation and Characterization of Cellulose Degrading Bacteria of Termite Gut from North Eastern Region of India South Asian Journal of Experimental Biology5(6): 283-290 (Special Issue)

26. Gupta et al 2012. Isolation of Cellulose-Degrading Bacteria and Determination of Their Cellulolytic Potential International Journal of Microbiology pp 1-5.

27. Dantur et al 2015. Isolation of Cellulolytic bacteria from the intestine of Diatraea saccharalis larvae and evaluation of their capacity to degrade sugarcane biomass. *AMB Express*5:15 1-11.

28. Sreedevi et al 2013. Cellulase producing Bacteria from the wood yards on Kallai River Bank Advances in Microbiology 3: 326- 332.

29. Eppinger et al 2011. Genome sequences of the Bacteriologically Important *Bacillus megaterium* strains QMB1551 and DSM 319 Journal of Bacteriology 193 (16): 4199-4213.

30. Baruzzi et al 2011. Antimicrobial Compounds produced by Bacillus sp. And applications in food. Science against microbial pathogens: Communication current research and technological advances A. Mendez- Vilas (Ed) 1102-1111.

31. Kimouli et al 2012. two cases of severe sepsis caused by *B.pumilis* in neonatal infants Journal of Medical Microbiology61: 596- 599.

32. Shivasharana, C.T., Naik, G.R. 2012. Ecofriendly applications of Thermostable alkaline protease produced from a *Bacillus* sp. JB- 99 under Solid State Fermentation. International Journal of Environmental Science3(3): 956-964.

33. Banerjee et al 1999. Thermostable alkaline protease from Bacillus brevis and its characterization as a laundry detergent additive. Process Biochemistry 35 pp 213- 219.