MOLECULAR IDENTIFICATION OF CELLULOLYTIC BACTERIAL ISOLATES FROM THE GUT OF THE TERMITE *MACROTERMES GILVUS**

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Abstract

The present work was conducted to investigate the identification and phylogenetics of the cellulolytic bacteria from the gut of termite. The termite specimens were collected from termite mounds in campus of University of Yangon and Hlawga Wildlife Park in Yangon Region. Study period was from June, 2018 to December, 2018. Six gram negative bacterial isolates from the termite gut were identified by 16S rRNA gene sequencing. Phylogenetic analysis and BLAST homology and similarity search of GenBank based on mitochondrial 16S rRNA gene sequences indicated that all six were *Pseudomonas putida* (99%-100% homology).

Keywords: 16S rRNA gene, Phylogeny, *Pseudomonas putida*

Introduction

Termites are classified into lower and higher termites, and they contain diverse microbes in their gut. Lower termite has protists and bacteria in their gut, although there is less information about the bacteria. However, higher termites lack protists and contain only prokaryotes (Ohkuma and Brune, 2011). The ability of termites to digest cellulose was collaborated with mutualistic symbiotic action of various microorganisms in the digestive tract (Ohkuma, 2003). Eutick *et al* (1978) stated the ability of termites, especially the caste of workers, in degrading cellulose is supported by the presence of cellulolytic bacteria and other enzymes in the digestive tract of termites. As a higher termite, *Macrotermes gilvus* possesses bacteria in its gut. These bacteria function as a second source of cellulolytic enzymes. The bacteria that have been identified from termite gut belong to the species of aerobes and facultative or strict anaerobes (Ramin *et al*., 2008). There were many previous works on gut of termites regarding their gut microflora and microfauna consisting cellulolytic and non- cellulolytic bacteria, protozoa or protists and fungi whose molecular identification had been done based on 16S rRNA gene sequences compared to related references in Gen Bank through phylogeny and Blast search (Wenzel *et al*., 2002; Mathew *et al*., 2012; Pourramezan *et al*., 2012; Upadhyaya *et al*., 2012; Ferbiyanto *et al*., 2015; Shinde *et al*., 2017). This study focused on molecular phylogeny and identification of the isolated cellulolytic bacteria from the gut of the higher termite *Macrotermes gilvus*, to identify selected cellulolytic bacteria based on 16S ribosomal RNA (rRNA) gene sequences and to determine the phylogenetic and similarity relationship of the cellulolytic bacterial species.

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Materials and Methods

Bacterial isolation

Termites were collected from termite mounds in campus of University of Yangon and Hlawga Wildlife Park in Yangon Region. Termites were washed with distilled water and dried on filter papers. Subsequently the specimens were sterilized externally by 70% ethanol and washed in distilled water for 1 min. Each termite specimen was then put on a glass slide and separated into head and body by a forceps. After removing the heads, the bodies (n= 10) were put into a 10 mL test- tube and crushed with a glass rod and 1mL of sterile distilled water was added into the tube. This mixture was centrifuged at 4000 rpm for 10 min to remove large debris. The supernatant was serially diluted ten fold down to 10^{-5} level. And then 1 mL supernatant of each dilution was transferred to a Petri-dish and 20mL of autoclaved plate count agar (PCA) was added to the supernatant, and mixed by gentle rotation and allowed to solidify according to pour plate method (Dubey and Maheshwari, 2002). The plates were incubated at 37°C overnight. Grown bacteria were purified on Carboxy methyl cellulose (CMC) plate medium. Morphological determination of bacterial colony was based on Handbook of Microbiology (Bisen and Verma, 1998).

Biochemical identification and *in vitro* cellulolytic activity tests of isolates

KB009A HiCarbo™ Kit and KB009C HiCarbo™ Kit (HiMedia, India) were used to determined biochemical reactions for identification of the isolated bacteria. The clear zone called halo zones around colonies against the red color of Congo red indicated positive for cellulolytic activity. Cellulolytic activity was measured as a diameter of clear zone after the CMC plate was poured by 1% congo red reagent. The clear zone sizes, measured in diameters (mm), reflected degree of cellulolytic activity (Lu *et al*., 2004).

Genomic DNA extraction

DNA extraction was done using PureLink® Invitrogen Genomic DNA Mini kit, according to the manufacturer's instructions.

PCR amplification of 16S rRNA gene

The 16S rRNA gene of the bacterial isolates was amplified using universal primers, 20F (5′-AGA GTT TGA TCA TGG CTC -3′) and 1500 R (5′-GGT TAC CTT GTT ACG ACT T-3′) (Weisburg *et al*., 1991). Polymerase chain reaction (PCR) was performed in a Thermal- cycler (Proflex PCR System, ProFlex™ Base Block 33). A total volume of 20µL with mixed components of 10µL of master mix, 1µL of forward primer, 1µL of reverse primer, 2µL of DNA samples, and 6 µL of distilled water were used for PCR amplification. Thermo cycling conditions were set up as follows: initial denaturation at 94°C for 4 min, denaturation at 94°C for 40 sec, annealing and extension at 55°C for 1 min and 72°C for 1 min and 10 sec, respectively, and final extension was carried out at 72°C for 10 min. The whole process was carried out in a total of 35 cycles. The amplified products were fractionated on 1% agarose gel (stained with ethidium bromide) together with DNA ladder (100 bp) as size marker. The PCR products in the gel were visualized with a UV transilluminator and photographs of the gel was taken under UV light as records.

Sequencing PCR of 16S rRNA gene

The amplified 16S rRNA gene fragments were utilized for sequencing reactions in PCR, using the same primers of 20F and 1500R (Weisburg *et al*., 1991). A total volume of 20μL with mixed components of 2μL of 5X sequencing buffer, 4μL of Big dye kit, 1μL of forward/ reverse primer, 6μL of DNA samples, and 7μL of distilled water were used for PCR reaction. The sequencing reaction was carried out in a thermal-cycler (Proflex PCR System, ProFlex^{TM} Base Block 33). PCR conditions were initial denaturation at 96°C for 1 min, denaturation and annealing at 96°C for 10 seconds and 50°C for 5 seconds, respectively. Extension was carried out at 60°C for 4 seconds. The whole process was carried out in a total of 30 cycles.

DNA sequencing

ABI 3500 Genetic Analyzer autosequencer (Applied Biosystems) was used for DNA sequencing. The dried up PCR products were prepared by adding 20μL HiDi formamide, mixing well with pipetting. Subsequently the solution was transferred to the 96 wells plate and set in the autosequencer for sequencing. The sequenced data were downloaded from the ABI 3500 computer onto DVD discs and laptop computer using MEGA 7 software, and the sequences were aligned and analyzed.

Sequence analysis and phylogenetic analysis

Sequence analysis was done on 16S rRNA gene marker. Sequence alignment was performed with the ProSeq software. Then, the forward and reverse sequences of both samples were aligned using pairwise alignment tool in BioEdit. BLAST (Basic Local Alignment Search Tool) was used to search for similarity in the nucleotide sequence database of GenBank.

Phylogenetic analysis trees were constructed using Molecular Evolutionary Genetic Analyses (MEGA-7) software with Maximum-Likelihood (ML) and Neighbor-Joining (NJ) methods at 1000× bootstraps.

DNA extraction, PCR reaction, NanoDrop measurement, Gel electrophoresis, and DNA sequencing and analysis were all conducted in the Molecular Biology Laboratory, Zoology Department at Yangon University.

Results

Morphological and biochemical characteristics of gut cellulolytic bacteria

Six bacteria were isolated from the gut of worker *Macrotermes gilvus*, i.e. isolate code 1ND 7, 1ND 9, 1ND 12, 8ND 2, 8ND 7 and 10ND 3. All bacterial isolates were Gram negative and rod shaped (Plate 1). All bacterial colonies were cream colour and undulated.

All bacterial isolates were motile and showed positive reactions to biochemical assays of citrate utilization, and catalase activity except isolate 1ND 9. They all showed negative reactions to biochemical assays of methyl red, Voges-Proskauer, gas and indole production, urease and gelatinase activities, fructose, raffinose, melibiose, rhamnose, cellobiose, melezitose, α- Methyl-DMannoside, xylitol and sorbose tests. However, isolates 8ND2, 8ND 7 and 10ND 3 showed positive reactions to biochemical assays of xylose and mannose tests.

Results of congo- red assay for cellulolytic activity

Congo- red assay of all the isolates was done *in vitro* to determine their cellulolytic activity. The isolates that digest cellulose as 1% CMC in the media, produces clear zones. The diameter of clear zones were measured which corresponded to the cellulose digesting ability of the respective isolates. Cellulolytic activity test showed that 10ND3 isolate had the largest cellulolytic index (3.28) and 8ND 7 isolate had the smallest cellulolytic index (2.14) (Plate 2, Table 1).

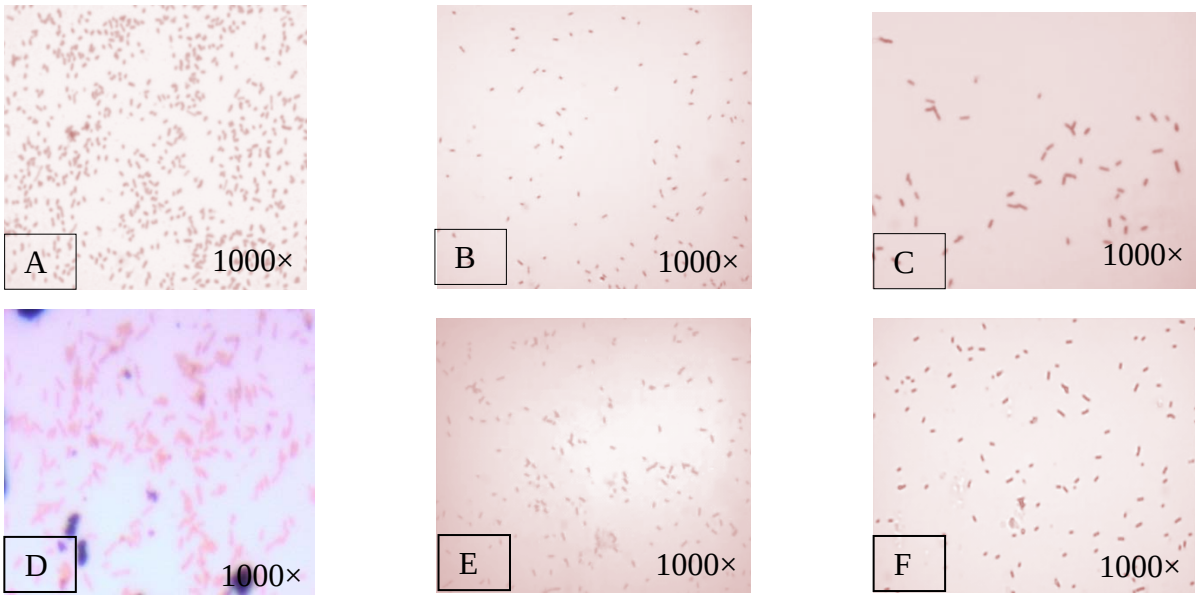

Plate 1 Gram staining of isolated cellulolytic gut bacteria. (A) 1ND 7, (B) 1ND 9, (C) 1ND 12, (D) 8ND 2, (E) 8ND 7 and (F) 10ND 3 bacterial isolates

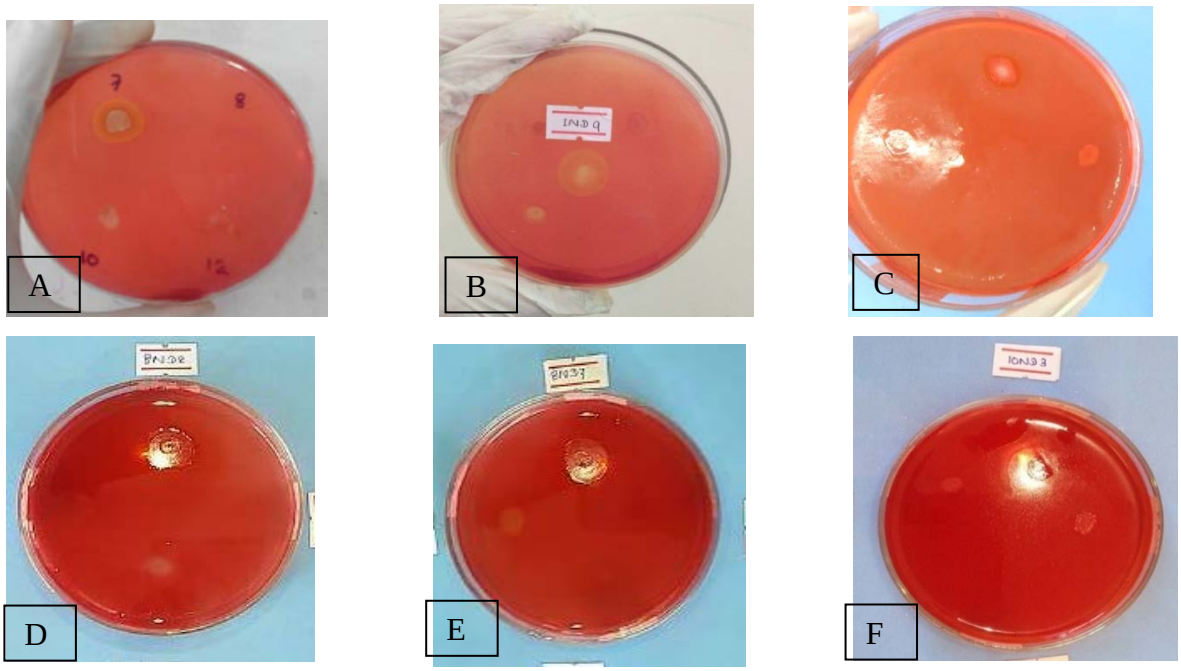

Plate 2 Clear zones are seen against the red colour of Congo red for the positive cellulolytic test. (A) 1ND 7, (B) 1ND 9, (C) 1ND 12, (D) 8ND 2, (E) 8ND 7 and (F) 10ND 3 bacterial isolates

Table 1 Cellulolytic index of the isolated gut bacteria

Sr. No.	Isolate code	Isolate species (Tentative ID)	Zone diameter (Zd) (mm)	Colony diameter (Cd) (mm)	Clear zone diameter (mm)	Ratio Zd/Cd
1.	1ND7	*Pseudomonas putida*	21	9	12	2.33
2.	1ND9	*Pseudomonas putida*	17	6	11	2.83
3.	1ND12	*Pseudomonas putida*	16	7	9	2.28
4.	8ND2	*Pseudomonas putida*	22	7	15	3.14
5.	8ND7	*Pseudomonas putida*	15	7	8	2.14*
6.	10ND3	*Pseudomonas putida*	23	7	16	3.28**

(**) = Highest, (*) = Lowest activity index

BLAST sequence similarity search for cellulolytic bacterial isolates

The similarity search of reference sequences in GenBank for isolate identification confirmed the cellulolytic isolates 1ND7, 1ND9, 1ND12, 8ND2, 8ND7 and 10ND3 as *Pseudomonas putida* (Genus ID similarity of 100%; Species ID similarity of 99% - 100%).

Table 2 Gen Bank BLAST search results for molecular similarity for identification

Sr. No.	Isolate code	Species (Confirmed ID)	Genus Identification (%)	Species Identification (%)
1.	1ND7	*Pseudomonas putida*	100%	99%
2.	1ND9	*Pseudomonas putida*	100%	100%
3.	1ND12	*Pseudomonas putida*	100%	100%
4.	8ND2	*Pseudomonas putida*	100%	100%
5.	8ND7	*Pseudomonas putida*	100%	100%
6.	10ND3	*Pseudomonas putida*	100%	100%

16S rRNA gene, 630 bp alignment (Gram negative)

Phylogenetic relationship of the gram- negative isolates obtained from the termite

ML tree analysis of six identified cellulolytic Gram- negative isolates (1ND9, 8ND7, 8ND2, 1ND12, 10ND3 and 1ND7) revealed all six isolates formed a distinct cluster (99% confidence) in a clade (100% confidence) also containing a different cluster of reference *Pseudomonas putida* in ML tree (Figure 1). Likewise, similar results were also found in NJ tree (Figure 2). The results for ML and NJ tree indicated the six isolates of the present study to be *Pseudomonas putida*. The Gram-positive out group of reference *Bacillus* spp. and *Cellulomonas* spp. diverged clearly indicating the six isolates to be Gram- negative.

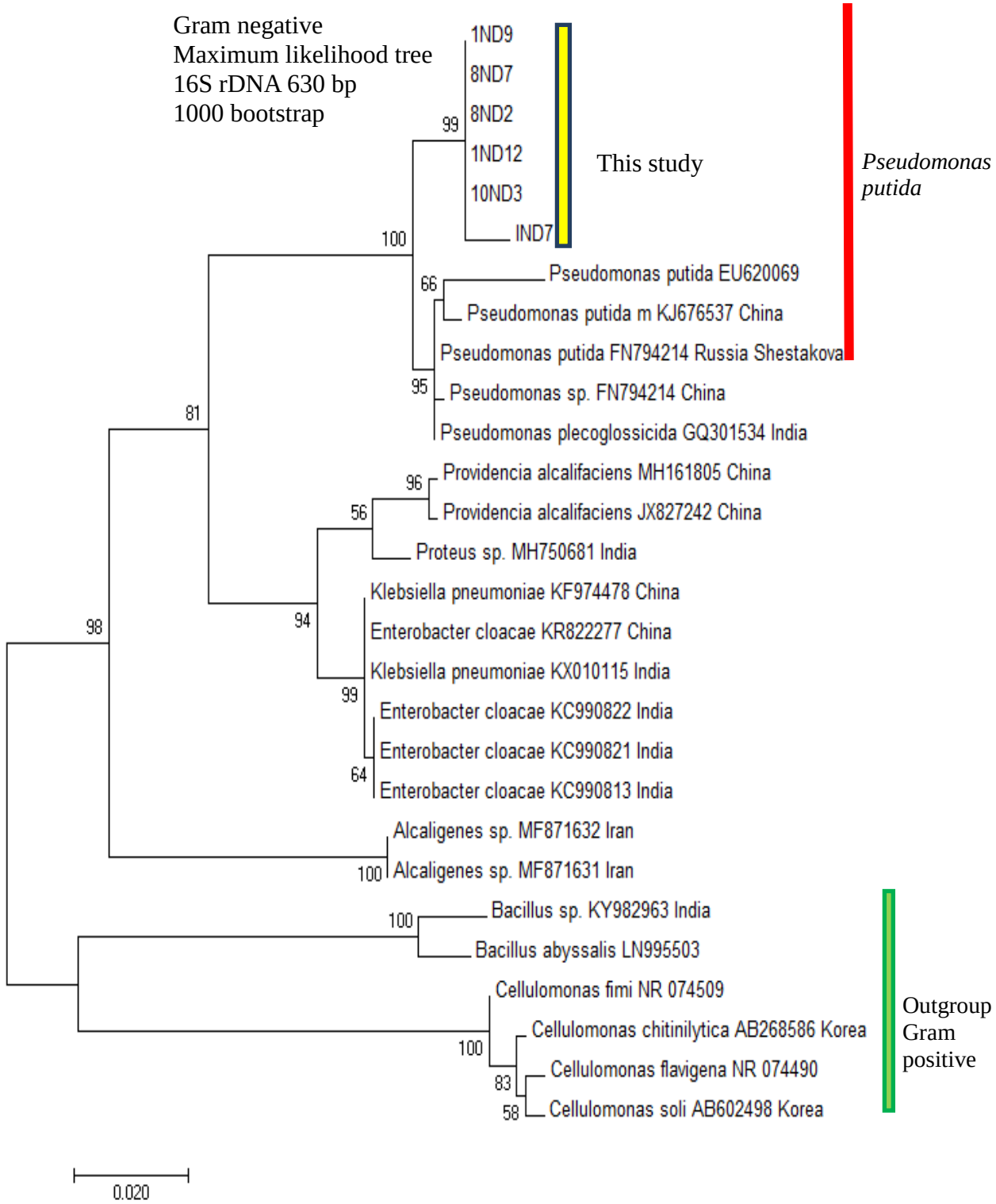

Figure 1 Molecular phylogenetic analysis by Maximum likelihood method for cellulolytic Gram negative isolates 1ND9, 8ND7, 8ND2, 1ND12, 10ND3 and 1ND7 using 630 aligned basepairs. Scale bar indicate nucleotide mutation/ site/ m yr.

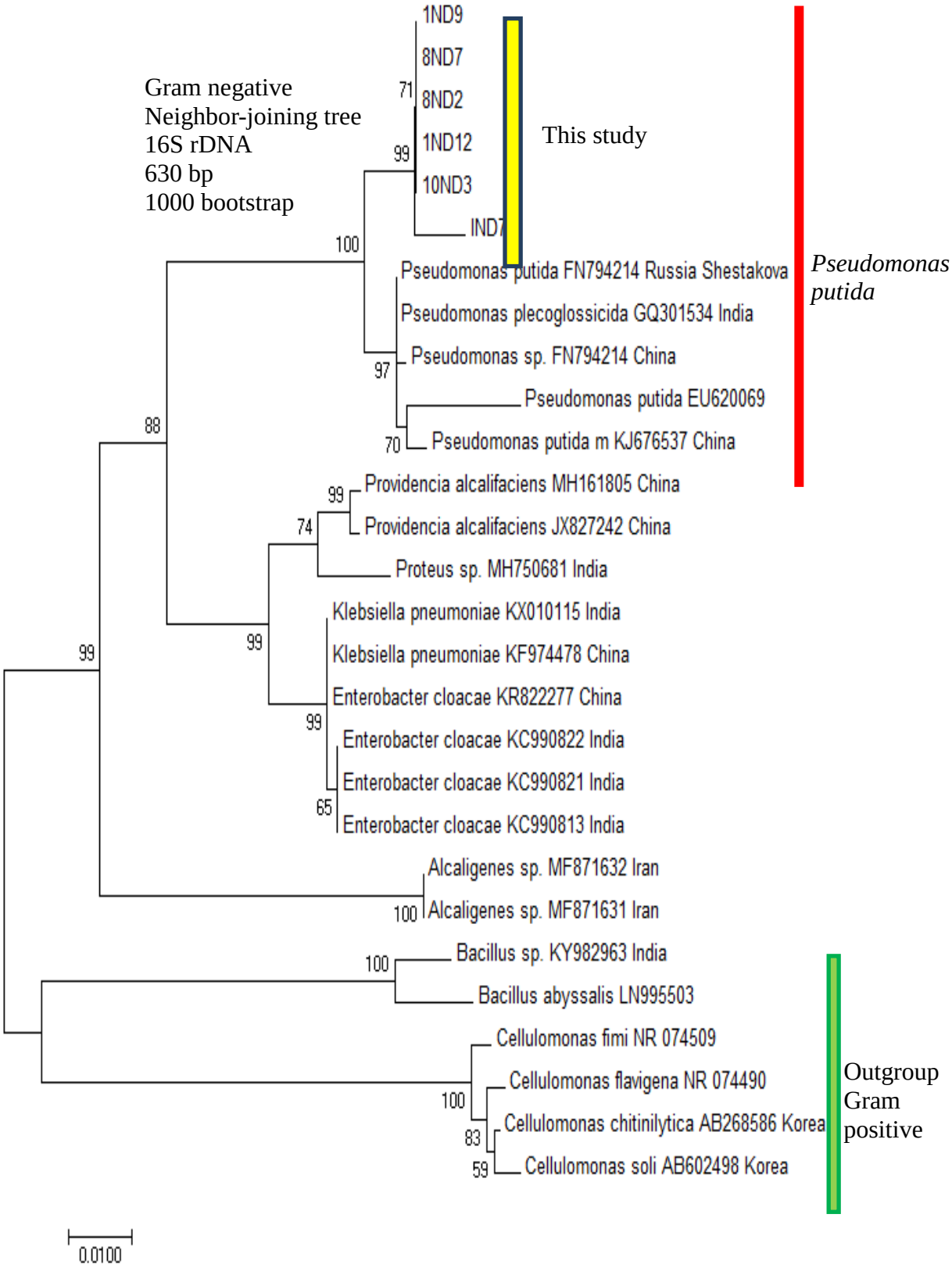

Figure 2 Molecular phylogenetic analysis by Neighbor-joining method for cellulolytic Gram negative isolates 1ND9, 8ND7, 8ND2, 1ND12, 10ND3 and 1ND7 using 630 aligned basepairs. Scale bar indicate nucleotide mutation/ site/ m yr.

Discussions and Conclusion

Termites thrive in great abundance in the terrestrial habitats by recycling cellulose from plants and wood. Termites have their own cellulolytic mechanism, but also harbor gut microbes which assist them to degrade cellulose (Upadhyaya *et al*., 2012).

The presence of discoloration of congo red around the colony on CMC agar indicates the cellulase activity of the bacteria (Lu *et al*, 2004). The diameter of clear zone is a measure of degree of cellulose digesting ability of bacterial strains (Upadhyaya *et al.*, 2012). The cellulase activity of the isolates can be known by measuring the ratio of the total zone diameter to colony diameter on the agar plate.

Regarding *in vitro* cellulolytic activity, highest activity was found in *Pseudomonas putida* (isolate code 10ND3) with Zd/Cd ratio of 3.28 and *Pseudomonas putida* isolate (8ND7) showed the lowest cellulolytic activity at ratio of 2.14. Out of these 15 only 2 bacterial isolates showed zone diametre to colony diametre ratio (Zd/Cd) below 2.0. Out of 15 isolates, 11 isolates showed cellulolytic activity in the range of Zd/Cd ratio 2.0 - 3.0 and only 2 which were showing highest activity above 3.0 (Nidhi Kakkar, 2015). Our finding was similar to the previous study.

The molecular identification was done for the six gram negative cellulolytic bacteria isolates from termite gut. Based on molecular identification of 16S rRNA gene, isolates 1ND9, 1ND12, 8ND2, 8ND7 and 10ND3 had 100% similarity with *Pseudomonas putida* and 1ND7 has 99% similarity with *Pseudomonas putida*.

According to Weisburg *et al* (1991) the amplification of 16S rRNA gene sequences with primer 20F and 1500R showed 1500bp DNA amplicons. In this study, the results revealed that, the primers could successfully isolate 16S rRNA gene but only partially (~850bp) with only 630bp successful alignment. Phylogenetic and Blast similarity analyses of the 16S rRNA gene sequences revealed the presence or occurrence gut bacteria *Pseudomonas* sp. and *Bacillus* sp. in *Macrotermes* sp. termite (Muwawa *et al*., 2016); *Bacillus megaterium* and *Pseudomonas spingomonas* in the termite *Zootermopsis angusticollu* (Wenzel *et al*., 2002); *Bacillus megaterium* in *Macrotermes gilvus* (Ferbiyanto *et al*., 2015).

The results of the present phylogenetic analysis indicated isolates 1ND7, 1ND9, 1ND12, 8ND2, 8ND7 and 10ND3 were closely clustered with reference *Pseudomonas putida.* Some members of the genus *Pseudomonas*, such as *P. fluorescens*, have been reported to produce cellulase enzymes (Hall, 1995). *Pseudomonas putida* was reported to be found in the gut of termites based on 16S rRNA gene phylogenetic and Blast analyses in India (Shinde *et al.*, 2017).

Species such as termites and crayfish produce their own cellulases that are different from those produced by their indigenous microflora (Ohkuma, 2003).

The results of the present study, it is hoped, would contributed to application of cellulase producing bacteria in various industries like production of fruit juices, biofuels, detergents, alcohols and other fermentation technologies.

Hence, the data generated in the present study could serve as a basis for further microbiological and molecular phylogenetic research on cellulolytic gut bacteria and their various species of termite host.

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References

Bisen, P. S., and Verma, K., (1998). *Handbook of Microbiology*. CBS Publishers and Distributors. Dehli.

Dubey, R. C., and Mahenshwari, D. K., (2002). *Practical Microbiology*. S. Chandand Company Ltd. Ram Nagar, New Delhi- 110 055.

Eutick, M., O'Brien, R., Slaytor, M., (1978). Bacteria from the gut of Australian termites. *Appll. and Environ. Microbiol*. 35 (5): 823-828.

Ferbiyanto, A., Rusmana, I., Raffiudui, R., (2015). Characterization and identification of cellulolytic bacteria from gut of worker *Macrotermes gilvus*. HAYATI *J. Biosciences*. 22: 197-200.

Kakkar, N., Gupta, S.K., and Saharan, B.S., (2015). Studies on Cellulolytic Activity and Structure of Symbiotic Bacterial Community in *Odontotermes parvidens* Guts. *Int. J. Curr. Microbiol. App. Sci.* 4(10): 310-315.

Lu,W. J., Wang, H. T., Nie, Y. F., Wang, Z.C., Huang D.Y. and Chen J.C., (2004). Effect of inoculating flower stalks and vegetable waste with lignocellulolytic microorganisms on the composting process. *Journal of Environmental Science and Health, Part B*. 39(5-6), 871–887.

Muwawa, E. M., Budambula, L. M., Osiemo, Z. L., Boga, H. I., and Makonde, H. M., (2016). Isolation and characterization of some gut microbial symbionts from fungus- cultivating termites (*Macrotermes* and *Odontotermes* spp.). Vol.10(26), 994-1004.

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

Ohkuma, M., Brune, A., (2011). Diversity, structure, and evolution of the termite gut microbial community. *Biology of Termites: A Moderns Synthesis* (Bignell DE, Roisin Y and Lo N, eds), 413- 438. Springer-Verlag, Berlin.

Pourramezan, Z., Ghezelbash, G. R., Romani, B., Ziaei, S., and Hedayatkhah, A., (2012). Screening and identification of newly isolated cellulose degrading bacteria from the gut of xylophagous termite *Microcerotermes diversus* (Silvestri). *Microbiol*. 81 (6): 736-742.

Ramin,M., Alimon, A. R., and Abdullah, N., (2008). Identification of cellulolytic bacteria isolated from the termite *Coptotermes curvignathus* (Holmgren). *Journal of Rapid Methods & Automation in Microbiology*. 17, 103–116.

Shinde, V. S., Agrawal, T., Kotasthane, A. S., (2017). Molecular characterization of cellulolytic bacteria derived from termite gut and optimization of cellulase production. *Int. J. Curr. Microbiol. App. Sci*. 6(10): 2474-2492.

Upadhyaya, S. K., Manandhar, A., Mainali, H., Pokhrel, A. R., Rijal, A., Pradhan, B., Koirala, B., (2012). Isolation and characterization of cellulolytic bacteria from gut of termite. *Rentech Symposium Compendium*, Vol. 1, 14-18.