MadrasAgric.J.,2024; https://doi.org/10.29321/MAJ.10.300011

RESEARCH ARTICLE

The Microbiotic Environment of the Termite Gut *Odontotermes Wallonensis*: Morphological and Biochemical Assessment

Santhoshkumar M 1 , Premalatha K 2 *, Prithiva J N 1 , Sushruta Boruah 1 and Devindra Gundakanal 1

1 Department of Agricultural Entomology, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India- 641003.

* 2 Department of Forage Crops, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India- 641003

Received: 18 May 2024

Revised: 29 May 2024

Accepted: 11 Jun 2024

ABSTRACT

The dissected gut from worker termites of *Odontotermes wallonensis* in a specific medium was incubated, and the microbial colonies were purified for morphological and biochemical studies. There were ten purified isolates, and they were predominantly regular in shape rather than irregular. The color of the isolates was dull yellow, cream, pink and white. Smooth texture was dominant among the isolates. The isolates responded differently to varied biochemical tests. Among the ten purified microbial isolates, all were found to be gram-positive, anaerobic, and not producing hydrogen cyanide except the ninth isolate, which showed positive results on the HCN test. The utilization of nutritional source also differed with isolates. Gut microflora of the termite (*O. wallonensis*) was also characterized using 16S rRNA gene amplification, yielding DNA fragments of 1300-1500 bp. The sequences aligned with major bacterial phyla *i.e* Actinobacteria, Firmicutes, Planctomycetes, Bacteroidetes, and Proteobacteria.

Keywords : *Termites, Gut Microbes, Biochemical Test, Isolates, Nutritional Source.*

INTRODUCTION

Termites are predominant invertebrate fauna of pedolith, and they live a well-organized social life. They are the only social insects comprises king, apart from queen, workers and soldiers. Though they are reported to be an ecological engineer responsible for the formation of macroaggregates in soil and the decomposition of litter in the soil ecosystem, they are also the major pests of crops and wooden structures. Their ability to digest cellulose enables them to feed on many kinds of cellulosic materials. The symbiotic microbes present in the termite hindgut play a vital role in the biochemical fragmentation of cellulose fed by the termites. Termites are recently classified under the order Blattodea which also consists of wood eating cockroaches. The termite families Mastotermitidae, Termopsidae, Hodotermitidae, Kalotermitidae, Serritermitidae, and Rhinotermitidae have them, and Rhinotermitidae has a population of lower termites. The higher termites are of Termitidae, which has widely occurring species. The flagellates occupy the guts of lower termites, whereas it is absent in higher termites. The life of termites depends on gut symbionts, which can starve the termites in their absence. The gut microflora is diverse with termite species, and it depends on chance circumstances. Hence, this investigation was carried out to know the microbial diversification of termites which may be considered as a part of developing novel termite management strategies.

MATERIALS AND METHODS

Isolation of termite gut bacteria

The selective medium of 5g/L Carboxy Methyl Cellulose and 0.1g/L CaCo3 (pH 6.7) was used to isolate gut microbes of *O. wallonensis* and the culture was incubated for four weeks at 30°C. Then agar plates with medium were streaked with 1 mL of the culture and incubated for 24 hrs at 30°C. The single colonies were obtained and purified for subsequent studies.

*Corresponding author's e-mail:

Identification of termite gut bacteria

Morphological characterization

The shape, texture, margin elevation and colour of the isolates were observed under a light microscope as described by Shirling and Gottlieb (1966).

Biochemical characterization

Gram-type staining was done on 48-hour-old cultures of bacterial isolates colony by the standard staining technique (Hucker and Conn, 1987). A pure isolate colony was made on a clean glass slide, dried in air, and fixed by passing through the flame of a burner. The smear was stained with crystal violet, subsequently by gram iodine and safranin and water rinsing after each staining.

Simmons citrate agar test was carried out with the pure colonies of termite gut bacteria which were inoculated on nutrient agar for eighteen hours (Ayitso and Onyango, 2016). Then streaked using a sterilized inoculating loop on the surface of the simmons citrate agar plate. The plates were then incubated at 30°C for 48 hrs.

Nutrient agar containing 0.2 per cent soluble starch was used for starch hydrolysis test. Test cultures were spotted on the Petri plates and incubated for 48hrs. Then Lugol's iodine solution was added and a colourless zone around the bacterial growth in contrast to the blue background of the medium was observed as positive reaction (Schaad, 1992).

To identify the anaerobic microbial growth, bacterial cultures were inoculated into a tube containing glucose broth and incubated in an anaerobic jar. Alternatively, the broth was overlaid with sterile mineral oil and incubated at 24ºC to observe the bacterial growth (Schaad, 1992).

The methyl red test broth (peptone 7g, dextrose 5g, potassium phosphate 5 g and distilled water 1000 mL) was prepared, dispensed in test tube and autoclaved at 15 lb pressure for 15 minutes. The gut microbes were inoculated in test tube and incubated at 35° C for 48 hrs. Then five drops of methyl red was added and colour change was observed after five minutes (Aneja, 1993).

The ability of microbes to utilize carbon compounds was tested by inoculation of bacterial culture on tryptic soya agar medium. Filter paper disc of 1.5 cm diameter was soaked in picric acid solution (picric acid 2.5 g, Na2CO3 12.5 g and distilled water one litre) and placed in the upper lid of each Petri dish (Miller and Higgins, 1970). Dishes were sealed with parafilm and incubated for four days. Hydrogen cyanide (HCN) production was assessed by the presence of a coloured zone around the bacteria and the yellow colour of the filter paper turning brown to reddish brown.

The ability of microbes to utilize carbon compounds was tested by using seven carbon sources *viz.,* dextrose, fructose, lactose, carboxyl methyl cellulose, manitol, sucrose and D-maltose which were separately incorporated at one per cent level and starch at 0.2 percent level into the basal medium consisting of 1.0g ammonium di-hydrogen phosphate, 0.2g potassium chloride, 0.2g magnesium sulphate and one litre distilled water and finally the pH was adjusted to 7.0. Durham's fermentation tube was filled with the medium, dropped into a test tube each containing eight mL of the medium with the bacterial isolate, incubated for seven days at room temperature of 28±2°C and examined for growth.All the tests un-inoculated tubes served as control (Schaad, 1992).

DNA Extraction from termite gut bacterial isolates

The termite gut bacteria were cultured in nutrient agar broth medium containing 5g of peptone, 3g beef extract, 5g Nacl and 5g dextrose. Total DNA was prepared with the HiPurA™ bacterial Genomic DNA Purification Kit (Mumbai-india).

PCR amplification of bacterial 16SrRNA genes

The PCR primers used to selectively amplify the bacterial 16S rRNA gene were 41F (5'-GCTCAGATTGAACGCTGGCG-3') and 1389R (5'-ACGGGCGGTGTGTACAAG-3') (Hongoh, 2003). The amplification reaction mixture (30 µL) contained 2.4 ng of total DNA, 3.0 µL of 10×PCR buffer, 2.4 µL of dNTP mixture (2.5 mmol/L), 0.15µL of Taq DNA polymerase, 0.3 µL of each primer, and sterilized distilled water. The reaction was performed as follows: initial denaturation at 94°C for 5 min, 26 cycles of 94°C for 30 s, 50°C for 1 min, and 72°C for 1.5 min, and a final extension at 72°C for 10 min. The amplified PCR products in agrose gel were visualized with a UV trans-illuminator and photographed using gel documentation system.

RESULT AND DISCUSSION

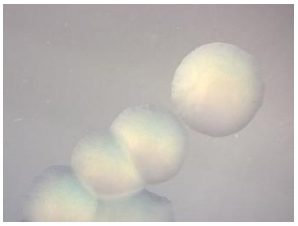
The culturing of termite gut microbes in a specific medium resulted in ten isolates. The morphological

characters of microbes revealed that the isolates IS1, IS3, IS5, IS7, and IS10 showed regular shapes whereas IS2, IS4, IS8, and IS9 were irregular. Dull white colour was observed in colonies of IS1, IS7 and IS10 and cream colour was in IS3, IS5, IS6 and IS8. IS2 and IS4 shown white and dull white colours respectively and the pink colour colonies were found in IS9. Smooth and flat texture was observed in almost all the isolates. Rough texture and raised texture was found in IS 2 &4 and IS2 & IS9 respectively (Plate No. 1).

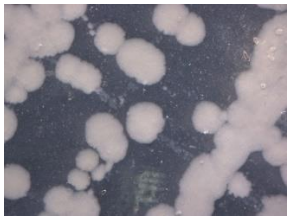
The biochemical characteristics of ten isolates showed that all the ten isolates were gram-positive and anaerobic (Fig. 1). The gram-positive bacterium was observed in *Macrotermes michaelseni* (Ntabo *et al*., 2012). The report on anaerobic bacteria of formosan termite was given by Adams and Boopathy (2009). Facultative anaerobes also reported from subterranean termites by Azizi-Shotorkhoft *et al*. (2016). The symbiotic bacterial community in *O. parvidens* gut isolates, which showed pink colour pigmentation, was studied by Kakkar *et al.* (2015).

Plate No. 1.Different morphological characters of gut bacterial isolates in *O. wallonensis*

Isolate 1 IS 1

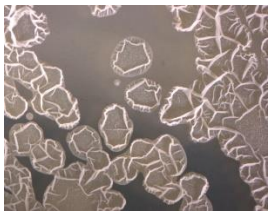


Isolate 2 IS 2



Isolate 3 IS 3

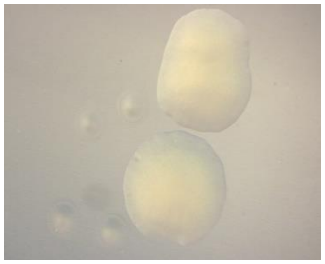
Isolate 4 IS 4



Isolate 5 IS 5

Isolate 6 IS 6

Isolate 7 IS 7

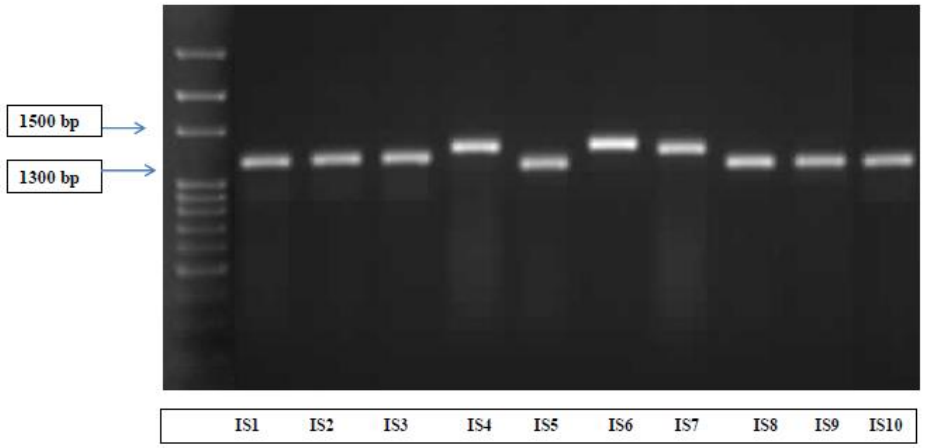


Isolate 8 IS 8

Isolate 9 IS 9

Isolate 10 IS 10

Plate No. 2. Laboratory experiment on molecular characterization of termite (O. wallonensis: Termitidae, Isoptera) gut bacterial isolates

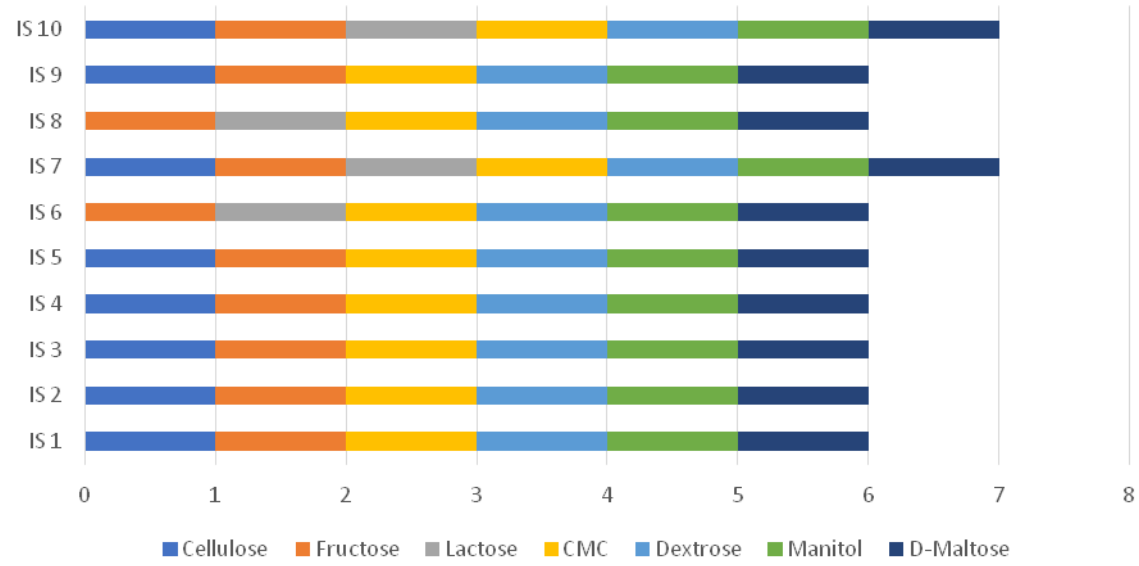


The positive reaction of the methyl red test shows the glucose utilization of microbes which were of IS1, IS2, IS5, IS8, IS9, and IS10. Similarly starch hydrolysis test was positive in the colonies of IS1, IS2, IS4, IS7 and IS9. The isolates IS1, IS3, IS5, IS6, IS7, IS8, and IS10 showed a positive reaction to Simmons citrate test, which indicated the ingestion of citrate by the microbes. The bacterial isolates utilizing citrate source may be considered as *Salmonella* sp. (Muwawa *et al*., 2016) which falls in protobacteria. The citrate utilization of microbes of *O. formosanus* was reported by Kavitha *et al.* (2014). Though most of the isolates react positively to the tests, HCN production was observed only in IS9. The fructose, CMC, dextrose, mannitol, and D maltose were taken by all the ten isolates, whereas cellulose was taken by IS1, IS2, IS3, IS4, IS5, IS7, and IS10. Lactose was utilized only by the isolates of IS6, IS7, IS8, and IS10 (Fig. 2). Nutritional source differences based on the geographical distribution of termites cause microbial difference (Husseneder *et al*., 2005).

Isolate IS5 and IS10 were similar in all morphological and biochemical characters except in colour and lactose compound utilization. IS 1 and IS 5 may be of Actinobacteria group which are cellulolytic bacteria earlier reported by Paul *et al.* (1993). Cellulolytic activities from few species of termites report was also given by Muwawa *et al.* (2016) who reported the microbiota of *Macrotermes* and *Odontotermes* gut. The cellulolytic activity of gut microbes was recorded in in *O. parvidens* (Kakkar *et al.,* 2015).

Figure1. Biochemical Characterization of termite gut microbiota *O. wallonensis*

Figure 2.Utilization of different carbon sources by termite gut microbes of *O.wallonensis*



The PCR products subjected to Agrosegel electrophoresis resulted in the amplification of DNA fragments ranging from 1300-1500 bp. Termites harbor a symbiotic gut microbial community that is responsible for their ability to thrive on recalcitrant plant matter. Based on molecular identification of gut microflora of termite (*O. wallonensis*) by using 16S rRNA gene amplification with primer 41F and 1389R showed that 1300- 1500 bp DNA amplicons (Plate No. 2). All fragment range grouped into one of five major bacterial phyla: Actinobacteria, Firmicutes, Planctomycetes, Bacteroidetes and Proteobacteria. The results are in similar with the findings of Zhu *et al.* (2012) who studied the phylogenetic analysis of the gut bacterial microflora of the fungus-growing termite *Macrotermes barneyi* by using amplification of 16S rRNA gene with primer 41F and 1389R and the result showed that many of the clones were derived from three phyla within the domain bacteria: Bacteroidetes, Firmicutes and Proteobacteria. In addition, a few clones derived from Deferribacteres, Actinobacteria and Planctomycetes were also found.

CONCLUSION

The goal of the study was to isolate and characterize *O. wallonensis* gut bacteria with an emphasis on the diverse microbes associated with termite digestion.

The isolation process generated ten distinct isolates, exhibiting diverse morphological features and biochemical characteristics. All isolates were identified as gram-positive and anaerobic, consistent with previous findings on termite gut microbiota. Particularly, the diverse utilization of carbon sources highlights the metabolic flexibility of these microbes, chiefly in cellulose digestion. DNA extracted from termite gut bacterial isolates showed diverse 16S rRNA gene fragments, aligning with major bacterial phyla typical of termite symbionts. These outcomes support current studies on termite gut microflora, emphasizing Actinobacteria, Firmicutes, Planctomycetes, Bacteroidetes, and Proteobacteria as significant contributors to termite digestion.

REFERENCES

Adams, L. and Boopathy, R. 2005. Isolation and characterization of enteric bacteria from the hindgut of Formosan termite. *Bioresour. Technol.*, **96**: 1592 – 1598. doi: 10.1016/j. biortech.2004.12.020. Epub 2005 Feb 26.

ANeja, K.R. 1993. Experiments in microbiology, plant pathology and tissue culture. Wishwa prakashan, New Delhi. Ayisto, A. S. and Onyango, D. M. 2016. Isolation and Identification by morphological and biochemical methods of antibiotic producing microorganisms from the gut of *Macrotermes michaelseni* in Maseno, Kenya. *J. Appl. Biol. Biotechnol*. **4**: 27-33. DOI:10.7324/JABB.2016.40105

Azizi-Shotorkhoft, A., Mohammadabadi, T., Motamedi, H., Chaji, M. and Fazaeli, H. 2016. Isolation and identification of termite gut symbiotic bacteria with lignocellulose-degrading potential,

and their effects on the nutritive value for ruminants of some by-products. *Anim. Feed Sci. Technol.*, **221**, 234-242. https://doi.org/10.1016/j.anifeedsci.2016.04.016

Hongoh, Y., Ohkuma, M. and Kudo, T. (2003). Molecular analysis of bacterial microbiota in the gut of the termite *Reticulitermes speratus* (Isoptera: Rhinotermitidae). *FEMS Microbiol. Ecol.*, **44**(2): 231-242. https://doi.org/10.1016/S0168-6496(03)00026-6

Husseneder, C.L.A.U.D.I.A., Wise, B.R. and Higashiguchi, D.T. 2005. Microbial diversity in the termite gut: a complementary approach combining culture and culture-independent techniques. Pp 189-195. in Chow-Yang Lee & William HR. (eds), *Proceedings of 5th International Conference on Urban Pests*, Perniagaan Ph'ng @ P&Y Design Network, Malaysia. https://www.icup.org.uk/media/4ipjeerf/icup030.pdf

Hucker, G. J. and Conn, H. J. 1987. Method of Gram staining. *N. Y. State Agric. Exp. Stn. Tech. Bull.*, **93**: 3 – 37. https://hdl.handle.net/1813/30787

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. Appl. Sci.,* **4**: 310 – 315. http://www.ijcmas.com/

Kavitha, D., Vijayarani, K. and Kumanan, K. 2014. 16S rRNA typing of cellulolytic bacteria from the termite, *Odontotermes formosanus*. *IJVASR*, **43**: 359 – 368. http://www.tanuvas.tn.nic.in/ijvasr/vol43(5)/359-368.pdf

Miller, R.L. and Higgins, V.J. 1970. Association of cyanide with infection of bird's food trefoil by Stemphylium rot. *Phytopathology*, **60**: 104 – 110. doi:10.1094/Phyto-60-104

Muwawa, E.M., Budambula, N.L., 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.). *Afr. J. Microbiol. Res.*, **10**: 994 – 1004. https://doi.org/10.5897/AJMR2016.8060

Ntabo, R., Boga, H., Muigai, A. and Mwirichia, R. 2012. Isolation and characterization of bacteria isolates from soil feeding termites and soil from Juja and Kakamega forest in Kenya. Pp 5-18 in *Proceedings of scientific technological and industrialization conference*. 17-19th November 2010, Jomo Kenyatta University of Agriculture and Technology, Kenya. http://journals.jkuat.ac.ke/index.php/jscp/article/view/675

Paul, J., Saxena, S. and Varma, A. 1993. Ultrastructural studies of the termite (*Odontoterme sobesus*) gut miclofora and its cellulolytic properties. *World J. Microbiol. Biotechnol.*, **9**: 108-112. doi: 10.1007/BF00656529.

Schaad, N. W. 1992. Laboratory guide for identification of plant pathogenic bacteria. *International book distributing*. Co., Lucknow, 44 – 58. https://www.sciepub.com/reference/186042Shirling, E. B. and Gottlieb, D. 1966. Methods of characterization of *Streptomyces sp. Int. J. Syst. Bacteriol.*, 61: 313 – 340. https://doi.org/10.1099/00207713-16-3-313

Zhu YunHua, Z.Y., Li Jian, L.J., Liu HuHu, L.H., Yang Hui, Y.H., Xin Sheng, X.S., Zhao Fei, Z.F., Zhang XueJia, Z.X., Tian Yun, T.Y. and Lu XiangYang, L.X., 2012. Phylogenetic analysis of the gut bacterial microflora of the fungus-growing termite *Macrotermes barneyi*. *Afr. J.Microbiol. Res.*, 2071-2078. DOI: 10.5897/AJMR11.1345