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Eubacterial Diversity and Oxalate Metabolizing Bacterial Species (OMBS) Reflect Oxalate Metabolism Potential in *Odontotermes* Gut

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Oxalates are toxic secondary metabolites found in a variety of plant taxa and are consumed by numerous organisms. Termites have a heterogeneous food habit and consume oxalate producing plants. Intestinal microbes have been reported to be instrumental in metabolizing oxalate in mammals. The present study dwells on the idea that termite gut microbiota plays crucial role in detoxifying oxalate compounds. To implore upon this hypothesis, we investigated gut eubacterial community structure of *Odontotermes* species through constructing and analyzing 16S rRNA gene clone library. Total of twenty four bacterial genera belonging to ten bacterial phyla were detected. In addition, three bacterial isolates having oxalate metabolizing ability were isolated through enrichment that belonged to *Citrobacter* species (OX_T1) and two *Rhizobium* species (OX_T2 & OX_T3), these isolate showed promising potential for oxalate metabolism. Further, metabolic prediction using PICRUSt, illustrated a variety of genes participating in the oxalate degradation pathways in termite gut. The present study attempts to catalogue *Odontotermes* gut microbiome and their plausible role in oxalate degradation and detoxification.

Keywords: Termite gut, bacterial diversity, oxalate metabolism.

Animals, plants and other living forms lack the ability to oxidize oxalate and therefore, oxalate acts as toxin to most forms of life, especially herbivores[1]. Hence, the presence of oxalate in food material is considered as anti-nutritional factor. The major source of oxalate in mammals including humans is plant based food habit. In Plant kingdom, oxalate is a common photosynthetic by-product[2] and many plants are known to synthesize calcium oxalate crystals which are stored in leaves and organ cells like idioblasts[3]. Such oxalogenic plants accumulate calcium oxalate crystals in the range of 3 to 80% (w/w) that accounts as much as 90% of the total calcium of a plant body[4]. Stored calcium oxalate plays an important role in calcium regulation and protection against herbivory[3]. Since mammals lack the ability to oxidize oxalate, they have to depend on certain bacteria that can metabolize oxalate and overcome their effects[5, 6].

Termites like *Odontotermes* are known to specifically feed on diet comprising of wood or grass, due to this heterogeneous food habits, termites often consume reasonable amount of plant

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derived oxalate. This accumulated oxalate in termite gut is toxic and affects renal tubular cells at supraphysiologic concentrations, hence oxalate is a part of chemotherapy as anti-termite dose[7]. Oxalate clearance from the mammalian gut is mainly achieved by microbes present in the gut which have capacity to metabolize and/or transform oxalate into detoxified compounds[1]. Such microbes are often termed as oxalate metabolizing bacterial species (OMBS) or oxalotrophic bacteria which are the major players in oxalate-carbonate pathway and their presence in various ecological niches indicate the significance of such pathways[8].

The oxalate-carbonate pathway demonstrates the bioconversion of toxic oxalate to carbonate molecules by biological actions and contributes to the major event in carbon cycle, importantly in terrestrial habitat. Since oxalate metabolizing bacteria thrive in a variety of ecological niches and perform the detoxification events, they have been previously isolated from various aquatic, terrestrial and gastro-intestinal habitats. The gastro-intestinal environment of termite is one such habitat that may be harbouring oxalate metabolizing bacteria. According to Pearce (1997)[9], termites play an important role in changing soil structure by the removal of herbaceous matter (up to 100%) in terrestrial habitat. Metabolic processes in termite gut ranges from digestion of organic food content, methane emission to xenobiotic detoxification. It has been proven that such metabolic processes in the termite gut are carried out by gut inhabiting bacteria[10].

In the view of described possibility, we hypothesize that the oxalate detoxification in termites is driven by gut bacteria and may act as potential contributor in termite gut ecology (Fig. 1). To address this hypothesis, we first characterized the termite gut total bacterial diversity by amplification and sequencing of 16S rRNA gene clone library that was subsequently used to estimate the abundance of oxalate metabolizing genes by deriving metagenome analysis by imputation. Then oxalotrophic bacteria from termite gut were enriched, isolated and identified by culture dependent approach. Further, oxalotrophic potential of the isolated bacteria was tested by estimating their ability to metabolize oxalate *in vitro*.

MATERIALS AND METHODS

Collection of termites

A single colony of wood feeding termite was collected in sterile polypropylene containers from botanical garden near National Centre for Cell Science, Pune, Maharashtra, India (Latitude: 18°342 N, Longitude: 73°582 E). From colony, worker-caste termites were identified, selected and used for further studies. For the molecular identification of termite, cytochrome oxidase I (COI) gene with HCO (5' TAAACTTCAGGGTGACCAAAAAATCA 3') and LCO (5' GGTCAACAAATCAT AAAGA TATTGG 3') primers was amplified, sequenced and BLASTn searched. Briefly, homogenate was prepared by crushing termite legs and used for isolation of host genomic DNA. The host genomic DNA was then used as template in PCR reaction for amplification and sequencing of cytochrome oxidase I (COI) gene. For isolation of oxalotrophic bacteria, the identified termites (n=8) were surface sterilized by washing with 70% ethanol (v/v) followed by thorough washing with sterile double distilled water for 4-5 times. The surface sterilized termites were then microscopically dissected under sterile condition to separate guts and other body parts. The dissected gut portions were homogenised by crushing in sterile plastic homogenizer and used for enrichment of oxalotrophs and isolation of total bacterial community genomic DNA.

16S rRNA gene clone library construction and analysis

The total bacterial community structure and composition in termite gut was analyzed by constructing and analyzing 16S rRNA gene clone library. Briefly, 16S rRNA gene was amplified from community DNA using a set of universal primers[11] 8F (5' AGAGTTTGATCCTGGCTCAG 3') and 907R (5' CCGTCAATTCCTTTRAGTTT 3') (Frank et al. 2009), then purified by gel elution using Gene Elute Gel Extraction Kit (Sigma-Aldrich, St Louis USA), ligated into pCR4® TOPO vector supplied with the TOPO TA cloning kit (Invitrogen, San Diego, USA) and transformed into One Shot TOPO10 electro competent cells of E. coli (Invitrogen, San Diego, USA) following the manufacturer's instructions. Sterile LB agar with 50 µg/ml (w/v) of kanamycin was used for selection of the transformed cells, which were incubated for 16 h at

37°C. M13F (5' GTAAAACGACGGCCAGT 3') and M13R (5' GCGGATAACAATTTCACACAGG 3') primers were used for screening and sequencing of the clones. The bidirectional sequencing was performed on ABI 3730 XL DNA analyser (Applied Biosystems Inc, USA) using the ABI Big-Dye terminator version 3.1 sequencing kit as per the manufacturer's instructions. The forward and reverse sequence reads were assembled and contigs were obtained using ChromasPro software (www.technelysium.com.au/ChromasPro.html). All sequences were checked for chimeric artefacts by Mallard[12] program, using *E. coli* U000096 as a reference sequence. The sequences were aligned using CLUSTAL X2[13] followed by manual sequence editing for removal of ambiguous sequences using DAMBE[14]. These sequences were then pooled into single FASTA file and analyzed using QIIME software (Quantitative Insight into Microbial Ecology) version 1.8.0 as described earlier[15]. Briefly, UCLUST[16] was used to cluster sequences to obtain OTUs at 97% similarity. Representative sequence from each OTU group was selected, aligned using PyNAST[17] and OTU sequences were assigned to taxonomy using RDP classifier. Finally, OTU table was generated by tabulating the number of times an OTU was found with its taxonomic identification.

Putative oxalotrophic gene abundance in predictive metagenome

For inferring the predictive metagenome of bacterial community, we utilized a computational approach: PICRUSt (phylogenetic investigation of communities by reconstruction of unobserved states) [18]. For PICRUSt, reference based OTU picking was performed within QIIME environment and resulting *.biom file was utilized for functional gene predictions using online tool http://huttenhower.sph.harvard.edu/galaxy/. Functional gene predictions were made using KEGG Orthology (KO) database with default settings and resulting data was used for identification of oxalate bioconversion pathways present in termite gut microbiome.

Enrichment, isolation and identification of oxalotrophic bacteria

For enrichment of oxalotrophic bacteria, method described by Sahin et al (2004) [19] was used with modifications. Briefly, 200 µl of gut homogenate was inoculated in 50 ml Nutrient Broth supplemented with 0.5% sodium oxalate (w/v) and incubated aerobically at 28 ºC for 72 h. After 72 h, enriched culture was serially diluted up to 10^{-3} dilutions and 20 µl of aliquots from each dilution were spread on Schegels Mineral agar media supplemented with 200 mM sodium oxalate and 1 g/L (w/v) calcium chloride. The cultures were incubated at 28 ºC for 72 h. The oxalotrophic bacteria were identified and selected based on their ability to degrade calcium oxalate precipitate in the medium. This was determined by measuring the clear zone of degradation around colonies. Positive cultures were re-streaked till pure cultures were obtained, then preserved and treated as oxalotrophic bacteria. For molecular identification of oxalotrophic pure cultures, genomic DNAs were extracted from pure cultures and used for amplification and sequencing of 16S rRNA gene described earlier[20]. Briefly, 16S rRNA gene was amplified using universal bacteria-specific primers pair 27F (5' AGAGTTTGATCMTGGCTCAG 3') and 1492R (5' TACGGYTACCTTGTTACGACTT 3'). The 16S rRNA gene sequences from oxalotrophic isolates were assembled and identified using eztaxon server at http://www.ezbiocloud.net/eztaxon. The evolutionary history (tree) was inferred using the Neighbour Joining method. The percentage of replicate trees in which the associated taxa were clustered together in the bootstrap test (1,000 replicates) is recorded next to the branches. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree using Molecular Evolutionary Genetics Analysis (MEGA, V. 5) software[21].

Estimation of oxalate metabolising potentials

In vitro oxalate utilizing activity of isolates was determined by titrometric estimation using potassium permanganate ($KMnO_4$) method described by Justice KE (1985) [22] with modifications. Briefly, 100 ml Schegels mineral broth supplemented with 200 mM sodium oxalate in 250 ml capacity flasks was inoculated with each isolates and subjected for growth. The cultures were incubated aerobically at 28 ºC on a rotary shaker operating at 120 rpm. The oxalate utilizing potentials of each isolate was estimated at every 24 h intervals till 120 h. Oxalate content in each sample were precipitated by the addition of 4 g/L $CaCl_2$, centrifuged and then obtained precipitate

was dissolve in deionized water. It was then titrated with 6 N H_2SO_4 at 70 to 90 °C temperature till pink colour developed. Oxalate-degrading capacity over the time of incubation was estimated by the standard graph of different percentage solution of oxalate with positive and reagent control titrations.

RESULTS

Host termite identification

In the present study, wood feeding termites were collected from natural terrestrial ecosystem and identified by both morphological characteristics and sequencing of mitochondrial cytochrome oxidase I (COI) gene. The analysis of morphological data and COI sequence indicated that the termites used in this study were *Odontotermes* species. The COI sequence from termites was deposited in NCBI GenBank database with accession- KR003406.

Total bacterial diversity in wood feeding termite

Approximately 300 clones were randomly picked and sequenced of which 237 reads were longer than 750 base pairs and satisfied all the quality criterions. Total of 33 OTUs were obtained that could be assigned to seven bacterial phyla namely Actinobacteria, Bacteroidetes, Firmicutes, Planctomycetes, Proteobacteria, Spirochaetes and Synergistetes with twenty four bacterial genera (Fig. 2a). Among the observed phyla, members of Bacteroidetes were predominant and accounted for 37.5% of the total numbers of OTUs followed by members of phylum Firmicutes that accounted for 6.2% of total numbers of OTUs observed. Among the members of various observed genera, members of genus *Dysgonomonas* were most dominants and accounted for 16.9% followed by members of *Treponema* that accounted for 8 % (Fig. 2b). 16S rRNA amplicons were deposited at NCBI GenBank (KF257128 - KF257363).

Putative bacterial oxalotrophic gene abundance in bacterial diversity

Using PICRUSt, we inferred KEGG Orthologs (KOs) from termite gut microbiota at 3 hierarchal levels. From these KOs, we were able to identify five different oxalate degrading pathways in termite gut microbiota leading to formation of varied by-products such as formate, carbon dioxide and hydrogen peroxide (Fig. 3). High abundance of some these KOs suggest that termite gut has

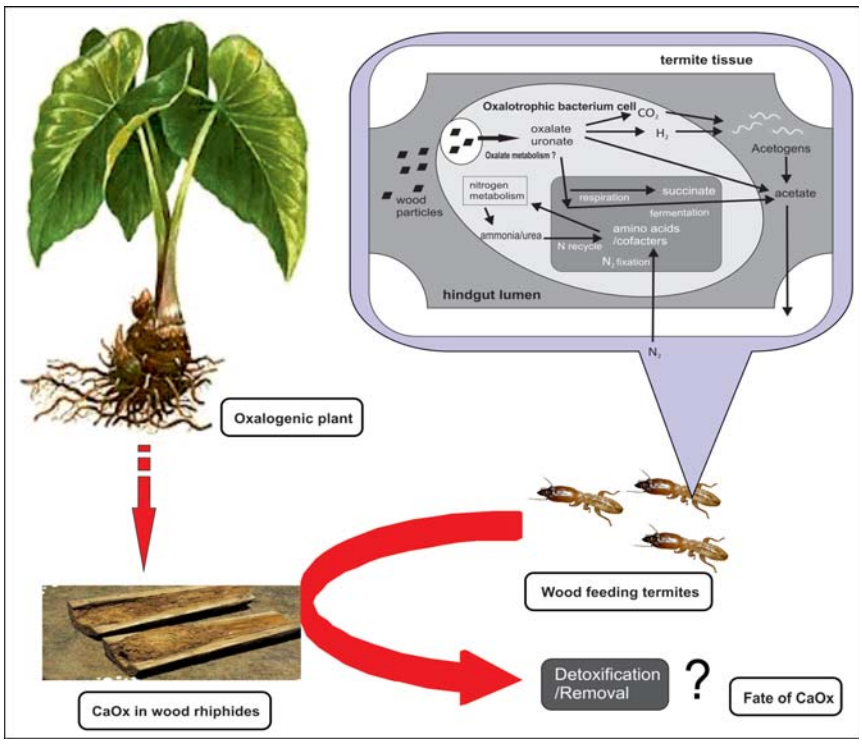

Fig. 1. Proposed mechanism of microbe mediated degradation in termite gut

acquired varied microbial lineages responsible for oxalate metabolism (Fig. 4).

Isolation and identification of Oxalotrophic bacteria

When oxalotrophic bacterial community from termite gut homogenate was enriched, serially diluted and plated, the total viable count of oxalotrophic bacteria varied in the range 9.1 x 10^4 to 4.7 x 10^5 cfu/ml. Based on visual inspection of colony morphologies, initially three oxalotrophic bacteria were selected and purified on plate containing mineral media with calcium oxalate as a sole carbon source. The NCBI BLASTn dependant search of 16S rRNA gene sequences from those

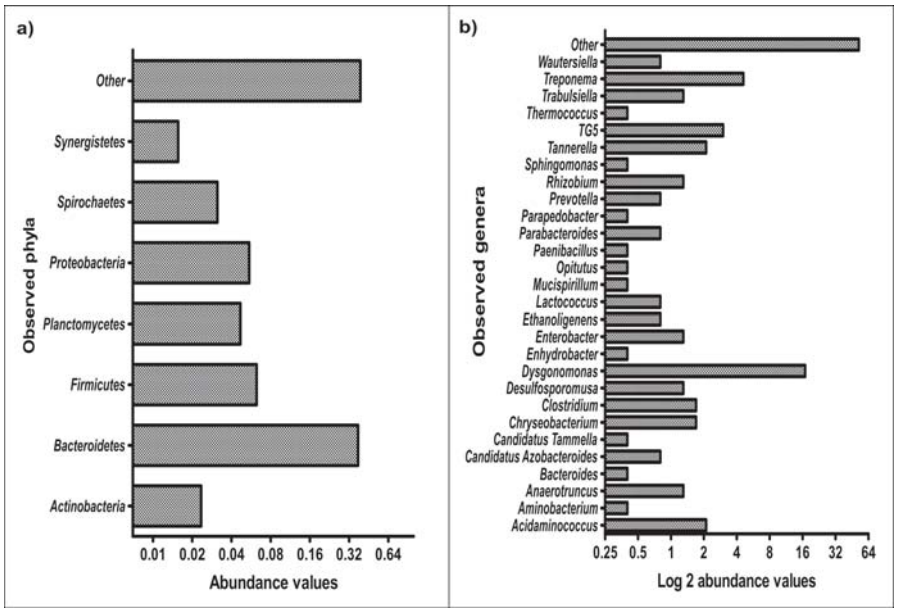

Fig. 2. Bacterial diversity of wood-feeding termite (Odontotermes sp.) gut (a) Phylum level abundance (b) Genus level abundance

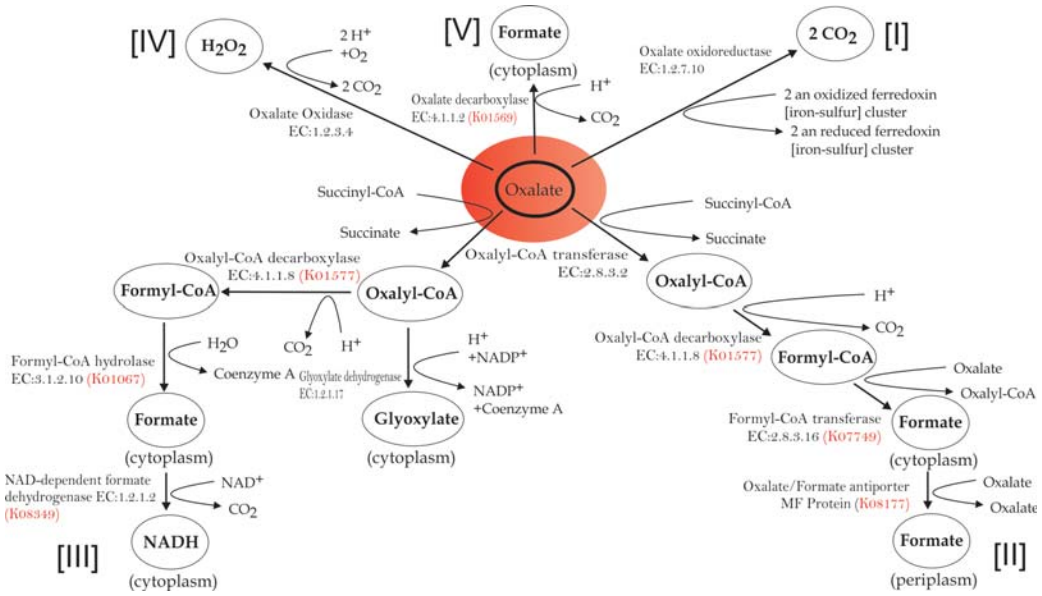

Fig. 3. Putative oxalate degradation pathways derived from the enzymes obtained from KEGG Orthology Maps

isolates and analysis of phylogenetic tree (Fig. 5) indicated that all three isolates were the members of Proteobacteria and two genera namely *Citrobacter* and *Rhizobium*. The isolate OX_T1 was identified as *Citrobacter* species whereas isolates OX_T2 and OX_T3, both were identified as *Rhizobium* species. These three isolates were able to utilize calcium oxalate as a sole carbon source as evident from their ability to grow when they were repeatedly streaked on calcium oxalate as a sole carbon source in selected mineral media.

Oxalate metabolising potentials of oxalotrophic isolates

For each of the three isolates obtained, *in-vitro* oxalate utilization ability was tested over the incubation period of 120 hrs. Steadily increased oxalate metabolism was observed in case of all three isolates, isolate OX_T1 was found most promising and utilized maximum 14.8 mM oxalate after 120 h of incubation, whereas isolates OX_T2 and OX_T3

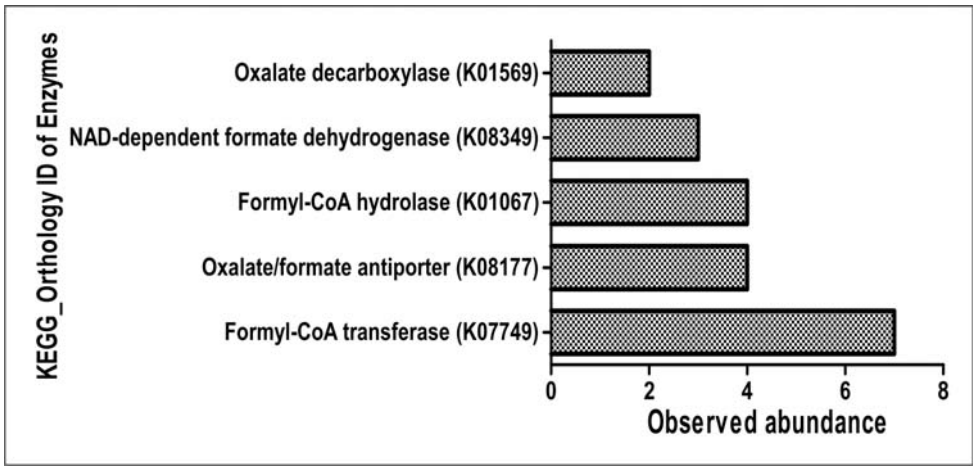

Fig. 4. Observed abundance of enzymes involved in oxalate metabolism in termite gut as obtained through KEGG Orthology

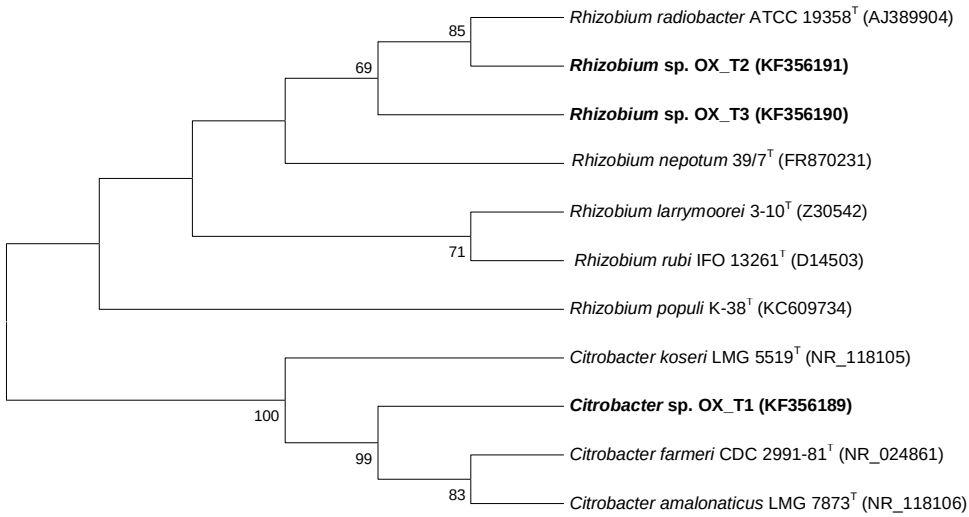

Fig. 5. Taxonomic position of Oxalate Metabolizing Bacterial Species (OMBS) isolates from termite gut. Topology of phylogenetic tree inferred using Neighbor-Joining with Kimura 2- parameter method. Whereas total of each 16S rRNA gene sequence 1339 bp were used with 1000 replicates in bootstrap test for analyses in MEGA5

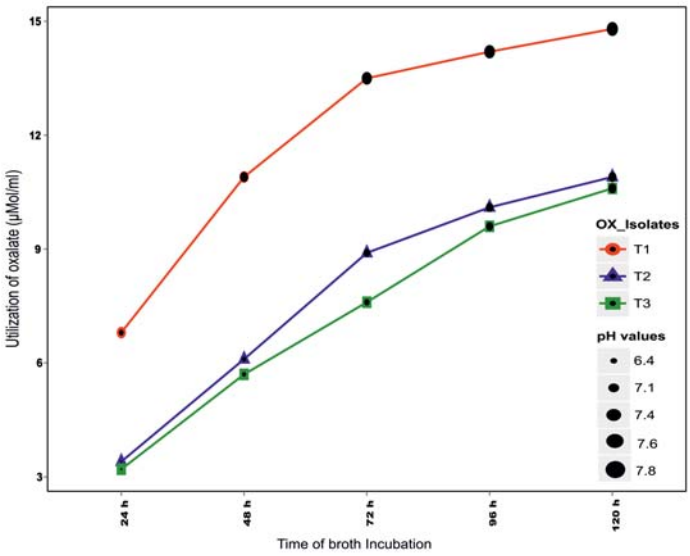

Fig. 6. Oxalate utilization potential through time of three oxalotrophic bacterial isolates obtained from wood feeding termite Odontotermes sp. gut

utilized maximum of 10.9 mM and 10.6 mM of oxalate respectively. In this experiment, we also monitored the change in pH of the medium over the incubation period of 120 h which revealed the increase in pH from 6.3 (at 25 h) to 7.8 (at 120 h). The increase in the pH of the medium clearly indicated the formation of carbonate as a consequence of utilization of oxalate. These results clearly confirmed the potential of the three isolates for the removal of oxalate from tested media with increase in pH (Fig. 6)

DISCUSSION

Wood-feeding termites have long been documented for their enormous influence on the decomposition of plant matters. However, factors playing role in oxalate degradation/metabolism within this system remains yet to be elucidated. The present study attempts to identify microbial key players in oxalate degradation in termite guts.

On terrestrial habitat, oxalogenic plants not only accumulate oxalate which cannot be catabolized and used for energy production but also make oxalate toxic to most forms of life, especially herbivorous mammals[1]. Consumption of such oxalogenic plant, especially by humans results in an accumulation of oxalic acid that leads to number of pathologic conditions such as hyperoxaluria, urolithiasis and sometime renal failure[23]. Pathological consequences were demonstrated with oxalate toxicity on renal epithelial cells, with unregulated gene expression and with necrotizing events on excretory systems.

The community structure of the wood feeding termite *Odontotermes* was illustrated by investigating the 16S rRNA genes from a PCR-based clone library. The termite gut microbiota was found to be dominated by the members of Bacteroidetes followed by Firmicutes. The two phyla have been found to be dominant in fungus feeding termite, *Odontotermes* spp. gut microflora[24, 25]. Different physiological conditions in arthropods' gut such as pH, redox potential and temperature determine distribution of microflora and thus provide a suitable niche for various microbial activity[26]. A recent study by Miller and co-workers[27] showed presence of OMBS consortia including genera like *Enterococcus*, *Lactococcus* and *Clostridium* in Woodrat gut. Presence of similar genera in termite gut in the present study, further strengthen the fact that arthopods gut (such as termites) is enriched with oxalate metabolizing microbes. Whereas such oxalotrophic bacteria

hypothesised to have the oxalate detoxification for Varroa destructor mites in host survival issues[28].

In the present study, *Citrobacter* species and *Rhizobium* species were obtained as the two most efficient oxalotrophs from the *Odontotermes* gut microflora. *Citrobacter* species has been observed to colonize in oxalate containing kidney stone nidus[29] (Okeke 2011) whereas, *Rhizobium*, a soil inhabiting diazotroph has also been associated with oxalate metabolism[30, 31]. This was further confirmed by *in vitro* assays for oxalate metabolising potentials of these oxalotrophic isolates. The results clearly indicated that isolates obtained in this study indeed had ability to metabolize oxalate and might be playing an important role in removal or detoxification of oxalate in the termite gut. Interestingly, absence of *Oxalobacter* in termite gut which is a predominant oxalate degrader present in human gut[32] suggest that oxalate degrading ability is widespread in microbial world and we hypotheses that host type might be playing an important role in selecting these microbes in their gut. Previously, the presence of these oxalotrophic bacteria clearly indicated the existence of oxalate- carbonate pathway in termite gut environment[33]. The utilization of oxalate leads to formation of carbonates that subsequently elevate the pH in termite gut[8]. In congruence with this fact, we also observed the increase in pH of the medium when three isolates were tested for their in vitro potential to utilize oxalate.

Several studies in bacteria have reported the presence of oxalate-metabolizing proteins such as Oxalate antiport transport protein (OxIT), Formyl CoA transferase (FRC) Oxalate decarboxylase (OXDC), oxalyl CoA decarboxylase (OXC) and oxalate oxidoreductase (OOR) [34,35,36,37]. The presence of these proteins and genes encoding them make bacteria capable of metabolizing oxalate. Therefore, harbouring these genes may act as the marker for oxalate carbonate pathway in prescribed niche, whereas the presence of Formyl CoA transferase (FRC) gene indicates the active involvement of observed bacteria in oxalate metabolism[33]. The presence of oxalate metabolizing genes and their bacterial origin indicates that oxalate detoxification termite gut may be driven by gut inhabitant bacterial flora. PICRUSt analysis for determining the putative bacterial oxalotrophic gene abundance, illustrated gene families associated with the known oxalate degradation pathways. However, the gene families identified in the present study corroborated to oxalate degradation pathways II, III and V.

Oxalate degradation has been a topic of interest for nephrologists as oxalate accumulation in kidney leads to kidney stones. Recent studies[38] have advocated the use of oxalate degrading microbes as probiotics to facilitate oxalate metabolism in the gut and subsequent management of the kidney stone diseases. Hence, it is imperative to identify prospective microbial candidates for oxalate degradation in the gut. The present study also demonstrates the potential of *Citrobacter* and *Rhizobium* in oxalate degradation.

Present study catalogues the microbial diversity in termite gut and explores its possible role in oxalate metabolism. The study suggests existence of oxalate metabolising proteins in termites gut microbiota which are part of oxalate-carbonate pathway. Hence, provides the new avenues for isolation of oxalate metabolizing microbes and their potential use in medicine.

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