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Identification and Preliminary Characterization of Enteric Bacteria of Termite *Odontotermes obesus* (Rambur) from Tea Gardens of Darjeeling Foothills

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A B S T R A C T

Eight facultative anaerobic bacteria were isolated from the gut of termite, *Odentotermes obesus* (Rambur). The isolates were initially identified as *Streptococcus, Pseudomonas* and *Enterobacter* sp. by using selective media. Study established their role in cellulose degradation. The isolates E3A, E3D of *Enterobactor*, S2B, S2Dand S2F of *Streptococcus* showed positive growth on cellulose Congo-Red agar media. Cellulose degrading potential of the isolates was qualitatively estimated by calculating their hydrolyzing capacity. The total cellulose activity on filter paper was found to be highest with 0.410 IU/ml for E3D and the lowest was 0.250 IU/ml for S2F.

***Key words*:** Termites, Enteric bacteria, *Odontotermis obesus*, Darjeeling foothills, Tea plantation

Tea [*Camellia sinensis* (L.) O. Kuntze] is an evergreen plant that is cultivated in tropical and sub-tropical climates for its leaves, which are dried, fermented, processed and consumed as much-loved beverage. In fact, after water, tea is the second most consumed beverage in the world [1]. In North East India, tea is planted in the Brahmaputra and Barak Valleys of Assam, plains of the Dooars and Terai and Darjeeling hills in northern part of West Bengal. In Brahmaputra Valley, it is planted in plain lands at elevations ranging from 50 to 120 m above msl [2]. Tea is grown as a perennial monoculture crop hence attracts a large array of arthropod pests leading to a substantial crop loss. According to Hazarika *et al*. [3], globally there are 1031 arthropod species associated with tea, out of which 300 species of insect pests are recorded from India and specifically 167 species from North-East India [4]. Pests which attack the shoot system of tea bush deserve more attention because of their perceptible and permanent damage symptoms. Therefore, acting against them with appropriate control measures is imperative. There are pests like termites or cockchafer grubs who often surreptitiously attack tea plants underground without facing any trouble from planters. Termites are dominant soil dwelling animals and play a significant role in tropical terrestrial ecosystems, especially in the decomposition process [5-8]. Termites are diverse in their feeding habits that lead to diverse microbiotas. They play an important role in maintaining carbon balance in nature and in the turnover and mineralization of complex biopolymers. Biopolymers like cellulose, hemicellulose and lignin primarily form the rich source of nutrients for termites [9]. However, for digesting these complex compounds these tiny bio-reactors / digestors require help of even tinier symbionts.

There are two groups of termites – old world termites and new world termites. First group harbor flagellate protozoans and bacteria for cellulose metabolism and mainly consists of all other families of termites except Termitidae; whereas, members of Termitidae (New World Termites) have only bacteria as symbiont for cellulose digestion [10-13]. To digest cellulose, termites have cellulose enzyme which in turn are provided by termite symbionts [14]. Cellulose is a linear polymer of glucose linked by β-1,4-glycosidic linkages. Cellulase which consists of various enzymes such as endoglucanase, exoglucanase and β-glucosidase, hydrolysaes the linkages present in cellulose [15]. The gut environment is in anaerobic condition as such it supports anaerobic microbes [16-18]. But some of the studies had also shown that the gut can contain aerobic and facultative anaerobic bacteria that help in scavenging the oxygen that may permeate to the gut and therefore effectively keep the gut environment anaerobic [19]. Such a gut environment also helps in the hydrolyzing of cellulosic biomass [20]. Cellulases enzyme have diversity of applications [21-22]. However, the major drawback of cellulase in industry is the high cost of the enzyme production. Therefore, much cost reduction may be possible by exploring the microorganisms that produce cellulolytic enzymes. It is therefore necessary to look for

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microorganisms that have a high rate of cellulase production [23].

Although, cellulase research has been focused mostly in fungi but cellulase production by bacteria are also catching eyes of researchers [24]. Bacteria which have a high growth than fungi are potential candidates to be used in cellulase production. Though, bacterial cellulases are more effective catalysts. However, bacteria are not widely used for cellulase production. Isolation of a novel microbial strain, having higher productivity of cellulase makes the process economically viable for industries [25].

The gut microbiota enables termites to efficiently hydrolyze cellulose. The cellulase activity of termite hindgut is attributed to cellulose degrading bacteria [26].

Earlier investigations have suggested that the gut microbes selectively help in cellulose degradation. Only a few cellulose-degrading bacteria could be isolated and identified from some termite species, such as *Clostridium termitidis* [27], *Micromonospora propionici* and *Clostridium sp*. [28], *Streptomyces sp*. and *Micromonospora sp*. [29], *Staphylococcus saprophyticus* [30], *Micrococcus luteus* and *Micrococcus roseus* [31], *Micromonospora acetiformici* [32], *Arthrobacter* sp. [33] out of many attempts [34-37].

Based on the above information, the objective of the present study was to isolate, identify and characterize enteric bacteria from the gut of termite, *Odontotermes obesus* (Rambur) (Fig 1) occurring in the sub-Himalayan terai-dooars tea plantations and their role in cellulose decomposition. Cellulose degrading potential of the positive isolates was also qualitatively estimated by calculating hydrolysis capacity.

Fig 1 Commonly occurring castes (forms) and a fungal garden found inside the mound of *Odototermes obesus*

MATERIALS AND METHODS

Specimen collection and isolation of enteric bacteria

Termites (*O. obesus*) were collected from the tea garden of Darjeeling Himalayan foothills. They were maintained with their own fungal garden material in buckets in BOD incubators at temperature 26 °C ± 2 °C and relative humidity 75± 5%. The termites (worker caste) were surface sterilized [38] and dissected in a sterile condition. The 30µl of macerated gut solution of termite was inoculated in the three selective media viz. MacConkey agar, Pseudomonas agar and Streptococcus agar. The cultures were incubated for 2-3 days in an incubator at 37 °C.

Characterization of the bacterial isolates

The isolates were grown overnight in nutrient broth and serial dilutions were made and then were plated and incubated at 37 °C for 48 hours. The single colonies of each isolate were selected for further microscopic observation and identification using Gram's staining, determining the generation time [39].

Gram stain

Smear of each bacterium was taken on clean glass slide and was allowed to air-dry and then heat fixed. Smear was gently flooded with crystal violet and was let to stand for 1 minute. It was gently washed with tap water and was counterstained with safranin for 45 seconds, followed by gentle wash with tap water and subsequent blotting it dries with filter paper for microscopic examination under oil immersion.

Biochemical tests

The biochemical tests such as starch hydrolysis was conducted as per the methods described by Cowan and Steel [40]. Additional biochemical tests were performed by using the Hi25™ Enterobacteriaceae Identification Kit (Himedia, KB003-20KT). The results were compared with Bergey's Manual of Determinative Bacteria [41].

Screening of cellulose degrading bacteria among the isolates

The cellulose degrading ability of the bacterial isolates were performed by streaking on the cellulose Congo-Red agar clearing zone assay. The zone of cellulose hydrolysis can be seen as a clear area [42-43]. The ratio of the clear zoneto colony diameter was measured in order to select with highest cellulase activity [44].

Enzyme production

The isolates were cultured in a shaker incubator at 37 °C at 150rpm in an enzyme production media containing Whatman filter paper No.1 (1x6 cm strip, 0.05 g per 20mL) and pH 6.8–7.2]. Broth culture after three days of incubation period was subjected to centrifugation at 5000 rpm for 15 min at 4 °C. Supernatant was collected and stored as crude enzyme preparation at 4 °C for further enzyme assays. Pellet recovered after centrifugation of broth culture was subjected to gravimetric analysis in order to determine the residual cellulose of filter paper [45].

Enzyme assay

Total cellulose activity was determined by measuring the amount of reducing sugar formed from CMC or filter paper through DNS (dinitrosalicylic acid) method. Endoglucanase activity was determined by incubating 0.5mL of supernatant with 0.5mL of 2% amorphous cellulose in 0.05m sodium citrate buffer (pH 4.8) at 50 °C for 30 min. After incubation for an hour at 50 °C, the reaction was terminated by adding 3mL of 3, 5-dinitrosalicylic acid (DNS) reagent to 1mL of reaction mixture. In the test, reducing sugars were estimated spectrophotometrically with 3, 5-dinitrosalicylic acid [46] using glucose as standards. The enzymatic activity of endoglucanase was defined in international units (IU).

Antibiotic tests

Antibiotic tests were done with the strains namely, P1A, P1F, S2B, S2D, S2F, E3A, E3D, using Icosa Universal-2(HIMEDIA). The antibiotics used were as follows:

Abbreviations	Full forms	Concentrations
AK	Amikacin	30mcg
AMP	Ampicillin	10mcg
AMX	Amoxycillin	10mcg
CFR	Cefadroxil	30mcg
CPZ	Cefoperazone	75mcg
CAZ	Ceftazimide	30mcg
CTR	Ceftriaxone	30mcg
C	Chloramphenicol	30mcg
CIP	Ciprofloxacin	5mcg

COX	Cloxacillin	1mcg
COT	Co-trimoxazole	25mcg
E	Erythromycin	15mcg
GEN	Gentamicin	10mcg
NA	Nalidixic acid	10mcg
NET	Netillin	10mcg
NIT	Nitrofurantoin	30mcg
NX	Norfloxacin	10mcg
P	Penicillin	10mcg
TOB	Tobramycin	10mcg
VA	Vancomycin	30mcg

RESULTS AND DISCUSSION

Eight facultative anaerobic bacteria were isolated from the gut of the termite, *Odentotermes obesus* (Rambur). Besides strict anaerobic bacteria, aerobic and facultative anaerobic microbes also occur in the gut of termites [47]. Strictly anaerobic cellulolytic bacteria were not investigated in present study. The isolates were identified as *Streptococcus*, *Pseudomonas* and *Enterobacter* sp. by culturing in selective media. Bacterial colonies (Fig 2A-C) showed differences in their generation time. Isolates of McConkey agar plates showed the generation time ranging between 42 to 60 minutes, whereas the isolates of Pseudomonas agar and Streptococcus selection agar showed the doubling time ranging between 66 to 96 minutes and 90 to 102 minutes respectively. The average density of the bacterial population was $36x10^4$ CFU/ml to $102.5x10^7$CFU/ml. The bacterial density obtained in the present study was quite high when compared with the population density of cellulolytic bacteria extracted from *Saperdavestita* (Coleoptera: Cerambycidae), *Ips pini* and *Dendroctonus frontalis* (Coleoptera: Curculionidae) which ranged between $2.4\ x10^5$ to $3.6\ x10^6$ CFU/gut [48] and that of bacterial isolates of the hindgut of *Odontotermes* sp. studied by Ngangi *et al*. [49] which ranged between $3.08x10^6$ to $5.01x10^6$ CFU/ml. These authors suggested that the termites that live in dead wood that have undergone a process of weathering in their digestive tract comprise of higher bacterial population than the termites living on live wood [49].

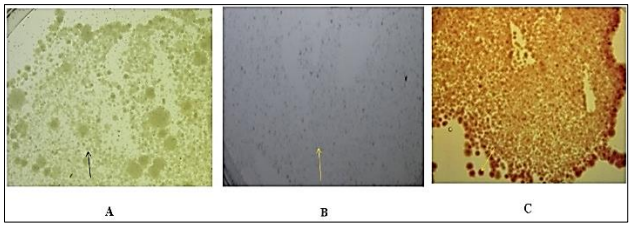

Fig 2 Colonies of isolated bacterial strains in selective media (A) *Streptococcus sp.*, (B) *Enterobacter sp.* and (C) *Pseudomonas sp.*

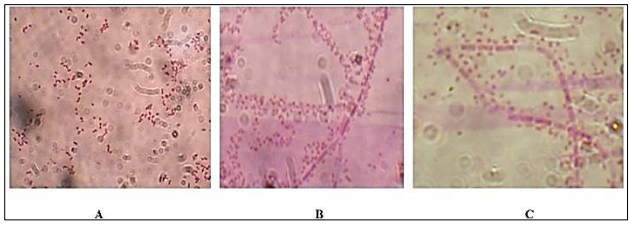

Fig 3 Microphotograph of bacterial strains (gram stained) (100X) (A) *Streptococcus sp.*, (B) *Enterobacter sp.* & (C) *Pseudomonas sp.*

All the present isolates were observed as Gram negative, rod-shaped and non-spore forming bacteria, further S2B, S2D and S2F were found to be chain forming bacteria. Identification results showed that P1A, P1D and P1F belonged to *Pseudomonas* sp., and S2B, S2D and S2F to *Streptococcus* sp., whereas E3A and E3D to *Enterobacter* sp. (Fig 3A-C).

Results of biochemical tests helped in further identification of bacteria species. All the isolates showed negative results for phenylalanine deamination, Indole test, starch hydrolysis and casein hydrolysis (Table 1).

Table 1 Morphological and biochemical characteristics of eight strains of enteric bacteria of *O. obesus*

Isolates	P_1A	P_1D	P_1F	S_2B	S_2D	S_2F	E_3A	E_3D
Gram staining	(-)ve	(-)ve	(-)ve	(-)ve	(-)ve	(-)ve	(-)ve	(-)ve
Cell morphology	Rod shaped	Rod shaped	Rod shaped	Chain forming	Chain forming	Chain forming	Rod shaped	Rod shaped
Starch hydrolysis	-	-	-	-	-	-	-	-
Casein hydrolysis	-	-	-	-	-	-	-	-
Catalase reaction	+	+	+	-	-	-	-	+
Anaerobic growth	-	-	-	+	+	+	+	+
ONPG	-	+	+	-	-	+	-	+
Lysine decarboxylase	+	+	+	-	+	+	+	+
Ornithine decarboxylase	-	+	-	-	+	+	+	+
Urease	-	-	-	+	-	-	+	-
Phenylalanine deamination	-	-	-	-	-	-	-	-
Nitrate reduction	+	+	+	-	+	+	-	+
H_2S production	-	-	-	-	-	-	-	-
Citrate utilization	-	+	+	+	+	+	+	+
Voges-proskaner	-	-	-	-	-	-	+	+
Methyl red	-	-	+	+	+	+	-	-
Indole	-	-	-	-	-	-	-	-
Malonate	+	+	+		+	+		
Esculine hydrolysis		+	+	+	+	+	+	+
Arabinose utilization	+	-	-	-	-	-	-	-
Xylose utilization	+	+	+	-	-	-	-	-
Adonitol utilization	-	-	-	-	-	-	-	+
Rhamnose utilization	-	-	-	-	+	+	-	+
Cellubiose utilization	+	+	+	-	-	-	-	-
Melibiose utilization	-	-	-	+	+	+	-	-
Saccharose utilization	-	-	+	+	+	+	-	-

Raffinose utilization	-	+	+	-	-	-	-	-
Trehalose utilization	-	-	-	+	+	+	-	-
Glucose utilization	+	+	+	+	+	+	-	-
Lactose utilization	+	+	+	+	+	+	-	+
Oxidase test	+	+	+	-	-	-	-	-

Table 2 Selected cellulolytic bacterial isolates of *O. obesus* gut with their hydrolysis capacity

Isolates	P_1A	P_1D	P_1F	S_2B	S_2D	S_2F	E_3A	E_3D
Cellulose degradation	-	-	-	+	+	+	+	+
Maximum clearing zone (mm)	-	-	-	36	40	39	50	53
Hydrolysis capacity	-	-	-	7.2	8.0	6.5	7.14	7.57

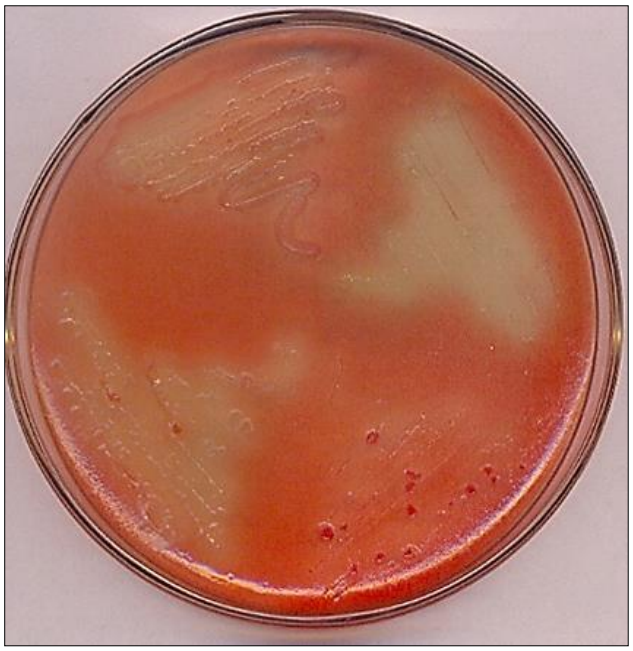

Fig 4 Cellulolytic bacterial growth on Congo-Red agar plate

The isolates E3A, E3D, S2B, S2D and S2F showed positive growth on cellulose Congo-Red agar media. The formation of a clear zone around the colony explains the secretion of extracellular cellulose (Fig 4) The measurement of isolates clear zone diameter showed that the highest clear zone was on E3D followed by E3A, S2D, S2F and S2B (Table 2). Thus, these bacterial isolates showed cellulolytic activity on Congo-Red test. Congo-Red test has been used previously by Upadhyaya *et al*. [50] to identify cellulolytic bacteria such as *Citrobacter*, *Enterobacter* and *Cellulomonas* strains. *Streptomyces*, *Pseudomonas*, *Acinetobacter*, and *Klebsiella*having cellulolytic activity also have been isolated and identified from some termite species [51]. Cellulase activity has also been shown in *Erwinia carotovora*a members of *Entrobacteriaceae* family [52]. However, cellulolytic staphylococci have also been isolated from termites [53].

The total cellulose activity on filter paper was found to be highest in E3D with 0.410 IU/ml and the lowest in S2F, 0.250 IU/ml. The histogram helps comparing the enzyme activities of other isolates (Fig 5). Similar findings were reported for *Acinetobacter anitratus* and *Branhamella* sp. grown in a basal salt medium with glucose and CMC as carbon source [54]. Results of Gravimetric analysis showed that maximum and minimum filter paper degradation ranged between 70.1% (E3A) and 63% (S2F) (Fig 6). Further Bichet-Hebe *et al*. [55] reported similar results, where bacterial populations in mixed culture showed filter paper degradation ranging from 31% to 60% by gravimetric analysis.

In antibiotics test it was found that isolates of *Psudomonas* sp., i.e., PIA, PID and PIF showed high resistance towards the antibiotics Vencomycin, Nitrofurantrin and Penicillin. Strains of *Streptococcus* sp. (S2B, S2D, S2f) and *Enterobacter* sp. (E3A, E3D) showed no resistance to any of the antibiotics used (Table 3).

Table 3 Reaction profile of gut bacterial isolates of *Odontotermes obesus* to antibiotics

Name of antibiotics	Strains of bacteria (results in mm)							
	P_1A	P_1D	P_1F	S_2B	S_2D	S_2F	E_3A	E_3D
AMX	14	20	_	35	19	33	40	17
AK	25	30	25	30	25	40	33	26
CAZ	10	35	37	14	17	-	15	-
NET	29	34	31	26	28	36	37	26
AMP	-	23	19	33	21	40	40	-
VA	10	10	12	24	10	11	25	12
E	_	14	17	33	14	29	24	_
CFR	_	21	13	35	_	38	26	_
CTR	21	40	32	30	31	29	22	26
GEN	34	33	30	29	28	38	28	29
CIP	40	40	23	38	_	40	20	40
NA	35	33	32	20	30	35	_	30
COX	29	19	_	37	20	31	24	37
CPZ	30	27	23	31	31	28	20	32
NIT	12	14	13	12	-	17	15	13
P	-	-	-	38	-	28	40	11
C	11	40	31	17	28	17	38	31
NX	40	35	19	34	34	34	29	30
COT	33	31	19	40	31	32	33	30
TOB	31	33	27	33	27	40	35	-

CONCLUSION

The present investigation established for the first time three strains of cellulose degrading bacteria from the gut of *Odontotermes obesus* occurring in the tea plantations of Darjeeling foothills. It can be concluded that in absence of cellulolytic flagellates, these cellulolytic bacteria take over the role of cellulose degradation.

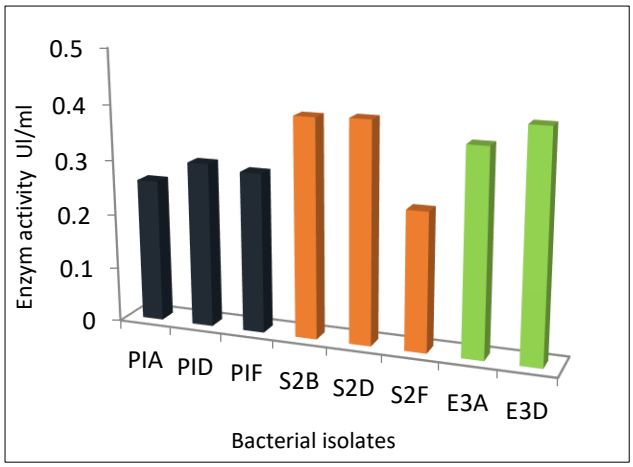

Fig 5 Endoglucanase activity of bacterial isolates of *O. obesus* gut with cellulolytic potential

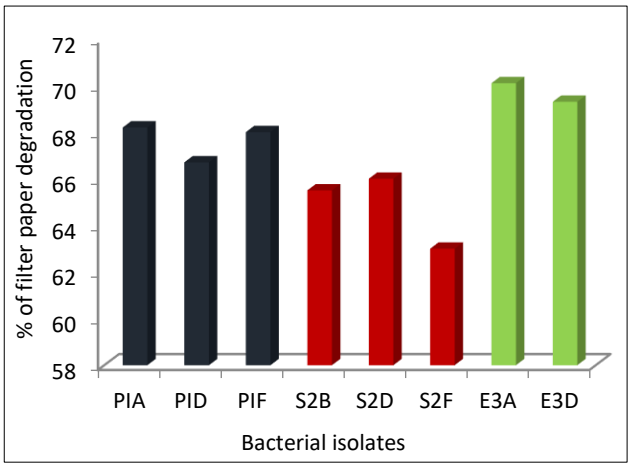

Fig 6 Percentage of filter paper degradation by bacterialisolates of *O. obesus* gut with cellulolytic potential

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