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Isolation and Characterization of Cellulase Producing Bacteria from the Gut of Termites (*Odontotermes* and *Heterotermes* Species)

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Authors' contributions

This work was carried out in collaboration between all authors. Author DS conceived and designed the study. Authors CPS and NKR carried out the experiments and performed the analysis of data. Author CPS wrote the first draft of the manuscript and managed literature searches. Author DS edited and proofread the final manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aim: To identify, characterize and compare the cellulolytic potentials of strains isolated from the gut of *Odontotermes* and *Heterotermes* species.
Methodology: Termites were collected, identified, surface sterilized and used as source of cellulase producers. Enrichment of cellulose utilizers were done using liquid media containing carboxy methyl cellulose (CMC) as the sole source of carbon and their cellulolytic potentials were confirmed using congo red plate screening method. The isolates showing considerable zone of clearance were biochemically characterized. Three effective isolates were further identified by 16S rRNA gene sequence analysis. Growth curves of the strains were constructed under six different conditions (static and shaking at 25°C, 37°C and 45°C). Their cellulase activities- endoglucanase, FPase and β-glucosidase, assayed at 18 hours of incubation were compared under the above mentioned conditions.
Results: Five isolates showing significant zone of clearance were selected, out of which three belonged to *Bacillus* and one each to *Staphylococcus* and *Enterobacter* sp. The three *Bacillus* sp.

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which were dominant cellulase producers were found to belong to *Bacillus cereus* (strain HT from *Heterotermes* sp. and ODO1, ODO2 from *Odontotermes* sp.). All the isolates showed high growth at 37°C under shaker condition. *Bacillus cereus* ODO2 displayed a higher cellulolytic potential compared to strain HT and ODO1. The endoglucanase, FPase and β-glucosidase activities of ODO2 were 5.06 U/mg, 2.52 U/mg and 6.01 U/mg respectively. ODO2 showed optimum specific activity at 37°C in shaker condition, whereas ODO1 and HT preferred static at same temperature.

Conclusion: The strains obtained in the present study are potent cellulase producers and thus can find application in food, animal feed, textile, fuel and chemical industries. Optimization of media and genetic modification of the strains can further improve their efficiency. All the three isolates are promising in view of use in future.

Keywords: Termite gut; cellulase; Bacillus cereus; 16S rRNA gene identification; Heterotermes sp.; Odontotermes sp.

1. INTRODUCTION

Increasing demand, limited supply and rising cost of fossil fuels has led to utilization of renewable resources like cellulose for the production of alternative energy [1]. Cellulose, a crystalline polymer composed of β-1, 4 linked D-glucose molecule is the most abundant and renewable biopolymer on earth [2]. The hydrolysis of cellulose to glucose and soluble sugars has thus become a subject of intense research and industrial interest [3]. Complete hydrolysis of cellulose to glucose requires the synergistic action of three enzymes. First, endoglucanase [EC 3. 2. 1. 4] nicks the internal cellulose chain, after which exoglucanase [EC 3. 2. 1. 91] attacks the ends of the crystalline structure and releases cellobiose processively or nonprocessively and finally, β-glucosidase [EC 3. 2. 1. 21] cuts cellobiose and cellooligosaccharide to produce glucose [4].

Cellulases are inducible enzymes obtained from fungi, actinomycetes and bacteria during their growth on cellulosic material. Though fungi are better cellulase producers than bacteria, there is increasing interest for bacterial cellulases as they are more effective biocatalysts stable under harsh conditions required for various industrial applications. Moreover, bacteria have a higher growth rate, and product recovery is simpler than from fungi [5]. Cellulase producers have been isolated and characterized from various sources like soil, decayed plant materials, hot springs, organic matters, composts, feces of ruminants and termites [6].

Microbes inhabiting the gut of organisms thriving on cellulosic biomasses as their major feed are a good source for cellulase enzyme extraction. Termites harbor a dense and diverse population of syntrophic symbiotic microflora in their guts that can degrade cellulose in wood, which has been partly degraded in the foregut by its own enzyme [7,8]. Termites, belonging to the order Isoptera, are mainly distributed in the tropical and subtropical regions where they play important role in biorecycling of lignocelluloses. Termites are classified into lower and higher termites based on their level of evolution, both in terms of behavior and anatomy [9]. The families *Mastotermitidae, Kalotermitidae, Termopsidae, Hodotermitidae, Serritermitidae* and *Rhinotermitidae* belong to lower termites whereas *Termitidae* to higher termites [10]. The genus *Heterotermes* (wood feeders) belong to *Rhinotermitidae* and *Odontotermes* (mound builders) belong to *Termitidae.*

Although, in recent years, large proportion of microorganisms have been isolated from termite gut, characterized and phylogenetically positioned, many microbial species remain uncultured and therefore uninvestigated [11-13]. Several *Bacillus* and *Paenibacillus* species have been detected in the termite gut, of which the genera *Bacillus* are predominant with titres of up to 10^7 ml^{-1} gut contents [14-16]. Species of *Staphylococcus, Streptococcus, Enterobacter and Flavobacterium* have also been isolated from termites [17]. In this study we compare the cellulolytic potentials of *Bacillus cereus* strains obtained from termites.

2. MATERIALS AND METHODS

2.1 Termite Collection and Identification

As a source of cellulase producing bacteria, termites were collected from nest or mound (*Odontotermes* sp. from the family *Termitidae*) and woody materials (*Heterotermes* sp. from the family *Rhinotermitidae*). The termites were identified at Department of Zoology, Payyanur

College. Before the enrichment and isolation steps, these termites were surface sterilized in 70% ethanol, and then washed in sterile normal saline solution.

2.2 Enrichment and Isolation of Cellulase Producers from Termites

Thirty surface sterilized termites were ground and aseptically transferred into a conical flask containing 50 ml sterile CMC (carboxy methyl cellulose) broth and incubated at 37°C for 3 days. CMC broth contained the following components dissolved in distilled water: CMC (10 g/l), NaCl (6 g/l), $(NH_4)_2SO_4$ (1 g/l), KH_2PO_4 (0.5 g/l), K_2HPO_4 (0.5 g/l), $MgSO_4$ (0.1 g/l), Yeast extract (1 g/l). The pH of the medium was adjusted to 7.0 [18]. A loop full of sample from the enriched culture medium was taken and streaked on to CMC agar plates and incubated at 37°C for 24-48 hours. All bacterial isolates were purified by re-streaking on to CMC agar plates.

2.3 Qualitative Screening of Cellulolytic Bacteria

All the pure isolates were spotted on CMC plates, flooded with Congo red solution (1 mg/ml in water) for 15 minutes, and then de-stained with 1M NaCl solution, for 10-15 minutes. Colonies showing a clear area in the otherwise congo red stained background on CMC agar plates were considered as cellulase positive [19]. Pure cultures of these microorganisms were maintained at 4°C.

2.4 Identification and Characterization of the Isolates

Colony morphology, staining properties, motility and biochemical tests were performed for bacterial isolates with measurable zone diameter. Only three effective isolates were selected for genomic DNA extraction and 16S rRNA gene analysis.

Genomic DNA was isolated using NucleoSpin® Tissue Kit (Macherey-Nagel) following manufacturer's instructions. The primers used were 16S-RS-F: 5'-CAGGCCTAACACATGCAAGTC-3' and 16S-RS-R: 5'-GGGCGGWGTGTACAAGGC-3'. PCR amplification reactions were carried out in a 20 µl reaction volume which contained 1X PCR buffer (100 mM Tris HCl , pH-8.3; 500 mM KCl), 0.2 mM each dNTPs (dATP, dGTP, dCTP and dTTP), 2.5 mM $MgCl_2$, 1 unit of AmpliTaq Gold DNA polymerase enzyme, 0.1 mg/ml BSA, 4% DMSO, 5 pM of forward and reverse primers and FTA disc as template. The PCR amplification was carried out in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) using hot-start procedure (95°C for 5 min). The conditions consisted of: Denaturation (95°C for 30 s), annealing (60°C for 40 s) and extension (72°C for 60 s) for 35 cycles followed by final extension step of 72°C for 7 min. PCR products were analyzed using agarose gel electrophoresis. The amplicons were purified and sequenced using the BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems, USA) on ABI 3730 DNA Analyzer (Applied Biosystems). The sequence quality was checked using Sequence Scanner Software v1 (Applied Biosystems). Sequence alignment and required editing of the obtained sequences were carried out using Geneious Pro v5.6. The edited sequences were blasted using the NCBI genomic BLAST program for comparing each sequence with all available 16S rRNA sequences.

2.5 Growth Curve Analysis

Each effective strain was inoculated into 50 ml of CMC broth and incubated at 25°C, 37°C and 45°C under both static and shaking conditions at 150 rpm in an orbital shaker. The samples collected at every 6 hours interval were analyzed for bacterial growth by measuring the absorbance at 650 nm. The growth curves were prepared by plotting bacterial cell concentration versus time.

2.6 Cellulase Activity Assay

To prepare crude enzyme for quantitative assay the strains were inoculated into 50 ml of CMC broth and incubated under different incubation conditions. The samples were taken from production media at 18 hours and centrifuged at 10,000 g for 10 min to separate the bacterial cells. The culture supernatants were used as crude enzyme for quantitative assay.

Endoglucanase assay was performed by incubating 1 ml of crude enzyme with 1 ml of 1% CMC in 50 mM sodium citrate buffer (pH 4.8) at 50°C for 30 min [4]. FPase assay was carried out using 50 mg strip of Whattman filter paper No. 1 (1 x 6 cm) under the same conditions with incubation time of 1 hour [20]. β-glucosidase activity testing was conducted in the same way as endoglucanase, replacing CMC by 1% cellobiose [21]. All the experiments were performed in triplicates and the average was

used to assess the enzymatic activities of the test isolates. Cellulase activities were calculated by measuring the amount of reducing sugars released by dinitrosalicylic acid (DNS) method [22] using glucose as the standard. One unit of cellulase activity is defined as the amount of enzyme required to liberate 1 µmol of reducing sugars per minute under the assay conditions. The amount of protein in culture supernatant was determined by using the method reported by Lowry et al. [23].

3. RESULTS

3.1 Isolation and Screening of Bacteria

Five bacterial strains were isolated from *Heterotermes* sp. and nine from *Odontotermes* sp. after enrichment in CMC broth. Only the isolates showing considerable zone of clearance were further selected and size of the zone recorded. The strains were designated as HT, HT1 from *Heterotermes* sp. and ODO1, ODO2, ODO3 from *Odontotermes* sp.

3.2 Identification of Selected Bacteria

Morphological and biochemical characteristics were investigated for each isolate and the results were compared with Bergey's Manual of determinative Bacteriology (Table 1). Three isolates HT, ODO1 and ODO2 belonged to *Bacillus* sp., whereas HT1 was a member of the genus *Enterobacter* and ODO3 was considered to belong to *Staphylococcus* sp. Among the five, the members of *Bacillus* sp., which displayed greater cellulose hydrolyzing capacities, were further identified by 16S rRNA gene sequence analysis. The BLAST results revealed 99% similarity with *Bacillus cereus* ATCC 14579 for strains HT and ODO1 and 100% similarity for strain ODO2. The sequences were deposited in Genbank database with accession numbers KP055077, KP055078 and KP055079 for strain HT, ODO1 and ODO2 respectively.

3.3 Growth Profile of the Strains *B. cereus* HT, ODO1 and ODO2

The strains were grown in different conditions and the growth curves were plotted (Figs. 1a, b and c). Even though the isolates showed growth at all conditions maximum cell density was found to be under agitation at 37°C for all isolates. Generally growth was found to increase till 18 hours and then remained almost constant. *Bacillus cereus* HT had a slightly higher growth rate under all the conditions.

Table 1. Morphological and biochemical characteristics of selected organisms

Termites	***Heterotermes* sp.**		***Odontotermes* sp.**		
Isolate code	**HT**	**HT1**	**ODO1**	**ODO2**	**ODO3**
Zone diameter (cm)	0.9	0.5	1.4	1.8	0.4
Morphological characteristics					
Shape	Long rods	Short rods	Long rods	Long rods	Cocci
Gram staining	+	-	+	+	+
Motility	+	+	+	+	-
Physiological characteristics					
Indole	-	-	-	-	-
Methyl-red	-	-	-	-	-
Voges-Proskauer	+	+	+	+	-
Citrate	+	+	+	+	+
Nitrate	+	+	+	+	-
Urease	-	+	-	-	-
Sugar utilization					
Glucose	+	+	+	+	+
Lactose	-	+	-	-	-
Sucrose	+	+	+	+	+
Mannitol	-	+	-	-	+
Maltose	+	+	+	+	+

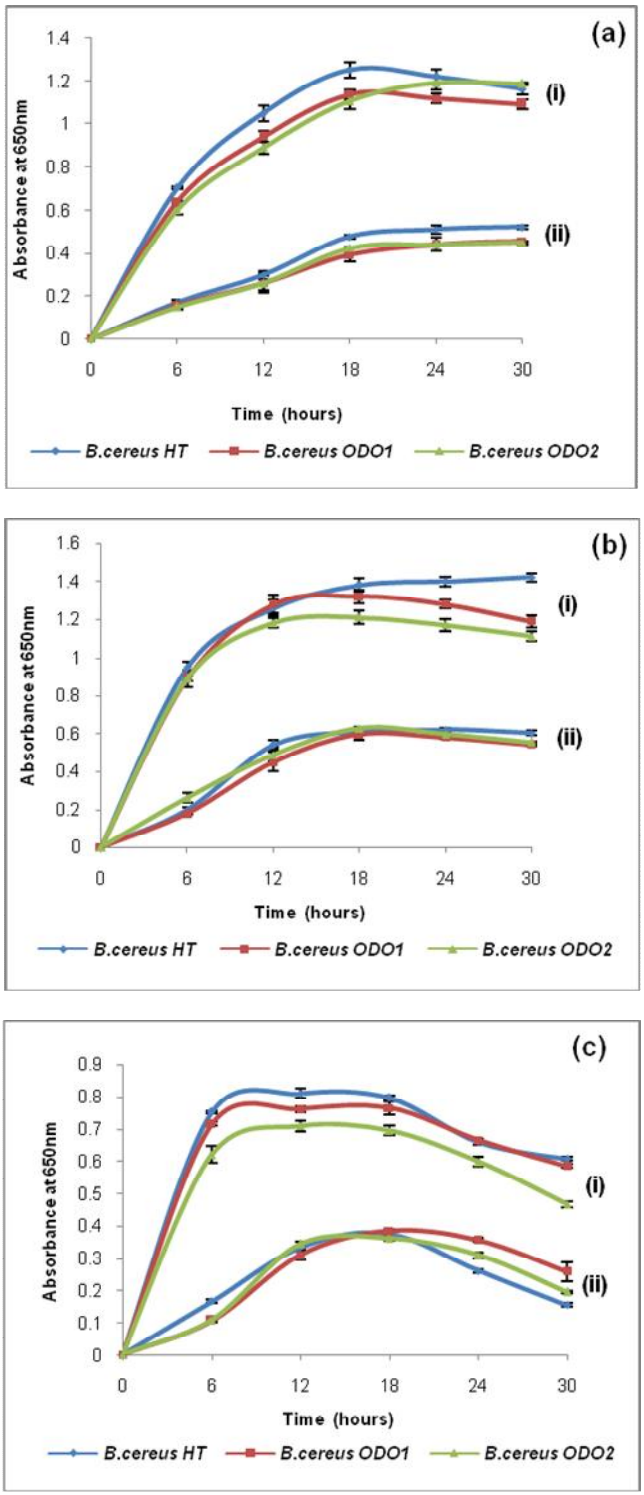

Fig. 1. Growth profile of strains *B. cereus* HT, ODO1 and ODO2 at (a) 25°C (b) 37°C and (c) 45°C. (i) Shaker (150 rpm) (ii) Static. Error bars indicate standard deviations of triplicate observations

3.4 Effect of Incubation Conditions on Cellulase Activities

Based on the growth curve analysis maximum cell density was achieved between 12-18 hours, thus the cellulase activities of the strains were examined at 18 hours. The CMCase, FPase and cellobiase assays were performed using carboxy methyl cellulose, filter paper, and cellobiose as substrates respectively. The cellulases from the three strains showed optimum activity at 37°C. *B. cereus* ODO2 opted shaking condition whereas HT and ODO1 preferred static condition. The highest CMCase activity (Fig. 2a) obtained for the isolates under their ideal conditions were in the order *B. cereus* ODO2 (5.06 U/mg), *B. cereus* HT (3.71 U/mg) and *B. cereus* ODO1 (2.98 U/mg). Fig. 2b compares the FPase activity displayed by the three strains. The highest FPase activity was observed for strain ODO2 (2.52 U/mg) followed by HT (1.98 U/mg) and ODO1 (1.62 U/mg). Cellobiase activity (Fig. 2c) showed the same pattern with highest for ODO2 (6.01 U/mg), HT (4.17 U/mg) and then ODO1 (3.42 U/mg).

4. DISCUSSION

Cellulose is the main building block of plants and one of the most abundant organic macromolecule in the ecosystem [24]. Bioconversion of cellulosic materials is a complex process and requires the participation of microbial cellulolytic enzymes. Habitats where these substrates are present are the best sources for isolation of cellulolytic microorganisms [25]. Termites were selected as the source for obtaining cellulase producers, as their gut contains large number of microorganisms that efficiently support lignocellulosic degradation [9]. Cellulose and hemicelluloses, but not lignin seems to be substantially degraded by organisms in termite gut [26].

In our study a total of about fourteen bacteria were isolated after enrichment in CMC broth from *Heterotermes* and *Odontotermes* sp. They were screened by congo red overlay method and five isolates were selected and biochemically identified. The bacteria were found to belong to the genus *Enterobacter, Staphylococcus* and *Bacillus.*

Heterotermes sp., of the *Rhinotermitidae* family, is a structure infesting subterranean termite that causes extensive damage to wooden structures. The strains isolated from these termites were named as HT and HT1. Isolate HT1 was identified as a member of the genus *Enterobacter.* According to Eutick et al. there appears to exist a correlation between the major gut bacterium and the family to which the termite belongs. Their studies showed that *Enterobacter* was the major gut bacterium in four species of termites belonging to the *Rhinotermitidae* [17]. Strain HT belonged to *Bacillus* species.

Odontotermes sp. belongs to the family *Termitidae* and subfamily *Macrotermitinae* (fungus-growing termites). More than half of the described species of this subfamily are assigned to the genus *Odontotermes.* These nest building species are the dominant invertebrate group in tropical and subtropical habitats [27]. The isolates from these termites were designated as ODO1, ODO2 and ODO3. ODO3 was found to belong to the genus *Staphylococcus*. Though cellulolytic activity is not a property commonly found in *Staphylococci,* cellulolytic strains have been isolated from termites [28]. ODO1 and ODO2 were members of the genus *Bacillus*.

Bacillaceae family has many cellulolytic members. Several studies have reported the isolation of cellulolytic bacilli from termites [16,29]. The *Bacillus* species obtained in this study were more potent than the other isolates and were thus phylogenetically analysed using 16S rRNA sequencing. All three strains were identified as *Bacillus cereus.* The sequences were deposited in Genbank database with accession numbers KP055077, KP055078 and KP055079 for strain HT, ODO1 and ODO2 respectively. Isolation of *Bacillus cereus* from the hind-gut of the termite *Reticulitermes hesperus* has been reported by Thayer [30].

The growth pattern and cellulase production of the three strains of *Bacillus cereus* (HT, ODO1 and ODO2), were compared under different incubation conditions (static and shaking at 25°C, 37°C and 45°C). Even though the strains showed an exponential growth between 6 to 18 hours under all conditions, the cell densities differed. The growth was more in agitated condition as compared to static. The cultures showed maximum optical densities in between 18 and 24 hours at 25°C and 37°C, after which they entered the stationary phase. At higher temperature the maximum density was attained between 12 and 18 hours. Population density, through quorum sensing, plays a critical role in

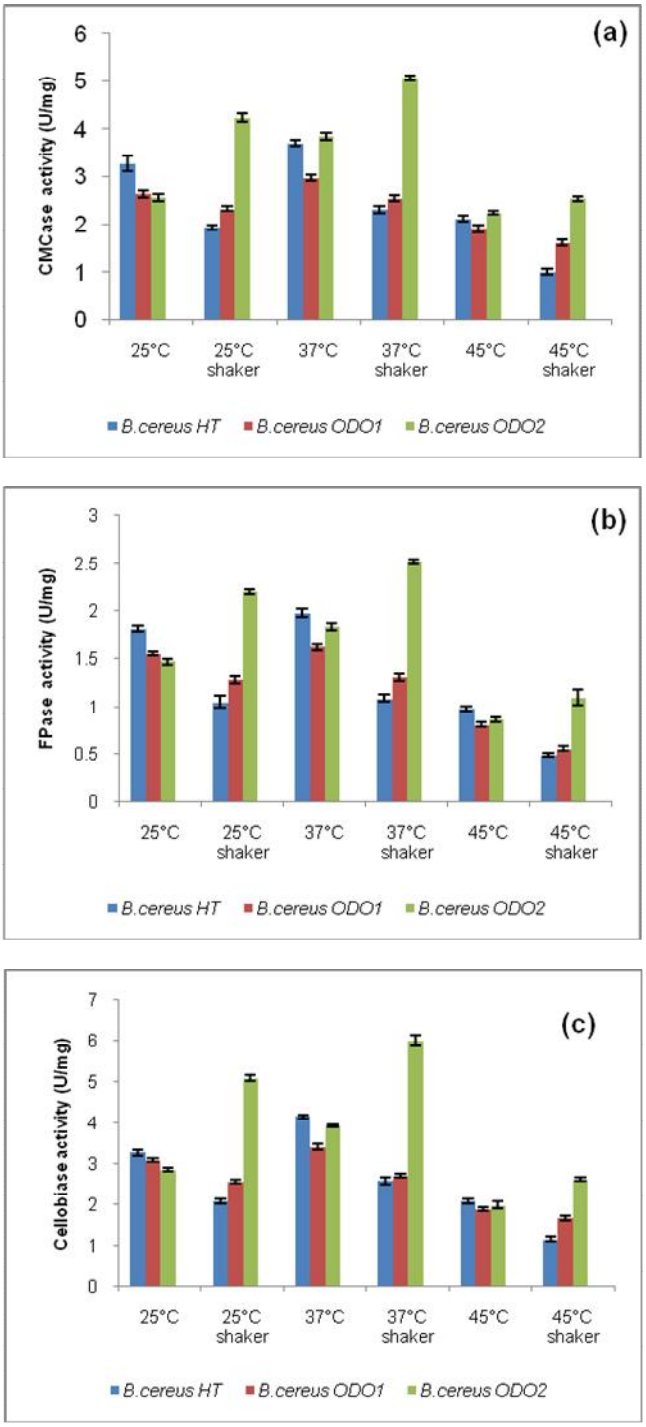

Fig. 2. Comparison of specific enzyme activities in U/mg of strains HT, ODO1 and ODO2 under different incubation conditions: (a) CMCase (b) FPase and (c) Cellobiase. Error bars indicate standard deviations of triplicate assays

the control of the synthesis of exoenzymes. The production of degradative enzymes normally occurs in the late logarithmic phase of growth, when the cell density is high [31,32]. Based on this fact, the ability of the isolates to utilize CMC, filter paper and cellobiose were determined after

18 hours of incubation. The organisms could utilize all three substrates but showed different activity under different conditions. All three isolates showed maximum activity at 37°C. The favorable conditions of the isolates differed from each other. *B. cereus* ODO2 displayed the highest cellulolytic potential (endoglucanase 5.06 U/mg, FPase 2.52 U/mg and β-glucosidase 6.01 U/mg) among the three and preferred agitation. This was followed by strain HT and ODO1 which chose static condition at 37°C. Elevated temperature retarded the production of the enzymes in all cases. All the strains were better β-glucosidase producers.

5. CONCLUSION

The endless search for economical and efficacious cellulases has led to the exploration of diverse environments like the gut of termites. In this study we were successful in isolating five effective cellulose degrading bacteria from termites of the genus *Heterotermes* and *Odontotermes.* Among the isolates, three belonged to the genus *Bacillus* and one each to *Staphylococcus* and *Enterobacter.* The most efficient cellulose degraders, all of which belonged to *Bacillus sp.* were identified as *Bacillus cereus* strain HT (*Heterotermes* sp.), ODO1 and ODO2 (*Odontotermes* sp.). All three strains showed high specific activities at 37°C; HT and ODO1 opted static condition, whereas ODO2 favored agitation. *Bacillus cereus* ODO2 displayed slightly higher cellulolytic potential than the others. The data obtained reveal that termites are indeed potential source of novel cellulolytic bacteria and that the isolates obtained in this study are promising candidates for applications in food, animal feed, textile, fuel and chemical industries.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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