Indian Journal of Biotechnology
Vol. 19, July 2020, pp 192-205

Identification and determination of cellulase activity of cellulose degrading microorganisms from earthworm species of different habitats of North East India

Surnalata Devi Hijam[1,2], Sailendra Goyari[1], Elizabeth Thokchom[1], Mohan Chandra Kalita[2] and Narayan Chandra Talukdar[1,3*]

[1]Microbial Resources Division, Institute of Bioresources and Sustainable Development, Takyelpat Institutional Area, Imphal, Manipur, India.

[2]Department of Biotechnology, Gauhati University, Jalukbari, Assam, India

[3] Institute of Advanced Study in Science and Technology (IASST), Paschim Boragaon, Assam, India

Received 25 April 2020; revised & accepted 20 May 2020

A total of 327 microbial strains isolated from the earthworm guts (EWGs) of five different species were comprised of 93 aerobic bacteria (AB), 78 facultative anaerobic bacteria (FAB), 72 actinobacteria and 84 fungi. Cellulolytic activity was determined both by qualitative and quantitative methods. Sixteen AB, 19 FAB, 26 actinobacteria and 23 fungi showed hydrolyzing zones on carboxymethyl cellulose medium and varied cellulase activity in the range of 0.0031 to 0.0263, 0.0022 to 0.013, 0.0022 to 0.0154 and 0.005 to 0.036 IU/ml, respectively. Cellulolytic bacterial and fungal isolates were identified based on 16S rDNA and ITS1-5.8S-ITS2 region, respectively. Nine cellulolytic bacterial (*Bacillus*, *Paenibacillus*, *Isoptericola*, *Cellulomonas*, *Clostridium*, *Enterobacter*, *Nocardia*, *Micromonospora* and *Streptomyces*) and ten fungal genera (*Aspergillus*, *Chaetomium*, *Acremonium*, *Fusarium*, *Penicillium*, *Purpureocillium*, *Epicoccum*, *Trichoderma*, *Talaromyces* and *Ascobolus*) were recorded in the EWGs. Cellulolytic microorganisms of EWGs exhibit taxonomic diversity and varying cellulase activity depending upon EW habitats. This is the first report on the cellulolytic bacterial and fungal diversity from the EWG of Imphal, Manipur, North East, India.

Keywords: Earthworm, cellulolytic microorganisms, cellulase activity

Introduction

The decomposition of lignocellulosic constituents of organic matter by the earthworms is known to be mediated with the help of the ingested microbiota in their guts[1-3]. Most EWGs and other soil animals do not produce cellulase of their own[4]. In 2009, the presence of an endogenous endocellulase gene in the earthworm *Pheretima hilgendorfi* (Megascolecidae) was reported for the first time[5]. However, native endocellulase alone cannot help the earthworm assimilate the lignocellulosic material they feed. Only the synergistic action of various enzymes namely, exocellulase, endocellulase, hemicellulase and lignin peroxidase can degrade lignocellulose efficiently[6]. The unique *in situ* conditions present in the EWG, which include mucus, high concentrations of organic substrates and anoxia enhance the proliferation of certain subset of ingested microorganisms to accomplish the desirable functions[7-8]. Many plant pathogenic microbes in the ingested organic matter get killed during the transit through the EWG[9] while some of the beneficial microbes get selectively proliferated and become predominant to take part in the digestion process[10]. The exoenzymes of the ingested microbial origin inside the EWG hasten the decomposition of the complex organic matter and thus, give rise to an enhanced assimilation in the gut through a mutualistic digestive system[11-12].

Diverse microorganisms are known to occur in the gut contents as well in the casts of earthworm[13-20]. Several factors including feed substrates, climate, soil type and organic matter determine the population and diversity of microorganisms within the EWG[21-22]. Variation in the occurrence of bacterial community in the gut wall of earthworms is reported to reflect a selection process in the order of ecological group > habitat > species[23]. Different environmental niches are unique in terms of distributions of microorganisms and therefore, the composition of the microbiota and their cellulolytic potential in the gut of the earthworms inhabiting different niches are expected

*Author for correspondence:
Tel: +91-361-2273061/7086012779, Fax: +91-361-2273062
talukdar_narayan@rediffmail.com

to be different. However, there are not many studies conducted in this direction[24].

The cellulose degrading microorganisms (CDMs) are important because they produce cellulases which not only help in the efficient bioconversion of cellulose - rich plant biomasses in ecosystems but also find application in biomass based renewable energy generation and food industry. Cellulases are a heterogeneous group of enzymes comprised of three different enzymes: exoglucanases (E.C. 3.2.1.176) (E.C. 3.2.1.91), endoglucanases (E.C. 3.2.1.4) and β - glucosidase (E.C. 3.2.1.21) which act synergistically for the complete hydrolysis of cellulose[25-26]. The cellulase enzymes are produced naturally by various microbial communities, including bacteria and fungi of diverse environmental origin[27]. Cellulases of fungi and bacteria in the genera of *Trichoderma* and *Bacillus* are the most intensively studied and commercially exploited for cellulase production[28]. Microbial cellulase enzymes can be produced in copious amounts using the established fermentation techniques[29]. Many studies put more emphasis on fungi as they produce relatively higher quantity of both cellulase and hemicellulase compared to other group of microorganisms[30-31] and fungal cellulases are easy to extract[32]. However, because of longer period for growth of cellulase producing fungi, many prefer bacterial cellulase over fungal, owing to the faster growth rate, expression of multienzyme complexes, resistance to extreme environments and higher compatibility and feasibility towards genetic engineering[33-35]. Thus, both fungi and bacteria have been heavily exploited for their abilities to produce a wide variety of cellulases. The stability and catalytic potential of cellulase enzymes derived from different microbial sources differ significantly leading to differences in the rate of cellulose breakdown[36-37]. Therefore, search for efficient microbial source for cellulase through conventional technique of microbial isolation remain as an important research activity[38]. Isolation of new cellulose - degrading strains also laid the foundation for molecular engineering strategies[39].

The objective of this study was to isolate and characterize cellulolytic aerobic bacteria (AB), facultative anaerobic bacteria (FAB), actinobacteria and fungi from the gut of four different indigenous earthworm species namely *Perionyx* sp. EM1, *Perionyx excavates* EM2, *Amynthas morissi* EM3 and *Drawida nepalensis* EM4 along with an exotic earthworm, *Eisenia andrei* EM6 prevalent in the Indian region of biodiversity hotspot.

Materials and Methods

Earthworm Sampling

Adult clitellate earthworms of four species found in different habitats of Imphal valley, Manipur, North East India and one exotic earthworm maintained in Indian Council of Agricultural Research Centre, Lamphelpat, Manipur, India were collected. The identification of the earthworms was done by sequencing the amplified sequence of mitochondrial cytochrome c oxidase subunit I genes.

Dissection of Earthworms

The earthworms were washed with tap water and then with sterile distilled water. They were anesthetized by keeping at -80^{o}C for 60 minutes and their body surfaces were sterilized by a brief rinse with 1.2% sodium hypochlorite solution followed by the repeated washing with sterile distilled water. The worms were then dissected using sterile blades, pins, scissors and forceps in the dissecting board. The gut contents were removed for microbial population analysis.

Isolation of AB, FAB, Actinobacteria and Fungi

One gram (1g) of the gut contents was suspended in 9 ml of sterile phosphate buffered saline (PBS) solution with vortex mixing for 5 minutes at maximum speed. The resulting suspension was serially diluted (10^{-1} to 10^{-7}) with PBS. One hundred microliter (100 µl) of 10^{-3} to $10^{-7}$and 10^{-3} to 10^{-4} dilution were spread onto the surface of Omeliansky's agar (OA) medium[40] supplemented with cellulose as the carbon source and Rose Bengal agar (RBA) medium with chloramphenicol (HiMedia) for the isolation of bacteria and fungi, respectively. The pH of the OA medium was 7.2 while that of RBA medium was adjusted to 5.3 by using 10% sterilized tartaric acid. The fungal plates were incubated for 5 days and the aerobic bacterial plates were incubated for 8 days at 30^{o}C to ensure growth of slow growing actinobacteria. For the isolation of FAB, the plates were incubated at 30^{o}C for 8 days. For all the incubations related to FAB, anaerobic jars along with anaero gas packs and indicator tablets (HiMedia) were used. Microbial colonies were counted using colony counter and colony forming unit (cfu) were expressed on the basis of per gram oven dried gut contents. The experiments were carried out with three replications for each sample. The morphologically distinct bacterial and actinobacterial colonies were purified to obtain single colonies by streaking on OA plates.

Stock cultures were made with Omeliansky's broth (OB) supplemented with 0.1% cellulose containing 30% (w/v) glycerol and stored at -80°C. The pure cultures of fungi were maintained on potato dextrose agar (PDA) slants at 4°C which served as the stock cultures for subsequent use. The colonies were sub-cultured after every 3 months.

Screening of CDMs

Isolates of four groups of microorganisms were screened for cellulase activity both qualitatively and quantitatively. In the qualitative plate assay for detecting extracellular cellulase activity, two chromogenic dyes namely Gram's iodine (GI)[41] and Congo red (CR)[42] dyes were used. The isolates were screened on carboxymethyl cellulose (CMC) agar plates (0.2% $NaNO_3$, 0.10% K_2HPO_4, 0.05% KCl, 0.05% MgSO4, 0.2% CMC, 0.02% peptone and 1.7% agar). The pH of the CMC agar plates was maintained at 7.2 and 5.2 for bacteria and fungi, respectively. A single colony was inoculated in 5 ml of Luria Bertani (LB) broth and grown at 30°C for 24 and 72 hrs for AB and actinobacteria, respectively in an incubator shaker at 180 rpm. For the screening of FAB, the 5 ml of LB broth was inoculated with a single bacterial colony and incubated at 30°C for five days. Then, 5 µl of the culture broth was spot inoculated on the CMC agar plates and incubated at 30°C for 48 hrs, 72 hrs and 5 days for AB, actinobacteria and FAB, respectively. For fungi, the isolates were pre-cultured on PDA at 30°C for 5 days and a mycelial disc of 4 mm diameter was punched using a sterile cork borer. The fungal mycelial disc was placed in the centre of the CMC agar plates using a sterile inoculating loop and incubated at 30°C for 72 hrs. After incubation, the plates were flooded with GI (2.0 g potassium iodide and 1.0 g iodine in 300 ml distilled water) for 3 to 5 mins and with 0.1% CR for 20 mins followed by washing the plates with 1 M NaCl for 15 to 20 mins. The plates were observed at different intervals for the formation of zone of clearance around the colonies. Cellulase activity of the isolates was measured qualitatively in terms of hydrolysis capacity on CMC agar i.e. the ratio of clear zone diameter to colony diameter[43-45]. The reference strains *Cellulomonas cellulans* (MTCC23), *Streptomyces albaduncus* (MTCC1764) and *Fusarium oxysporum* (MTCC1755) obtained from Institute of Microbial Technology (IMTECH) Chandigarh, India, were used as positive controls for cellulolytic activity.

The isolates which showed zone of clearance around the colonies in the plate assay were subjected to quantitative determination of reducing sugars by 3, 5-dinitro salicylic acid (DNS) method as a measure of cellulase activity[46].

Preparation of Crude Cellulase Enzyme Solution from the Cellulolytic Microorganisms

To prepare crude enzyme solution for AB and actinobacteria, a single colony was inoculated in 4 ml of OB supplemented with 0.1% cellulose and incubated at 30°C for 72 and 96 hrs at 180 rpm for bacteria and actinobacteria, respectively. One ml of the culture broth was transferred to 40 ml of the OB in 100 ml conical flask. The conical flasks containing the culture broth were allowed to grow at 30°C for 15 days in an incubator shaker at 180 rpm. For FAB, a single colony was inoculated with 4 ml of OB supplemented with 0.1% cellulose and incubated at 30°C for 5 days. Then, 100 µl of the 5 days old broth was transferred to 5 ml of the OB and incubated at 30°C for 15 days in the anaerobic jars. In case of fungal isolates, five 4 mm discs of pure fungal mycelia were inoculated with 40 ml of Czapek Dox broth (pH 5.2) supplemented with 0.1% cellulose and allowed to grow at 30°C for 15 days. The experiments were carried out in triplicates. The enzyme assay was carried out at different time intervals for all the four types of microorganisms.

Filter Paper Assay for Cellulase Enzyme

The Omeliansky's and Czapek Dox culture broth containing cellulose as carbon source were used for cellulase assay for bacterial and fungal isolates, respectively. Two ml of the culture broth was centrifuged at 10000 g at 4°C. The supernatant (0.5 ml) was diluted with 0.05 M sodium phosphate buffer (0.5 ml) at pH 7.0 in 50 ml falcon tube for bacteria and actinobacteria and with 0.05 M citrate buffer (0.5 ml) at pH 5.2 for fungi. Reagent blank was prepared by mixing 0.5 ml of water with 0.5 ml buffer. The substrate was provided as cut pieces of filter paper (32 mg). The enzyme activity was measured in terms of reducing sugars released and was quantified by DNS method. The assay was performed by incubating the mixture solution (0.5 ml of the crude enzyme extract with 0.5 ml buffer and filter paper) in a water bath maintained at 50°C for 30 mins. After incubation, 3 ml of freshly prepared DNS reagent was added and the reaction was terminated by boiling at 100°C for 10 minutes. After

boiling, 1 ml of 20% sodium potassium tartrate solution was added in order to maintain the stability of the color developed. The total volume was made up to 8 ml and the absorbance reading was measured at 540 nm. One unit (IU) of enzyme activity was defined as the amount of enzyme that released 1 µmol glucose per minute from the substrate under the experimental conditions.

Statistical Analysis

The values for the cellulase enzyme assay are presented as mean ±SD (standard deviation). All statistical significance tests and multiple comparisons between the enzyme activities of different isolates were determined by one way analysis of variance (ANOVA) supplemented with Tukey's HSD test using SPSS statistical software package, version 16.0. Values at $P<0.05$ were considered to indicate statistical significance.

DNA Extraction and PCR Amplification

Genomic DNA for cellulolytic bacteria and fungi were extracted using the protocols given by Sambrook and Cenis, respectively[47-48]. The 16S rDNA region of the bacterial isolates were amplified with the universal primers (27F 5′-AGAGTTTGATCMTGGCTCAG-3′ and 1492R 5′-TACGGYTACCTTGTTACGACTT-3)[49]. The amplification of the ITS1-5.8S-ITS2 region was carried out in a C1000™ Touch Thermal Cycler (BIORAD, USA) using the primer pair: ITS1 5′-TCCGTAGGTGAACCTGCGG-3′ and ITS4 5′-TCCTCCGCTTATTGATATGC-3′) according to standard protocol[50]. The run was programmed with an initial denaturation at 94°C for 10 min, followed by amplification for 35 cycles at the following conditions: 1 min 20 sec at 94°C, 1 min at 52°C and 1 min at 72°C. A final 7 min extension at 72°C and infinite hold time at 4°C completed the program run. The PCR amplified products were electrophoresed on 0.8% agarose gel stained with ethidium bromide using 0.5X TBE buffer for 45 min at 80V. The gel was observed using the ChemiDoc MP gel documentation system (Bio-Rad, Hercules, USA). DNA marker (GeNei, RMBD135) was included for the estimation of the size of the DNA fragments.

Sequencing and Phylogenetic Analysis

The PCR amplified products were sequenced in an automated ABI3100 genetic analyzer (Applied Bio-systems) in Merck specialists, Bangalore, India. The sequence electropherogram data for partial 16S *r*RNA gene were corrugated using Chromas LITE version 2.1.1 software. Determinations of the taxonomic identity of amplified query sequences were accomplished by comparing with the GenBank nucleotide database of National Centre for Biotechnology Information (NCBI) using the BLASTn protocol of BLAST tool. The nucleotide sequences were aligned using ClustalW implemented in BioEdit 7.0.5.3 using the default settings and were then manually corrected[51]. Using the BLAST tool[52], additional sequences were downloaded from the GenBank database in order to use as reference strains. Neighbor-Joining (NJ)[53] analyses were performed using Molecular Evolutionary Genetics Analysis (MEGA) software version 6.0[54] with Kimura's two parameter (K2P) model[55] for the construction of the evolutionary trees. The type strains *Pseudomonas aeruginosa* (LC069033) and *Rhizopus microsporus* (EU798703) were used as the out groups in the phylogenetic tree construction of bacteria and fungi, respectively. A bootstrap analysis with 1000 replications was performed. DNA sequences were deposited in GenBank under accession numbers KX138434-KX138449 (AB), KX242115-KX242133 (FAB), KX138411-KX138433 (fungi) and KX242134-KX242158 along with KU519614 (actinobacteria).

Results

Characterisation of the Earthworms by Sequencing

The indigenous earthworm species were assigned as EM1, EM2, EM3 and EM4 and the exotic one as EM6. The GenBank accession numbers for the deposited sequences are KT716821-23 and KT716825-26. *Perionyx* sp. EM1 and *Perionyx excavates* EM2 were inhabitants of cow dung heap and banana pseudostem, respectively. Earthworms *Amynthas morissi* EM3 and *Drawida nepalensis* EM4 were collected from the soil habitat. The exotic earthworm, *Eisenia andrei* EM6 was reared in organic matter prepared using cow dung and rice straw.

Population of the Microorganisms in the EWG

The microbial population (log cfu/g dry weight of the gut contents of the earthworm) was in the range of 6.345 ± 0.049 to 7.33 ± 0.009, 4.47 ± 0.13 to 6.053 ± 0.106 and 4.34 ± 0.093 to 6.13 ± 0.017 for AB, FAB and fungi, respectively (Table 1). A total of 327 isolates comprising 93 AB, 78 FAB, 72 actinobacteria and 84 fungi were obtained from the gut content of the five different earthworm species (Table 2).

Table 1 — Log cfu/gram dry weight of the gut contents of earthworms

SL. No.	Earthworm type	Aerobic bacteria	Facultative anaerobic bacteria	Fungi
1	*Eisenia andrei* EM6	7.33 ± 0.009	5.86 ± 0.06	5.93 ± 0.059
2	*Drawida nepalensis* EM4	6.75 ± 0.091	5.89 ± 0.091	4.81 ± 0.089
3	*Amynthas morissi* EM3	6.78 ± 0.115	4.47 ± 0.13	4.34 ± 0.093
4	*Perionyx exvavatus* EM2	6.345 ± 0.049	5.04 ± 0.073	4.48 ± 0.042
5	*Perionyx* sp. EM1	7.206 ± 0.047	6.053 ± 0.106	6.13 ± 0.017

Table 2 — Number of isolates which showed clearing zone around the colonies in the plate assay done by using Gram's iodine and Congo Red dyes

Earthworm type	Aerobic bacteria	Facultative anaerobic bacteria	Actino-bacteria	Fungi	Total
Eisenia andrei EM6	4	3	5	3	15
Drawida nepalensis EM4	3	4	8	4	19
Amynthas morissi EM3	4	3	5	6	18
Perionyx exvavatus EM2	3	3	5	4	15
Perionyx sp. EM1	2	6	3	6	17
Total	16	19	26	23	84

Qualitative Screening of the Microbial Isolates for their Cellulase Activity

Hydrolysis capacity varied among the different CDMs and out of 327 isolates, only 84 (16 AB, 19 FAB, 26 actinobacteria and 23 fungi) showed halo zones around their colonies which was a positive indication for the cellulase activity. The appearances of the bacteria, actinobacteria and fungi with halo zones around their colonies on CMC agar plates is shown in Figure 1. The hydrolysis capacity was found to vary in the range of 0.2 to 1.7 cm with GI and 0.7 to 2.2 cm with CR for AB, 0.2 to 3.2 cm with GI and 0.3 to 3.4 cm with CR for FAB, 0.3 to 2 cm with GI and 0.3 to 2.1 cm with CR for actinobacteria and 0.2 to 2.2 cm with GI and 0.2 to 2.7 cm with CR for fungi.

Quantitative Screening of the Microbial Isolates for their Cellulase Activity

The 84 isolates exhibiting hydrolysis capacity were subjected to quantitative screening. Cellulase activity was found in the range of 0.0031 to 0.0263, 0.0022 to 0.013, 0.0022 to 0.0154 and 0.0056 to 0.036 IU/ml for the AB, FAB, actinobacteria and fungi, respectively (Figs. 2-5). Among the AB, *Paenibacillus barcinonensis* CB10 showed the highest activity (0.0263 IU/ml) followed by *Paenibacillus* sp. CB2 (0.0133 IU/ml) and *Cellulomonas* sp. CB11 (0.0087

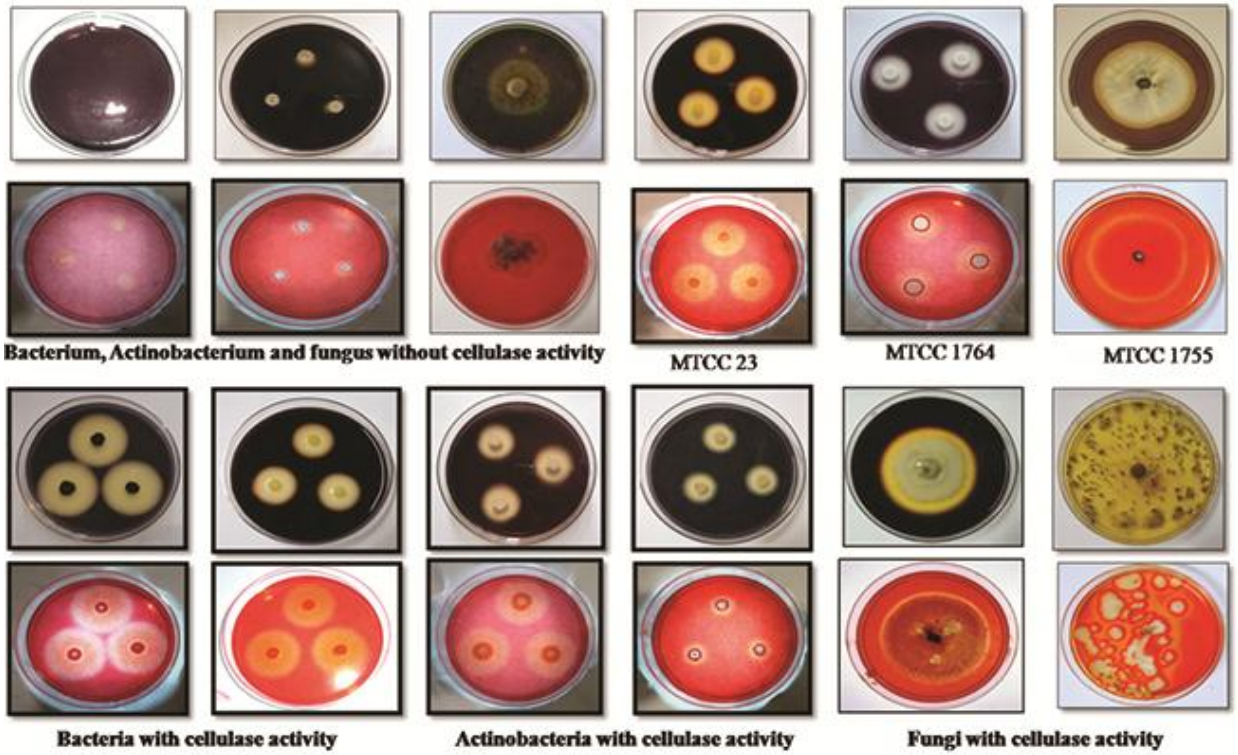

Fig. 1 — Formation of clearing zones around the colonies of microorganisms in the CMC agar medium after pouring Gram's iodine and Congo red dyes.

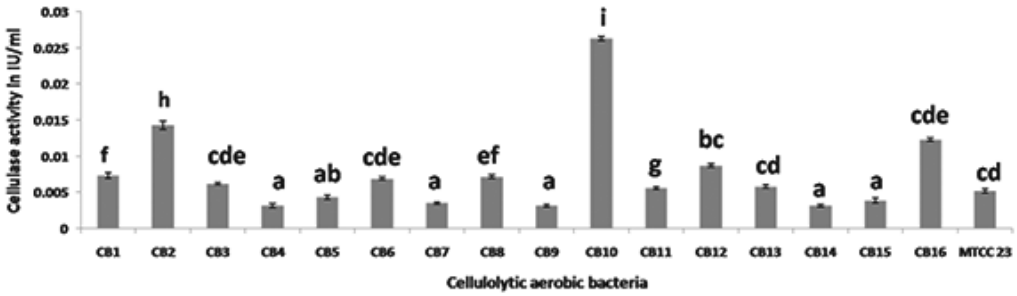

Fig. 2 — Cellulase activity of the cellulolytic aerobic bacterial isolates determined on 5th day based on DNS assay (FPase IU/ml). Each bar indicates the mean ± S.D (n=3). Bars marked without a common letter are significantly different ($P < 0.05$).

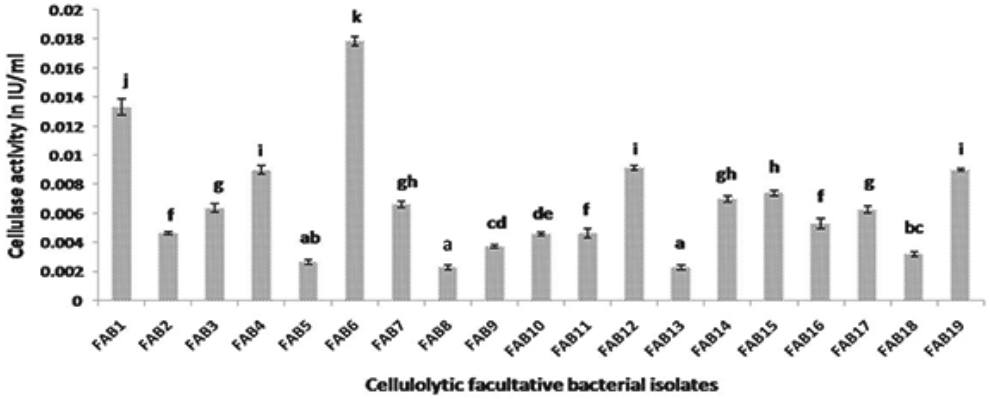

Fig. 3 — Cellulase activity of facultative anaerobic cellulolytic bacterial isolates determined on 5th day based on DNS assay (FPase IU/ml). Each bar indicates the mean ± S.D (n=3). Bars marked without a common letter are significantly different ($P < 0.05$).

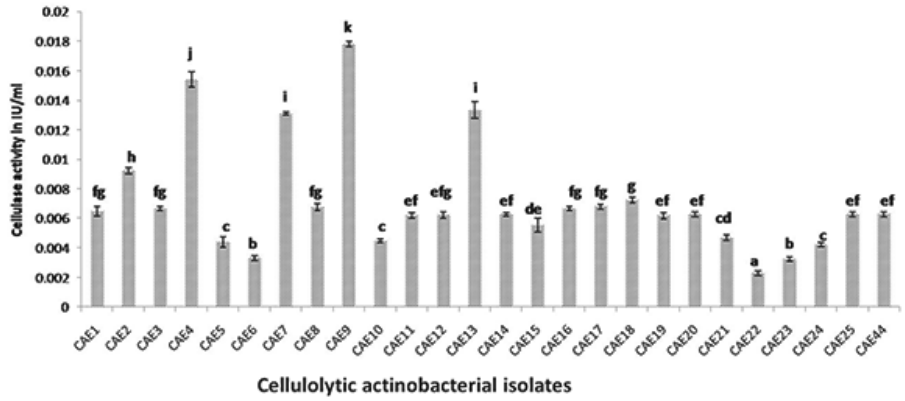

Fig. 4 — Cellulase activity of the actinobacterial isolates determined on 5th day based on DNS assay (FPase IU/ml). Each bar indicates the mean ± S.D (n=3). Bars marked without a common letter are significantly different ($P < 0.05$).

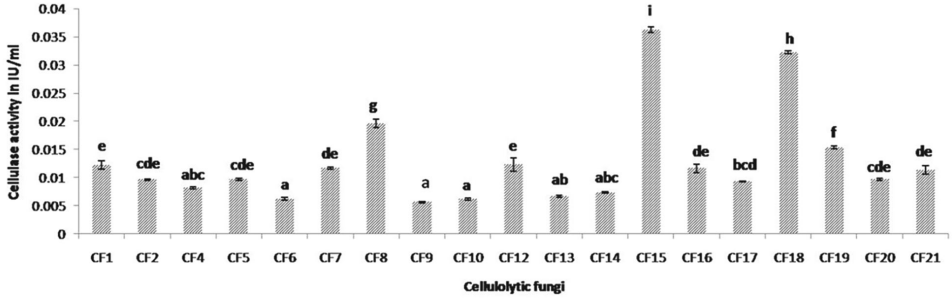

Fig. 5 — Cellulase activity of the fungal isolates determined on 5th day based on DNS assay (FPase IU/ml). Each bar indicates the mean ± S.D (n=3). Bars marked without a common letter are significantly different ($P < 0.05$).

IU/ml). *Bacillus* sp. CB4 which was morphologically similar to CB7, 9 and 14 showed the least enzyme activity of 0.0031 IU/ml. Among the FAB, *Clostridium* sp. FAB6 showed the highest activity (0.0178 IU/ml) followed by *Clostridium* sp. FAB1 (0.0133 IU/ml) and *Clostridium algidixylanolyticum* FAB4 (0.009 IU/ml) which was morphologically similar with FAB12 and FAB19. *Enterobacter* sp. FAB8 which was morphologically similar to FAB13 showed the least enzyme activity of 0.0022 IU/ml. Among the actinobacteria, *Streptomyces* sp. CAE9 showed the highest activity (0.0178 IU/ml) followed by *Streptomyces* sp. CAE4 (0.0154 IU/ml) and *Streptomyces* sp. CAE13 (0.0133 IU/ml). *Nocardia* sp. CAE22 showed the least enzyme activity of 0.0022 IU/ml. Among the fungi, *Aspergillus terreus* CF15 showed highest activity (0.036 IU/ml) followed by *Trichoderma* sp. CF18 (0.032 IU/ml) and *Fusarium* sp. CF8 (0.0196 IU/ml) and the least enzyme activity of 0.0056 IU/ml was shown by *Purpureocillium lilacinum* CF9. In general, fungal isolates showed higher cellulase activity compared to bacterial and actinobacterial isolates.

Molecular Characterization of the CDMs and Phylogenetic Analysis

The bacterial and fungal isolates which were found to be positive for cellulase activity determined by both qualitative and quantitative screening methods were characterized based on 16S rRNA gene and ITS1 - 5.8S - ITS2 region, respectively. The cellulase producing AB were assigned as CB1 to CB16, FAB as FAB1 to FAB19, fungi as CF1 to CF23 and actinobacteria as CAE1 to CAE25 along with CAE44. The designated microbial isolates for cellulolytic AB, FAB, actinobacteria and fungi of different categories are shown in the phylogenetic NJ trees constructed using MEGA version 6 (Figs. 6-9).

Discussion

Large volume of research data is available on the occurrence of cellulolytic microorganisms in different habitats such as soil[56], compost systems[57] sediments[58], rotting wood samples[59] and inside the gut of *Holotrichia parallela* larvae[60]. Occurrence of cellulolytic microorganisms inside the earthworm gut is advantageous for efficient degradation of organic matter. However, compared to general microbial diversity present in the gut of different earthworm species as reported by researchers[61] the occurrence and distribution of the cellulolytic microbial component in the gut of different earthworm species is not understood well. The decomposer microbes present in the gut of the earthworms function as key detritivores and enhance the efficiency of the organic matter recycling functions in either mineral soils or surface accumulated organic debris of soil habitats[62-63]. This research has generated comprehensive data on diversity of cellulolytic microorganisms in four different indigenous earthworm species of the Imphal valley, Manipur, India along with an exotic species, *Eisenia andrei* EM6 using classical and molecular techniques.

Different isolates of AB, FAB, actinobacteria and fungi were screened for qualitative and quantitative cellulase activity by using chromogenic dyes (Gram's iodine and Congo red) and DNS method, respectively. Since the number of the CDMs was large, optimization of enzyme production at different levels of various factors like temperature, pH, incubation time etc. were not investigated. Thus, the enzyme activities reported in this study were obtained from an un-optimised medium with cellulose as the substrate. Among the CDMs, *Aspergillus terreus* CF15 showed the highest cellulase activity of 0.036 IU/ml while *Enterobacter* sp. FAB8 and *Nocardia* sp. CAE22 showed the least enzyme activity of 0.0022 IU/ml. A similar range of cellulase activity was also reported from earlier studies[64-65]. Cellulase production is significantly influenced by nutritional composition and physical factors such as temperature, pH, incubation period and agitation speed[66-67]. By optimizing both nutritional and physical parameters, cellulase enzyme production of the isolates can be improved. The aerobic *Paenibacillus* sp. CB10 which was isolated from the gut of *Drawida nepalensis* EM4 displayed a significantly higher cellulase activity than reference strain *Cellulomonas cellulans* MTCC 23 as well as other bacterial and actinobacterial isolates and thus appears to be a potential candidate for cellulase production. Recently, a novel β-glucosidase was reported from *Paenibacillus* sp. strain MTCC 5639[68].

Since the earthworm gut has anoxic conditions, it was expected to harbor mainly the facultative anaerobes in the gut. However, many aerobic microorganisms were found to occur inside the gut. Similar findings were also reported by other research workers[69]. Diversity of different groups of CDMs in the gut of the earthworms was remarkable, although cellulolytic yeast, protozoa and strictly anaerobic bacteria were not investigated in this study. The Gram-

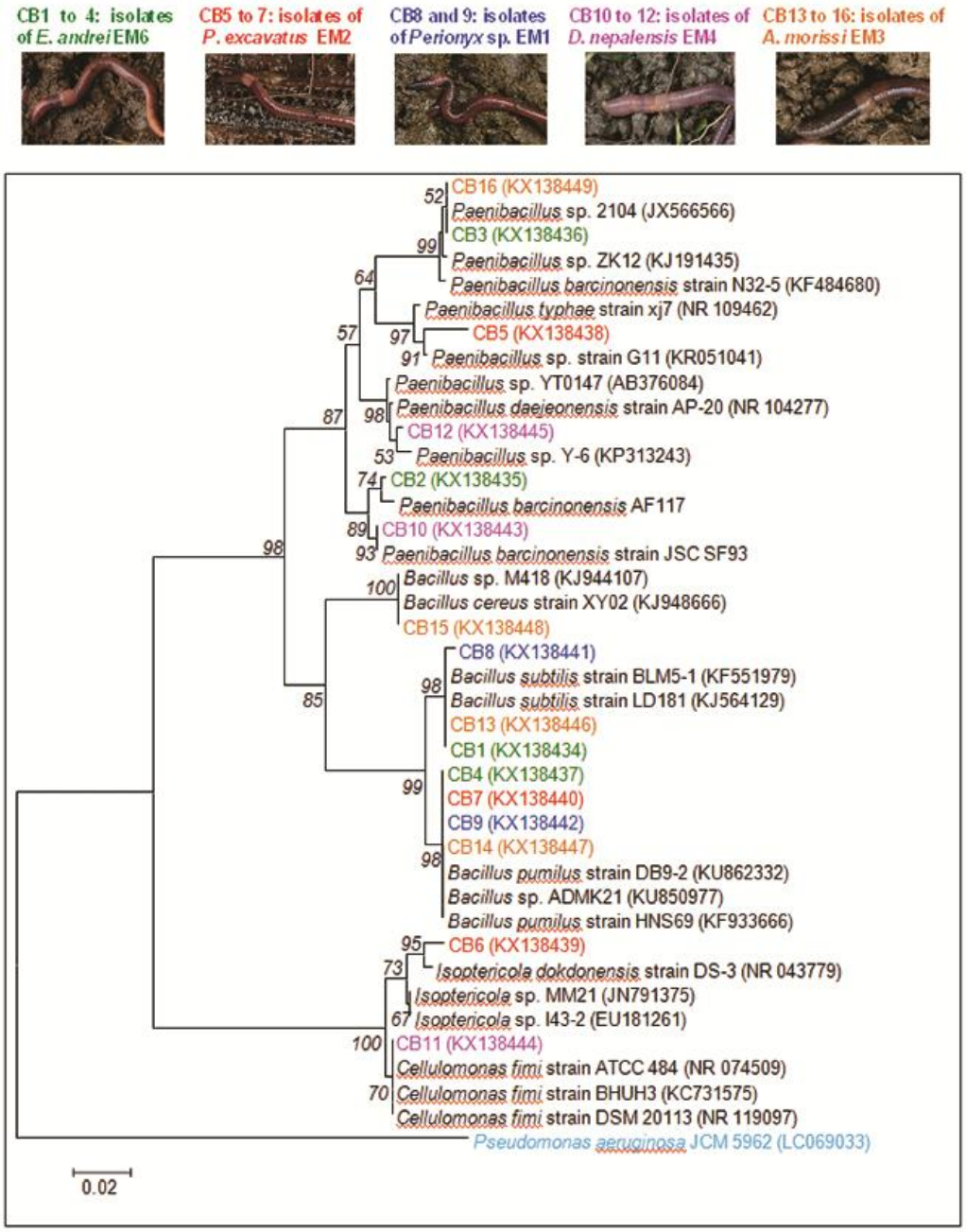

Fig 6. — NJ phylogenetic tree of the cellulolytic aerobic bacterial isolates based on 16S rRNA gene sequence.

positive bacteria within the order Bacillales and belonging to the genera of *Bacillus* and *Paenibacillus* formed the main component of cellulolytic aerobic bacteria. A similar observation was made in a previous study conducted in the gut of the termite *Zootermopsis angusticollis*[70]. *Bacillus pumilus* was found to be present in the gut of all the earthworms except *D. nepalensis* EM4. *Bacillus subtilis* was present in the gut of *Eisenia andrei* EM6, *Perionyx* sp. EM1 and *A. morissi* EM3 while *Bacillus cereus* was present in the gut of *A. morissi* EM3 only. Various species within the *Paenibacillus* genus were found to be present in the gut of all the earthworms. In case of the cellulolytic FAB, genus *Clostridium* was predominant. The occurrence of several species of *Clostridium* (*C. paraputrificum*, *C. butyricum* and *C. beijerinckii*) in the gut of *Eisenia fetida* had also been reported[71]. *Bacillus cereus* and *C. algidixylanolyticum* were found in the gut of *Perionyx* sp. EM1, *Perionyx excavatus* EM2 and *A. morissi* EM3. *Paenibacillus polymyxa* was found in

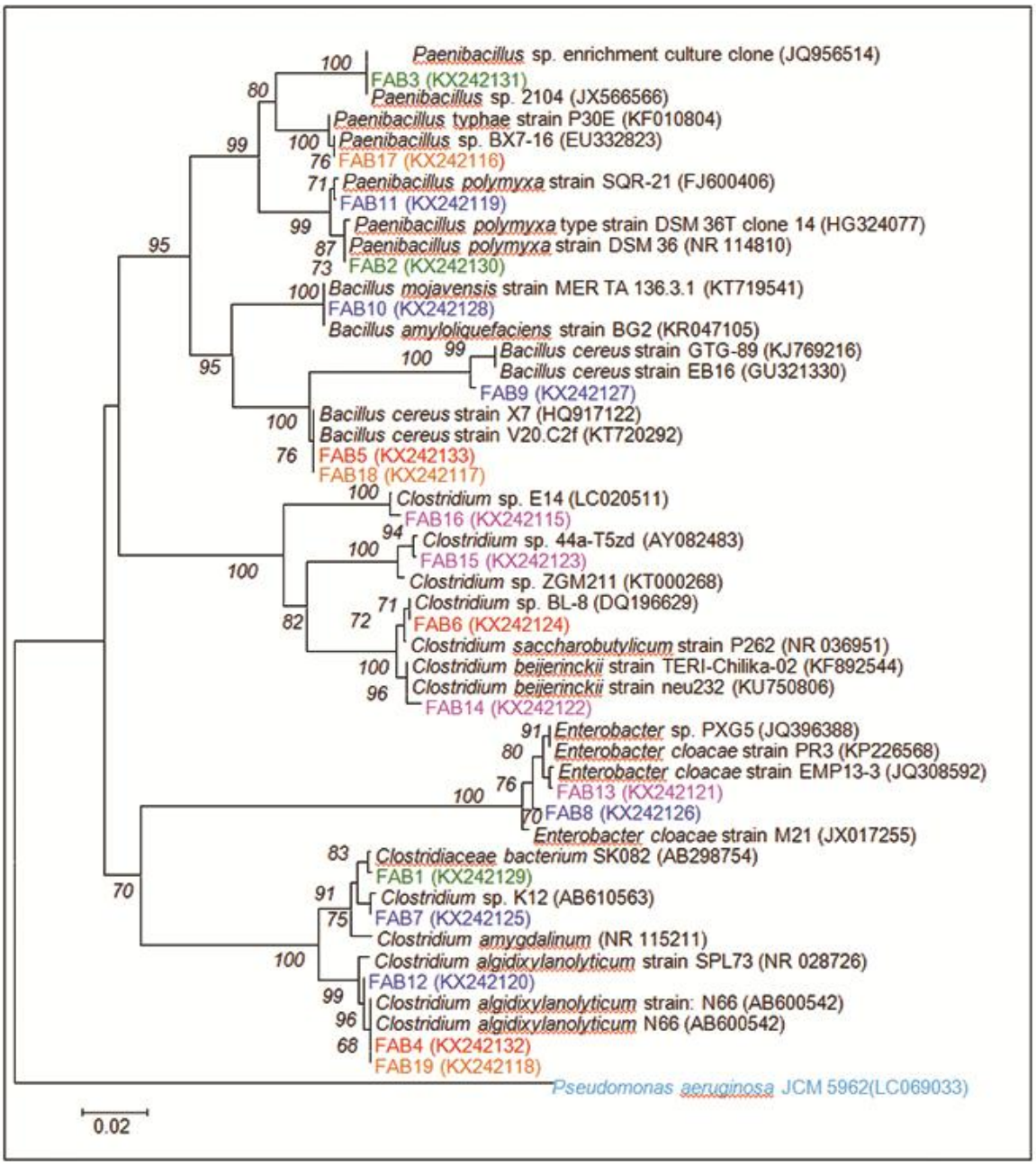

Fig. 7 — NJ phylogenetic tree of the cellulolytic facultative anaerobic bacterial isolates based on 16S rRNA gene sequence.

E. andrei EM6 and *Perionyx* sp. EM1. *Enterobacter cloacae* was found in the gut of *Perionyx* sp. EM1 and *D. nepalensis* EM4. *Chaetomium* and *Burkholderia* were found to be the dominant fungal and bacterial genera among the aerobic cellulolytic microorganisms present in the gut of *E. fetida* (Lumbricidae) and *Amynthas heteropoda* (Megascolecidae)[4]. Bacteria in the genus of *Bacillus*, *Clostridium*, *Enterobacter*, *Nocardia*, *Aeromonas*, *Vibrio*, *Serratia*, *Pseudomonas*, *Flavobacterium*, *Gordonia*, *Mycobacterium*, *Klebsiella* and *Azotobacter* were also reported to be present within the intestines of the earthworms (Brito-Vega & Espinosa-Victoria, 2009).

Several strains of the genus *Streptomyces* had been known to be cellulase producers. They were found as the largest number of cellulolytic species in the phylum actinobacteria and were also known to produce various types of unique cellulase enzymes[72]. In our study also, the homology search of the enquired 16S rRNA gene sequences of the cellulolytic actinobacteria in

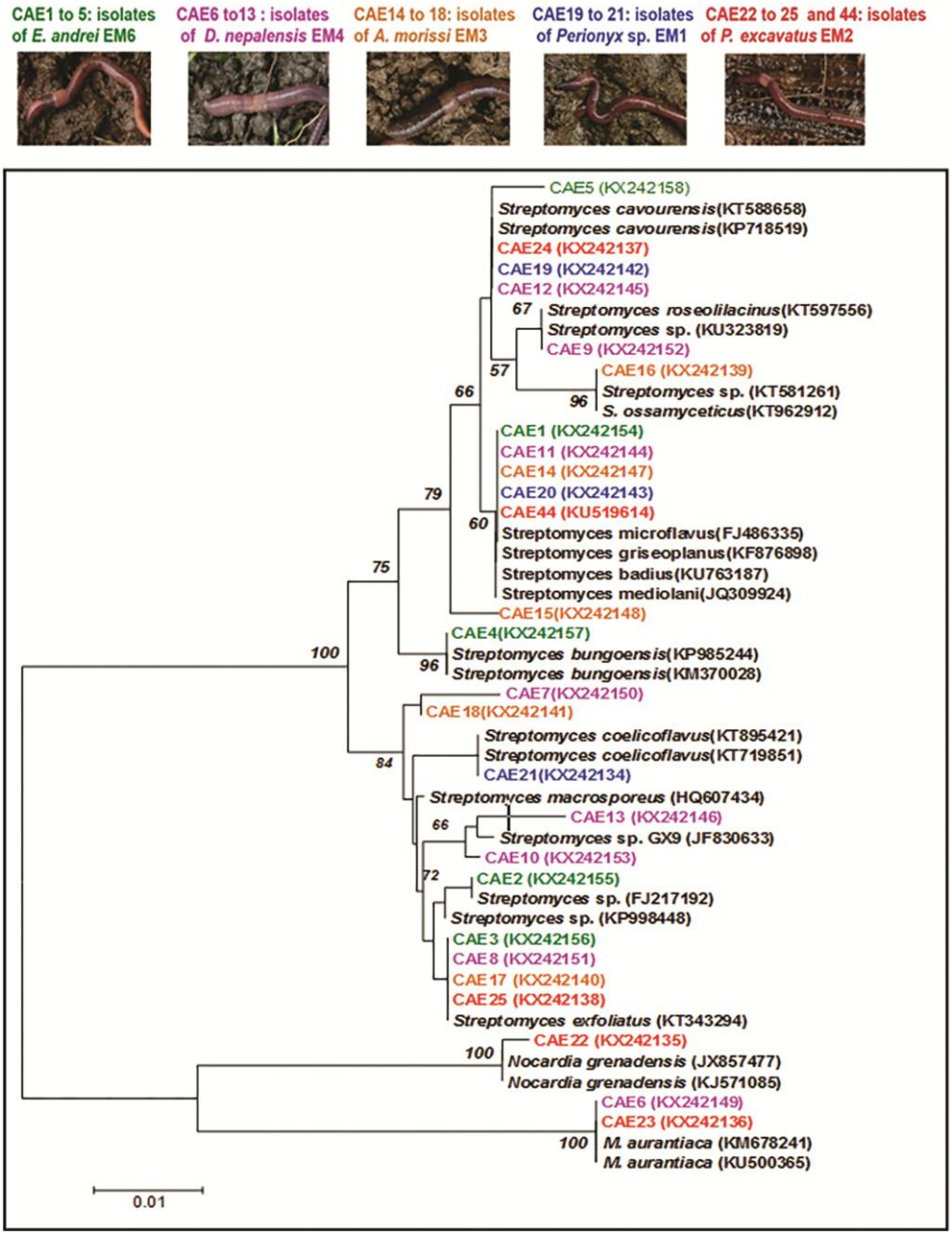

Fig 8. — NJ phylogenetic tree of the cellulolytic actinobacterial isolates based on 16S rRNA gene sequence.

NCBI BLAST showed the dominance of the *Streptomyces* genus in the earthworm gut. Out of 26 cellulolytic actinobacteria, 23 belonged to *Streptomyces.* Only one species of each of the two genera *Nocardia* and *Micromonospora* was found to be present in the earthworm gut. The occurrence of *Streptomyces* and *Nocardia* species were also reported in the gut of earthworms (Parthasarathi *et al.* 2007) The 23 species of cellulolytic fungi represent 10 genera and this result suggests that highest genetic diversity was in fungi among the cellulolytic microbes present in the earthworm gut. Fungi in the genera of *Aspergillus, Penicillium, Fusarium, Trichoderma* and *Chaetomium* are commonly known to be cellulolytic, but fungi in the genera *Acremonium, Arthrinium, Purpureocillium, Epicoccum* and *Ascobolus* were found to be cellulolytic in our study which are comparatively less common cellulolytic fungi. Fungi within the genera of *Chaetomium, Penicillium, Fusarium* and *Staphylotrichum* were reported to be

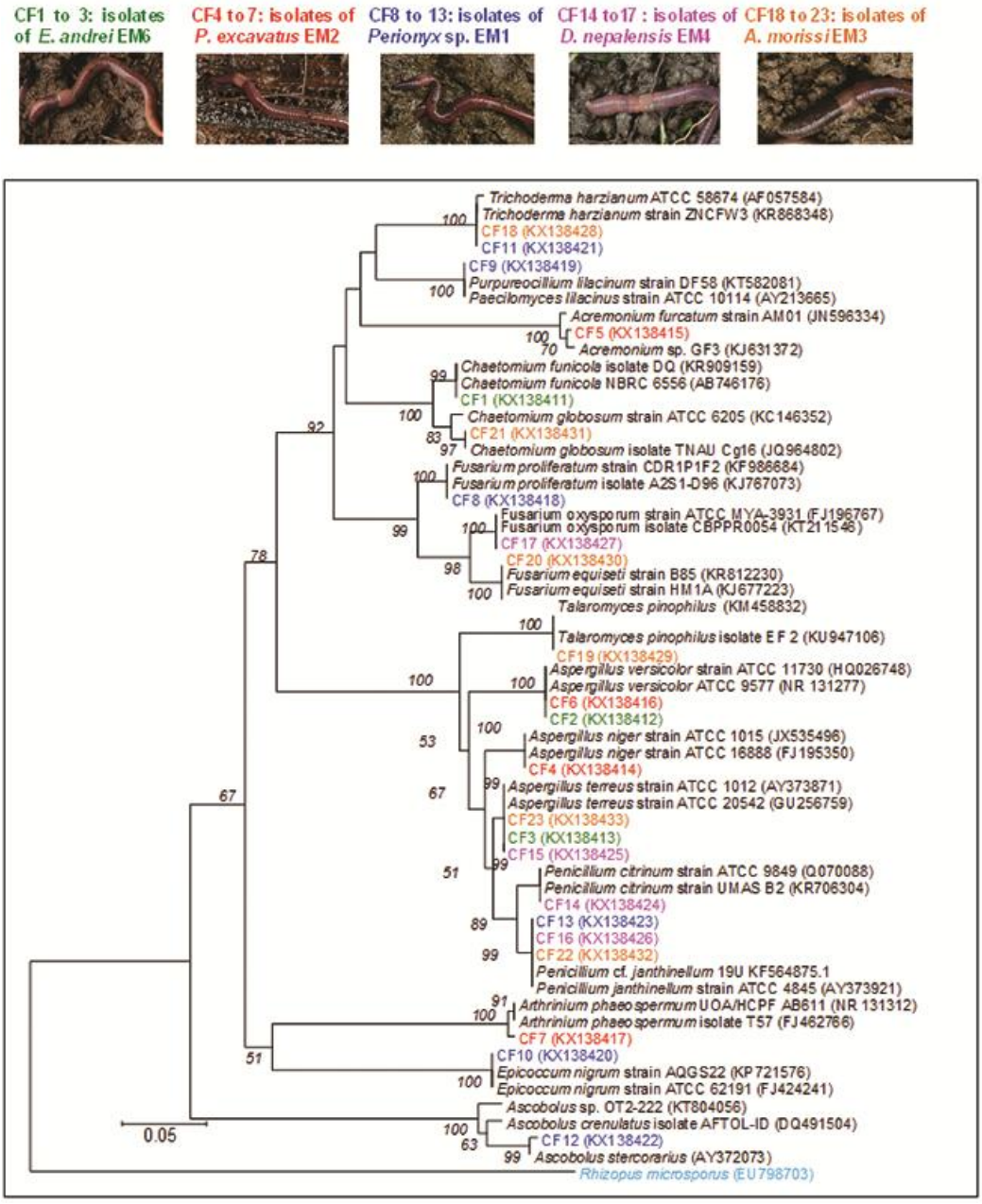

Fig. 9 — NJ phylogenetic tree of the cellulolytic fungal isolates based on ITS region

cellulolytic in the earthworm gut (Fujii *et al.* 2012). The differences in the microbial communities obtained by the different researchers might be due to the difference in the earthworm species or the diverse habitats which differ in biotic and abiotic factors or in the use of media and the carbon source for the isolation of microorganisms.

In the phylogenetic analyses, some cellulolytic AB (CB 2, 5, 6, 8 and 12), FAB (FAB 1, 7, 8, 9, 10, 11, 12, 13, 14, 15 and 16), actinobacteria (CAE 5, 7, 10, 13, 15, 18 and 22) and fungi (CF 5, 7 and 12) exhibited differences from the reference strain sequences and formed distinct genetic lineages. They could represent potentially new cellulolytic strains which should be confirmed in future through full length sequencing for 16S rRNA and ITS1-5.8S-ITS2 genes. The five actinobacterial isolates namely CAE 1, 11, 14, 20 and 44 were grouped into a single cluster with different reference strains namely *S. microflavus*, *S. griseoplanus*, *S. badius* and *S. mediolani*. This may be due to the fact that we did only reverse primer sequencing of the 16S rRNA gene

and hence the length of the sequence may be short to resolve these five isolates to species level and they may represent different species of the *Streptomyces* genus. The present study demonstrates that the gut of the different earthworm species feeding on various substrates harbors a diverse community of cellulolytic microorganisms and is the first report on cellulolytic microbial diversity of *Amynthas morissi*, *Drawida nepalensis* and a *Perionyx* sp. from North East India.

Acknowledgements

This research was financially supported by the Institute of Bioresources and Sustainable Development (IBSD), Department of Biotechnology, Government of India in the form of Junior Research Fellowship for the PhD program. We would also like to appreciate Mr. Dhaneshwor, Kiran Ningthoujam and Dilip for their support during sample collection.

References

1 Hornor S G & Mitchell M J, Effect of the earthworm *Eisenia foetida* (Oligochaeta) on fluxes of volatile carbon and sulphur compounds from sewage sludge, *Soil Biol Biochem*, 13 (1981) 367-372.

2 Lee K E, Earthworms: Their ecology and relationship with soil and land use. Australia: Academic Press, 1985.

3 Maboeta M S & Van Rensburg L, Vermicomposting of industrially produced woodchips and sewage sludge utilizing *Eisenia fetida*, *Ecotoxicol Environ Saf*, 56 (2003) 265-270.

4 Fujii K, Ikeda K & Yoshida S, Isolation and characterization of aerobic microorganisms with cellulolytic activity in the gut of endogeic earthworms, *Int Microbiol*, 15 (2012) 121-130.

5 Nozaki M, Miura C, Tozawa Y & Miur T, The contribution of endogenous cellulase to the cellulose digestion in the gut of earthworm (*Pheretima hilgendorfi*: Megascolecidae), *Soil Biol Biochem*, 41 (2009) 762-769.

6 Lynd L R, Weimer P J, Van Zyl W H & Pretorius I S, Microbial cellulose utilization: Fundamentals and biotechnology, *Microbiol Mol Biol Rev*, 66 (2002) 506-577.

7 Barois I, Mucus production and microbial activity in the gut of two species of *Amynthas* (Megascolecidae) from cold and warm tropical climates, *Soil Biol Biochem*, 24 (1992) 1507-1510.

8 Trigo D & Lavelle P, Changes in respiration rate and some physicochemical properties of soil during gut transit through *Allolobophora molleri* (Lumbricidae, Oligochaeta), *Biol Fertil Soils*, 15 (1993) 185-188.

9 Marks D H & Cooper E L, *Aeromonas hydrophila* in the coelomic cavity of the earthworms *Lumbricus terrestris* and *Eisenia foetida*, *J Invertebr Pathol*, 29 (1977) 382-383.

10 Contreras E, Studies on the intestinal actinomycete flora of *Eisenia lucens* (Annelida: Oligochaeta), *Pedobiologia*, 20 (1980) 411-416.

11 Brown G G, Barois I & Lavelle P, Regulation of soil organic matter dynamics and microbial activity in the drilosphere and the role of interactions with other edaphic functional domains, *Eur J Soil Biol*, 36 (2000) 177-198.

12 Owa S O, Olowoparija S B, Aladesida A & Dedeke G A, Enteric bacteria and fungi of the Eudrilid earthworm *Libyodrilus violaceus*, *Afr J Agric Res*, 8 (2013) 1760-1766.

13 Parle J N, Microorganisms in the intestines of earthworms, *J Gen Microbiol*, 31 (1963) 1-11.

14 Kris ˇtufek V, Ravasz K & Pizl V, Changes in densities of bacteria and microfungi during gut transit in *Lumbricus rubellus* and *Aporrectodea caliginosa* (Oligochaeta: Lumbricidae), *Soil Biol Biochem*, 24 (1992) 1499-1500.

15 Karsten G R & Drake H L, Denitrifying bacteria in the earthworm gastrointestinal tract and *in vivo* emission of nitrous oxide (N_2O) by earthworms, *Appl Environ Microbiol*, 63 (1997) 1878-1882.

16 Furlong M A, Singleton D R, Coleman D C & Whitman W B, Molecular and culture-based analyses of prokaryotic communities from an agricultural soil and the burrows and casts of the earthworm *Lumbricus rubellus*, *Appl Environ Microbiol*, 68 (200) 1265-1279.

17 Ihssen J, Horn M A, Matthies C, Goβ ner A, Schramm A *et al*, N_2O-producing microorganisms in the gut of the earthworm *Aporrectodea caliginosa* are indicative of ingested soil bacteria, *Appl Environ Microbiol*, 69 (2003) 1655-1661.

18 Singleton D R, Hendrix P F, Coleman D C & Whitman W B, Identification of uncultured bacteria tightly associated with the intestine of the earthworm *Lumbricus rubellus* (Lumbricidae; Oligochaeta), *Soil Biol Biochem*, 35 (2003) 1547-1555.

19 Horn M A, Ihssen J, Matthies C, Schramm A, Acker G *et al*, *Dechloromonas denitrificans* sp. nov., *Flavobacterium denitrificans* sp. nov., *Paenibacillus anaericanus* sp. nov. and *Paenibacillus terrae* strain MH72, N_2O-producing bacteria isolated from the gut of the earthworm *Aporrectodea caliginosa*, *Int J Syst Evol Microbiol*, 55 (2005) 1255-1265.

20 Byzov B A, Nechitaylo T Y, Bumazhkin B K, Kurakov A V, Golyshin P N *et al*, Culturable microorganisms from the earthworm digestive tract, *Microbiology*, 78 (2009) 360-368.

21 Parthasarathi K, Ranganathan L S, Anandi V & Zeyer J, Diversity of microflora in the gut and casts of tropical composting earthworms reared on different substrates, *J Environ Biol*, 28 *(*2007) 87-97.

22 Brito-Vega H & Espinosa-Victoria D, Bacterial diversity in the digestive tract of earthworms (Oligochaeta), *J Biol Sci*, 9 (2009) 192-199.

23 Thakuria D, Schmidt O, Finan D, Egan D & Doohan F M, Gut wall bacteria of earthworms: A natural selection process, *ISME Journal*, 4 (2010) 357-366.

24 Dey K K, Talukdar N C, Nongkhlaw F M W & Thakuria D, Isolation, characterization and practical significance of cellulose degrading bacteria from the gut wall of two ecologically distinct earthworms, *Curr Sci*, 114 (2018) 1474-1484.

25 Singhania R R, Patel A K, Sukumaran, R K, Larroche C & Pandey A, Role and significance of β-glucosidases in the hydrolysis of cellulose for bioethanol production, *Bioresour Technol*, 127 (2013) 500-507.

26 Zhang X Z & Zhang Y H P, Cellulases: Characteristics, sources, production, and applications, in: Bioprocess. bioprocessing technologies in biorefinery for sustainable Production of Fuels, Chemicals, and Polymers, (2013) 131-146.

27 Santhi V S, Bhagat A K, Saranya S, Govindarajan G & Jebakumar S R D, Seaweed (*Eucheuma cottonii*) associated

microorganisms, a versatile enzyme source for the lignocellulosic biomass processing, *Int Biodeterior Biodegradation*, 96 (2014) 144-151.
28 Kuhad R C, Gupta R & Singh A, Microbial cellulases and their industrial applications, *Enzyme Res*, 2011 (2011) 1–10.
29 Acharya S & Chaudhary A, Bioprospecting thermophiles for cellulase production: A review, *Braz J Microbiol*, 43 (2012) 844-856.
30 Dashtban M, Schraft H & Qin W, Fungal bioconversion of lignocellulosic residues: Opportunities and perspectives, *Int J Biol Sci*, 5 (2009) 578-595.
31 Khokhar I, Haider M S, Mushtaq S & Mukhtar I, Isolation and screening of highly cellulolytic filamentous fungi, *JASEM*, 16 (2012) 223-226.
32 Gusakov A V & Sinitsyn A P, Cellulases from *Penicillium* species for producing fuels from biomass, *Biofuels*, 3 (2012) 463-477.
33 Maki M, Leung K T & Qin W, The prospects of cellulase-producing bacteria for the bioconversion of lignocellulosic biomass, *Int J Biol Sci*, 5 (2009) 500-516.
34 Ponnambalam A S, Deepthi R S & Ghosh A R, Qualitative display and measurement of enzyme activity of isolated cellulolytic bacteria, *Biotechnol Bioinf Bioeng*, 1 (2011) 33-37.
35 Soares F L, Melo I S, Dias A C F. & Andreote F D, Cellulolytic bacteria from soils in harsh environments, *World J Microbiol Biotechnol*, 28 (2012) 2195-2203.
36 Liu J M, Xin X J, Li C X, Xu J H & Bao J, Cloning of thermostable cellulase genes of *Clostridium thermocellum* and their secretive expression in *Bacillus subtilis*, *Appl Biochem Biotechnol*, 166 (2012) 652–662.
37 Sindhu R, Kuttiraja M, Binod, P, Sukumaran R K & Pandey A, Bioethanol production from dilute acid pretreated Indian bamboo variety (*Dendrocalamus* sp.) by separate hydrolysis and fermentation, *Ind Crop Prod*, 52 (2014) 169-176.
38 Harshvardhan K, Mishra, A & Jha B, Purification and characterization of cellulase from a marine *Bacillus* sp. H1666: A potential agent for single step saccharification of seaweed biomass, *J Mol Catal B Enzy*, 93 (2013) 51-56.
39 Maki M L, Broere M, Leung K T & Qin W, Characterization of some efficient cellulase producing bacteria isolated from paper mill sludges and organic fertilizers, *Int J Biochem Mol Biol*, 2 (2011) 146-154.
40 Sadhu S, Saha P, Mayilraj S & Maiti T K, Lactose-enhanced cellulase production by *Microbacterium* sp. isolated from fecal matter of zebra (*Equus zebra*), *Curr Microbiol*, 62 (2011) 1050-1055.
41 Kasana R C, Salwan R, Dhar H, Dutt S & Gulati A, A rapid and easy method for the detection of microbial cellulases on agar plates using Gram's iodine, *Curr Microbiol*, 57 (2008) 503-507.
42 Teather R M & Wood P J, Use of Congo red–polysaccharide interactions in enumeration and characterization of cellulolytic bacteria from the bovine rumen, *Appl Environ Microbiol*, 43 (1982) 777-780.
43 Reese E T, Sui R G H & Levinson H S, The biological degradation of soluble cellulose derivatives and its relationship to the mechanism of cellulose hydrolysis, *J Bacteriol*, 59 (1950) 485-497.
44 Hendricks C W, Doyle J D & Hugley B, A new solid medium for enumerating cellulose-utilizing bacteria in soil, *Appl Environ Microbiol*, 61 (1995) 2016-2019.
45 Hankin L & Anagnostakis S, Solid media containing carboxy methyl cellulose to detect CM cellulase activity of microorganisms, *J Gen Microbiol*, 98 (1977) 109-115.
46 Miller G L, Use of dinitrosalicylic acid reagent for determination of reducing sugar, *Anal Chem*, 31 (1959) 426-428.
47 Sambrook J, Fritsch E F & Maniatis T, Molecular cloning: A laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y, USA, 1989.
48 Cenis J L, Rapid extraction of fungal DNA for PCR amplification, *Nucleic Acids Res*, 20 (1990) 2380.
49 Devi S S, Devi N G, Kalita M C & Talukdar N C, Isolation and selection of cellulose-degrading microorganisms for utilization along with earthworms in efficient conversion of municipality waste mix to compost, *Curr Sci*, 114 (2018) 1261-1274.
50 White T J, Bruns T & Lee S, Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (ed) PCR Protocols: a Guide to Methods and Applications. Academic, New York, 1990, 315-322.
51 Hall T A, BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT, *Nucleic Acids Symp Ser*, 41 (1999) 95-98.
52 Altschul S F, Thomas L M, Alejandro A S, Jinghu Z, Zheng Z *et al*, Gapped BLAST and PSI-BLAST: A new generation of protein database search programs, *Nucleic Acids Res*, 25 (1997) 3389-3402.
53 Saitou N & Nei M, The neighbor-joining method: A new method for reconstructing phylogenetic trees, *Mol Biol Evol*, 4 (1987) 406-425.
54 Tamura K, Stecher G, Peterson D, Filipski A & Kumar S, MEGA6: Molecular Evolutionary Genetics Analysis version 6.0., *Mol Biol Evol*, 30 (2013) 2725-2729.
55 Kimura M, A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences, *J Mol Evol*, 16 (1980) 111-120.
56 Skinner F A, The isolation of an anaerobic cellulose decomposing bacterium from soil, *J Gen Microbiol*, 22 (1960) 539-554.
57 Lu W J, Wang H T, Yang S J, Wang Z C & Nie Y F, Isolation and characterization of mesophilic cellulose-degrading bacteria from flower stalks-vegetable waste co-composting system, *J Gen Appl Microbiol*, 51 (2005) 353-360.
58 Masson R H, Bryder M J & Book N J, Isolation and characterization of an anaerobic cellulolytic bacteria, *Clostridium papyrosolvens* sp. nov, *Int J Syst Bacteriol*, 32 (1982) 87–91.
59 Paudel Y P & Qin W, Characterization of novel cellulase-producing bacteria isolated from rotting wood samples, *Appl Biochem Biotechnol*, 177 (2015) 1186-1198.
60 Huang S, Sheng P & Zhang H, Isolation and identification of cellulolytic bacteria from the gut of *Holotrichia parallela* larvae (Coleoptera: Scarabaeidae), *Int J Mol Sci*, 13 (2012) 2563-2577.
61 Pedersen J C & Hendriksen N B, Effect of passage through the intestinal tract of detritivore earthworms (*Lumbricus* sp.) on the number of selected Gram negative and total bacteria, *Biol Fertil Soils*, 16 (1993) 227-232.
62 Lavelle P, Bignell D, Lepage M, Wolters V, Roger P *et al*, Soil function in a changing world: The role of

invertebrate ecosystem engineers, *Eur J Soil Biol*, 33 (1997) 159-193.

63 Edwards C A, The importance of earthworms as key representatives of soil fauna. In: Edwards, C.A. (Ed.), Earthworm Ecology, CRC Press, Boca Raton, 2, 2004, 3-11.

64 Immanuel G, Dhanusha R, Prema P & Palavesam A, Effect of different growth parameters on endoglucanase enzyme activity by bacteria isolated from coir retting effluents of estuarine environment, *Int J Environ Sci Technol*, 3 (2006) 25-34.

65 Chantarasiri A, Boontanom P, Yensaysuk N & Ajwichai P, Identification of a cellulase-producing *Bacillus* sp. strain BR0302 from Thai coastal wetland soil, *KMUTNB, Int J Appl Sci Technol*, 8 (2015) 197-203.

66 Goyal V, Mittal A, Bhuwal A K, Singh G, Yadav A *et al*, Parametric optimization of cultural conditions for carboxymethyl cellulose production using pretreated rice straw by *Bacillus* sp. 313SI under stationary and shaking conditions, *Biotechnol Res Int*, 2014 (2014) 651839.

67 Yang W, Meng F, Peng J, Han P, Fang F *et al*, Isolation and identification of a cellulolytic bacterium from the Tibetan pig's intestine and investigation of its cellulase production, *Electronic J Biotechnol*, 17 (2014) 262-267.

68 Adlakha N, Sawant S, Anil A, Lali A & Yazdani S S, Specific fusion of β-1,4-endoglucanase and β-1,4-glucosidase enhances cellulolytic activity and helps in channeling of intermediates, *Appl Environ Microbiol*, 78 (2012) 7447-7454.

69 Karsten G R & Drak H L, Comparative assessment of the aerobic and anaerobic microfloras of earthworm guts and forest soils, *Appl Environ Microbiol*, 61 (1995) 1039-1044.

70 Wenzel M, Schönig I, Berchtold M, Kämpfer P & König H, Aerobic and facultatively anaerobic cellulolytic bacteria from the gut of the termite *Zootermopsis angusticollis, J Appl Microbiol*, 92 (200) 32-40.

71 Citernesi U, Neglia R, Seritti A, Lepidi A, Filippi C *et al*, Nitrogen fixation in the gastro-enteric cavity of soil animals, *Soil Biol Biochem*, 9 (1977) 71-72.

72 George J, Arunachalam R, Paulkumar K, Wesely E G, Shiburaj S *et al*, Characterization and phylogenetic analysis of cellulase producing *Streptomyces noboritoensis* SPKC1, *Interdiscip Sci*, 2010 (2010) 205-212.