Enrichment and Identification of Cellulolytic Bacteria from the Gastrointestinal Tract of Giant African Snail, *Achatina fulica*

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Abstract The cellulolytic bacterial community structure in gastrointestinal (GI) tract of *Achatina fulica* was studied using culture-independent and -dependent methods by enrichment in carboxymethyl cellulose (CMC). Culture-dependent method indicated that GI tract of snail was dominated by Enterobacteriaceae members. When tested for cellulase activities, all isolates obtained by culture-dependent method showed both or either of CMCase or avicelase activity. Isolate identified as *Citrobacter freundii* showed highest CMCase and medium avicelase activity. Sequencing of clones from the 16S rRNA gene clone library identified ten operational taxonomic units (OTUs), which were affiliated to Enterobacteriaceae of phylum Gammaproteobacteria. Of these ten OTUs, eight OTUs closely matched with *Enterobacter* and *Klebsiella* genera. The most abundant OTU allied to *Klebsiella oxytoca* accounted for 70 % of the total sequences. The members of *Klebsiella* and *Enterobacter* were observed by both methods indicating their dominance among the cellulolytic bacterial community in the GI tract of the snail.

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Introduction

Lignocellulosic plant biomass is an important renewable carbon resource for the biorefinery industry and is thus considered a sustainable and eco-friendly alternative to the current petroleum platform [1]. With the recent rise in oil prices, along with growing concern about global warming caused by carbon dioxide emissions, biofuels have been regaining popularity around the globe. Interest in bioenergy has been sharply increasing in recent years due to the necessity of sustainable economies and clean environments [2–4]. There is a strong impetus, both nationally and internationally for devising new, nonfossil-based fuels that are generated in a sustainable way with minimum greenhouse gas production. Cellulose, the major component of lignocellulosic biomass, can be hydrolyzed to glucose by cellulase enzymes. Intensive efforts have been made in recent years to develop efficient technologies for the pretreatment of lignocellulosic biomass and developments of enzymes for enhanced cellulose/hemicellulose saccharification [5]. In comparison to acid hydrolysis and thermochemical processes, enzymatic hydrolysis of lignocellulosic biomass is considered more economic and eco-friendly [6]. In nature, lignocellulosic biomass is degraded with the cooperation of many microorganisms, mainly including diverse fungal and bacterial genera producing a variety of cellulolytic and hemi-cellulolytic enzymes under aerobic and anaerobic conditions [7]. The biodegradation of cellulosic biomass through the use of microbial co-cultures or complex communities has been proposed as a highly efficient approach for biotechnological application, since it avoids the problems of feedback regulation and metabolite repression posed by isolated single strains [8–10]. Improvement of microbial and enzymatic processes on lignocellulosic biomass degradation is thus an important area of research in sustainable "green" biotechnology [11]. Isolation, screening and selection have resulted the discovery of several novel cellulase-producing bacteria from a wide variety of environments [12]. Some insects and molluscs are apparently cellulose-degrading organisms and degrade cellulose with the aid of symbiotic gut microorganisms.

Terrestrial gastropods like Giant African snail, *Achatina* (*Lissachatina*) *fulica* Bowdich, 1822, feed primarily on vascular plants [13]. This snail also participates, with other soil invertebrates, in the decomposition of leaf litter [14]. Consequently, a large set of cellulolytic bacteria inhabit the gastrointestinal (GI) tract of this snail. Recently, the bacterial diversity in different regions of GI tract of this snail was found to harbour high bacterial diversity [15]. The complexity of bacterial communities occurring in GI tract regions such as crop and intestine of the field-collected *A. fulica* were assessed and then compared with those from groups of snails that were reared in the laboratory on a sugarcane-based diet [16]. Also, recent metagenomic analysis of microbiota of crop of this snail revealed an abundance of sequences coding for oligosaccharide-degrading enzymes (36 %) as well as many novel cellulase and hemicellulase coding sequences [17]. These reports and the herbivorous pest nature of this snail led us to hypothesize that *A. fulica* is more likely to contain diverse cellulose-degrading bacteria.

To test our hypothesis, we investigated the whole GI tract of *A. fulica* as a source for isolation of cellulolytic bacteria which would thus be potentially useful for hydrolysis of

lignocellulosic substrates. The cellulolytic bacterial community enriched in carboxymethyl cellulose (CMC) was identified using culture-independent methods based on 16S rRNA gene clone library and sequencing.

Materials and Methods

Sampling of Snail and Dissection

For enrichment of cellulolytic bacterial community from snail gut, snails ($n=5$) in active state were collected from different locations around Pune, Maharashtra, India, and immediately brought to laboratory and processed as described previously [15].

Enrichment of Cellulolytic Bacterial Community

The enrichment of cellulolytic bacterial community was done in Berg's minimal salt (BMS) medium that contained the following salts in grams per litre: NaNO$_3$, 2 g; K$_2$HPO$_4$, 0.5 g; MgSO$_4$·7H$_2$O, 0.02 g; MnSO$_4$·7H$_2$O, 0.02 g; FeSO$_4$·7H$_2$0, 0.02 g and CaCl$_2$·2H$_2$O, 5 g. The gut tissues were weighed, homogenized in 0.8 % saline in a sterile glass homogenizer, and then, 5 ml of the gut homogenate was inoculated in 50 ml BMS supplemented with 0.5 % CMC (*w/v*). The inoculated media was then incubated on a shaker at 120 rpm, 37 °C for 3 days. After incubation for 3 days, 5 ml of 3-day-old culture was transferred to fresh BMS (45 ml) with 0.5 % CMC (*w/v*). The remaining 45 ml culture was used to monitor the cellulolytic activities by estimating the activities of enzymes such as CMCase and avicelase. These procedures of transfer to fresh medium and estimation of cellulolytic activities were repeated till 21 days (for seven transfers).

Enzyme Assays

Samples collected at the time of each transfer were centrifuged at 10,000 rpm for 10 min at RT. Endoglucanase (CMCase) and exoglucanase (avicelase) activities were determined according to the method described by Nitisinprasert and Temmes [18]. The estimation of proteins was carried out by Bradford method [19] using BSA as standard.

Isolation and Identification of Cellulolytic Bacteria from Enriched Culture

Isolation of cellulolytic bacteria was done from the enriched culture that showed maximum CMCase and avicelase activities. One millilitre of enriched culture was serially diluted up to 10^{-8}, and 100 µl of each dilution was plated on BMS agar medium with 0.5 % CMC. The plates were incubated at 37 °C for 2–3 days and observed for bacterial growth. Colonies were classified based on morphological parameters of shape, colour, margins, elevation and texture. Representatives of each colony morphology were purified by sub-culturing. Extraction of total genomic DNA from purified isolates, amplification of 16S rRNA gene and sequencing were performed as described previously [20]. The sequences representing each bacterial isolate were compared with NCBI database using BLASTn, and the closest matches to bacterial isolates were retrieved.

Screenings for Cellulolytic Activity of Bacterial Isolates

Screenings of the cellulolytic activity of the isolated bacteria were carried out by using plate-based technique. All the bacterial isolates obtained were patched (patch size ≤0.5 mm) separately on BMS Agar plates with 0.5 % CMC. After incubation for 3 days at 37 °C, plates were observed for proper growth and flooded with 5 ml of Gram's iodine (2.0 g KI and 1.0 g iodine in 300 ml of distilled water) for 5 min. The excess stain was removed, and plates were observed for zone of clearance around the colonies. Based on the diameter of the zone of clearance, cultures were identified as having low (0.5–2 cm), medium (2–4 cm) and high (>4 cm) cellulase activity.

Identification of Uncultured Cellulolytic Bacteria from Enriched Culture

Community genomic DNA from enriched culture was extracted by using DNeasy Blood Tissue Kit (Qiagen, UK) following the manufacturer's instructions. For construction of 16S rRNA gene library, genomic DNA was used as template in PCR reaction with the bacteria-specific primer pair 27F (5′-AGAGTTTGATYMTGGCTCAG) and 907R (5′-CCGTCAATTCMTTTGAGTTT) [21]. The 50-μl PCR reaction consisted of 5 μl (8 ng μl^{-1}) of template DNA, 5 μl of Taq buffer (Bangalore Genei, India), 0.25 mmol l^{-1} dNTPs (Bangalore Genei, India), 10 pmol of each primers and 0.6 U of Taq polymerase (Bangalore Genei, India). The thermal cycling conditions were set with the following program: an initial 5-min denaturation at 95 °C, 35 cycles of denaturation (1 min at 94 °C), annealing (1 min at 55 °C) and extension (1 min at 72 °C) and a final 7-min extension at 72 °C. PCR reaction was carried out in triplicate, and products were checked for size and purity on 1 % (w/v) agarose gels. The PCR product was purified using PEG–NaCl precipitation [22], and TA cloning was performed using a TOPO TA cloning kit (Invitrogen, Bangalore, India). One hundred and twenty-five clones were randomly picked, and sequencing was performed using the ABI Big-Dye version 3.1 sequencing kit as per the manufacturer's instructions, with both M13F and M13R primers. The generated sequences were analyzed using ChromasPro software (http://www.technelysium.com.au/ChromasPro.html) and compared with the current database of nucleotide sequences at GenBank and Ribosomal Database Project (RDP). Reference sequences were chosen on the basis of BLASTn similarities. All 16S rRNA gene sequences were checked for possible chimeric artifacts using the Pintail program [23] in conjunction with Bellerophon [24]. Multiple sequence alignments of 16S rRNA gene sequences were performed with ClustalX [25] and were edited manually using DAMBE [26] to obtain an unambiguous sequence alignment. Nucleotide distance matrices were constructed with DNADIST from PHYLIP version 3.61 [27] using the Kimura two-parameter model [28]. Operational taxonomic units (OTUs) were generated using the DOTUR program [29] at 97 % sequence similarity cutoff. The sequences representing each OTU were compared with NCBI database using BLASTn, and the closest matches to bacterial strains were obtained. Phylogenetic tree was constructed by the neighbour-joining method using Kimura 2 parameter distances in MEGA 5.0 software [30].

Nucleotide Sequence Accession Numbers

All the 16S rRNA sequences generated from cultured isolates and clone library from this study have been deposited at the NCBI GenBank database with the following accession numbers: clone library, KF434591-KF434600; isolates, KF434601-KF434614.

Results and Discussion

Despite the reports by Pawar et al. [15] and Cordoso et al. [17] on economically and ecologically important snail, *A. fulica*, little is known about the diversity and composition of cellulolytic bacterial flora within its gut.

Enrichment and Identification of Culturable Cellulolytic Bacteria

In this study, we investigated the cellulolytic bacterial community of this snail gut using both culture-dependent and culture-independent approaches. The cellulolytic bacterial community was enriched in CMC as substrate and characterized. During enrichment, the culture remained after sub-cultivation was used to monitor the progress of enrichment by estimating cellulolytic activities, bacterial load by total viable count (TVC) and 16S rDNA copy number by real-time PCR (qPCR) analysis. For estimation of TVC and 16S rDNA copy number by qPCR analysis, methods used previously were followed [15]. TVC and qPCR analysis indicated that culture had the highest bacterial load at first sub-cultivation which gradually decreased over the period of enrichment. The enriched culture contained 4.6×10^3 CFU and 2.4×10^4 copies of 16S rDNA/ml of enriched culture (Fig. 1). Maximum CMCase and avicelase activities were observed on 15th (before fifth sub-cultivation) and 18th (before sixth sub-cultivation) days of incubation, respectively (Fig. 2). Therefore, the enriched culturable cellulolytic bacterial community was grown, identified and screened after 15 days of incubation.

Of 72 isolates purified and screened initially, 14 distinct isolates were obtained (NCBI GenBank database accession numbers KF434601-KF434614). Out of these, 13 belonged to phylum Gammaproteobacteria and one to phylum Alphaproteobacteria. Based on the 16S rRNA gene sequences, isolates belonging to Gammaproteobacteria showed their closest matches to seven distinct genera, namely *Citrobacter*, *Escherichia*, *Klebsiella*, *Salmonella*, *Raoultella*, *Stenotrophomonas* and *Enterobacter*, whereas single isolate belonging to phylum Alphaproteobacteria was the member of
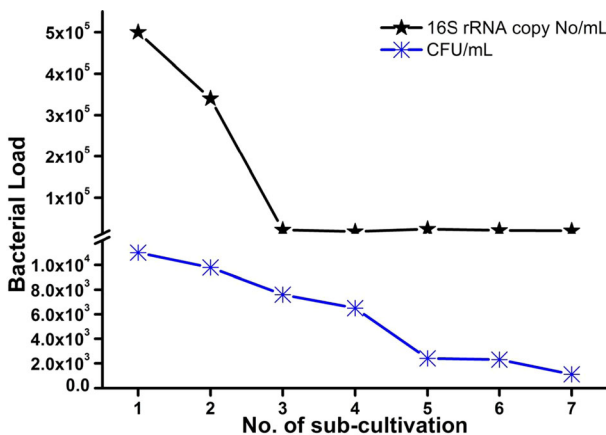

Fig. 1 Pattern of change in total bacterial load (total viable count and 16S rRNA copy number) over the period of enrichment. Total genomic DNA was isolated from liquid cultures sampled before each sub-cultuvation over the period of 21 days. Purified *Citrobacter freundii* DNA was used in 1:10 dilution series (10 pg–100 ng) as a standard. The CT values were determined on the basis of the fluorescence signals at the mean baseline during the early cycles of amplification. PCR efficiency was calculated by using the following equation: efficiency=10 (−1/ slope) −1
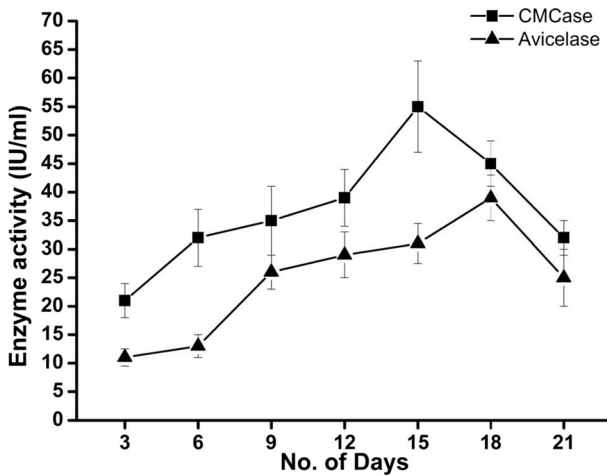
Fig. 2 Pattern of change in enzyme activities (CMCase and avicelase) over the period of enrichment. The progress of enrichment was monitored by estimating the CMCase and avicelase activities in cultures sampled before each sub-cultuvation over the period of 21 days

genus *Ochrobactrum* (Table 1). Thus, using culture-dependent approach, it was observed that the gut of snail was dominated by a diverse group of family Enterobacteriaceae of Gammaproteobacteria.

Cellulose Degradation Assay of Bacterial Isolates

Screenings of the cellulolytic activity of the isolated bacteria were carried out by using plate-based technique. All of the isolates showed both or either of CMCase and avicelase activity. Among 14 isolates, 11 isolates showed CMCase activity, four isolates showed avicelase activity whereas only two isolates showed both enzyme activities. As the enrichment was done in CMC, the abundance of bacteria with CMCase activity in enriched culture was obvious. Isolate identified as *Citrobacter freundii* showed highest CMCase and medium avicelase activity (Table 1). Plate-based CMCase and avicelase assays revealed that the snail gut bacteria were able to degrade cellulosic substrates such as CMC and avicel indicating their possible contribution to plant polymer degradation inside the gut (Suppl. Fig. S1).

Identification of Uncultured Cellulolytic Bacteria from Enriched Culture

For identification of uncultivable cellulolytic bacteria from enriched culture, 16S rRNA gene clone library was constructed from community genomic DNA isolated from the enriched culture. Isolation of genomic DNA, construction of 16S rRNA gene clone library, sequencing and phylogenetic analysis were done as described previously [15]. From the 16S rRNA gene clone library, 125 clones were randomly picked and sequenced. After removing 13 chimeric sequences, 112 good quality sequences were obtained and analyzed further. DOTUR analysis of these 112 sequences identified 10 OTUs, all of which were phylogenetically affiliated to family Enterobacteriaceae of phylum Gammaproteobacteria. All the 16S OTU sequences generated from clone library have been deposited at the NCBI GenBank database with the following accession numbers: KF434591-KF434600. Of these ten OTUs observed, eight showed their closest matches to two main genera, namely *Enterobacter* and *Klebsiella*, while

Table 1 Phylogenetic affiliation of representative isolates of bacterial isolates enriched from gut of *A. fulica* in CMC

Isolate code	Taxonomic affiliation (phylum)	Closest relative	NCBI accession no.	% similarity	CMCase activity	Avicelase activity
SNISO_A2	Gammaproteobacteria	*Citrobacter freundii* strain DSM 30039	NR_028894	99	[c]	[b]
SNISO_B12	Gammaproteobacteria	*Citrobacte rmurliniae* strain CDC 2970-59	NR_028688	99	[b]	ND
SNISO_C1	Gammaproteobacteria	*Klebsiella oxytoca* KCTC 168	NR_102982	99	[b]	ND
SNISO_C6	Gammaproteobacteria	*Salmonella bongori* strain BR 1859	NR_041699	99	[a]	ND
SNISO_D1	Gammaproteobacteria	*Raoultella ornithinolytica* B	NR_102983	95	[a]	[a]
SNISO_E1	Alphaproteobacteria	*Ochrobactrum anthropi* strain CNS 2-75	NR_026039	99	[a]	ND
SNISO_E3	Gammaproteobacteria	*Stenotrophomonas maltophilia* strain IAM 12423	NR_041577	99	ND	ND
SNISO_E12	Gammaproteobacteria	*Enterobacter asburiae* strain JCM6051	NR_024640	99	[a]	ND
SNISO_F2	Gammaproteobacteria	*Enterobacter cloacae* strain 279-56	NR_028912	99	[a]	ND
SNISO_F3	Gammaproteobacteria	*Enterobacter cancerogenus* strain LMG 2693	NR_04497	99	[a]	ND
SNISO_F4	Gammaproteobacteria	*Klebsiella pneumoniae* strain DSM 30104	NR_036794	99	ND	[a]
SNISO_I12	Gammaproteobacteria	*Citrobacteryoungae* strain GTC 1314	NR_041527	98	ND	[a]
SNISO_J2	Gammaproteobacteria	*Escherichia coli* O157:H7 str. Sakai	NR_074891	98	[a]	ND
SNISO_J4	Gammaproteobacteria	*Salmonella enterica* subsp. *Enteric* sero var Typhimurium str. LT2	NR_074910			
	98	[a]		ND		

ND activity not detected

[a] Low enzyme activity (zone of clearance between 0.5 and 2 cm)

[b] Medium enzyme activity (zone of clearance between 2 and 4 cm)

[c] High enzyme activity (zone of clearance >4)

remaining two OTUs were the members of unclassified Enterobacteriaceae. The most abundant OTU (SNGUT C4) was phylogenetically affiliated to *Klebsiella oxytoca* which accounted for 70 % of the total sequences. Besides, three next most abundant OTUs, namely SNGUT L5 (6 %), SNGUT L6 (6 %) and SNGUT L8 (5 %), together accounted for 17 % of the total number of sequences and were also related to members of genus *Klebsiella* (Fig. 3). This clearly indicated that the members of genus *Klebsiella* dominated the cellulolytic bacterial community in the snail gut.
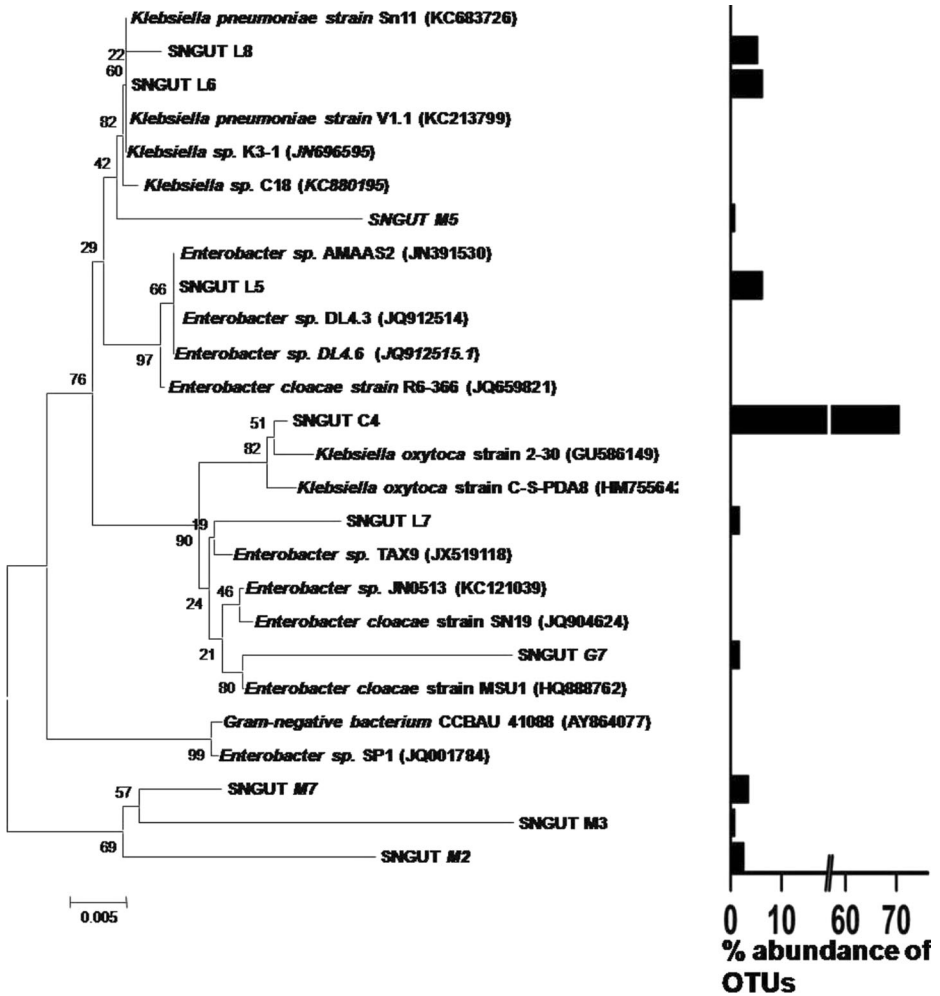
Fig. 3 Phylogenetic tree of the 16S rRNA gene and their closest related strains from the GenBank. The tree was generated by using the neighbour-joining method with Kimura 2 parameter distances in MEGA 4.0 software. *Numbers at nodes* indicate percent bootstrap values calculated based on 1000 replicates. The *bar* indicates the Jukes–Cantor evolutionary distance. Accession numbers of the nearest neighbours are given in parentheses. The *bar diagram* represents the percentage distribution of each OTU

In our study, the cellulolytic members of genera *Klebsiella* and *Enterobacter* were observed by both culture-dependent and -independent methods supporting further our observation that the members of these genera are dominating the cellulolytic community in the snail gut. The sequences related to members of other genera such as *Citrobacter*, *Salmonella*, *Raoultella*, *Ochrobactrum*, *Escherichia* and *Stenotrophomonas* were observed by only culture-dependent method. This indicated that the member of these genera made up a minor portion of the cellulolytic community; however, they might still be playing an important role in the cellulose degradation process. In comparison between the two approaches used to study the cellulolytic bacterial community structure, culture-dependent method was found more suitable since it isolated and identified the members that made up minor portion of the cellulolytic community.

Previously, Park et al. [31] reported that more than half of the bacteria isolated from the guts of Longicorn Beetle species were belonging to phylum Gammaproteobacteria. In the present study, Gram-negative members of the Enterobacteriacae predominated which are commonly reported in other Arthropods, particularly in saprophagous *Glomeris marginata* and *Oniscus asellus* [32, 33], wood-feeding termites *Reticulitermes flavipes* [34] and the phloem-feeding aphid *Buchnera* sp. [35].

The observation as Gammaproteobacteria being the most abundant phylum in snail gut is in congruence with our previous study in which, based on culture-dependent and culture-independent methods, sequences related to Gammaproteobacteria were observed most abundant in different gut regions [15]. However, the present study differs from our previous one in having studied the cellulolytic bacterial community enriched in CMC. To the best of our knowledge, this is the first report to have studied the cellulolytic bacterial community from the whole GI tract of the Giant African snail. The presence of only ten OTUs in the enriched culture is most likely not an artifact of limited sampling efforts as indicated by the rarefaction analyses of clone libraries (Suppl. Fig. S2). The cloning, sequencing and analysis were repeated, and similar results were observed indicating low diversity. The presence of few numbers of OTUs and CMCase activity shown by most of the isolates confirmed that cellulolytic bacterial community was indeed enriched by the method employed in this study. For this study, snails in active state ($n=5$) were collected from only one location. As indicated by Watkins and Simkiss, [36] that most of the snail's microflora is picked up from the environment during feeding, without discrimination, and modified further by environmental conditions such as starvation, hibernation or aestivation. Keeping this in view, it will be interesting and worth to investigate further how cellulolytic bacterial community structure differs along the different GI tract regions, different environments and in different physiological conditions such as starvation, hibernation or aestivation. Since this study focused on revealing the structure of cellulolytic community enriched in CMC, in future, we will focus on studying the structure of cellulolytic community enriched in other cellulosic and other agro-residue-based substrates such as grass straw, rice straw, wheat straw, rice husk, wheat husk and cotton. Moreover, few potential isolates with high lignocellulolytic activities will be evaluated and tested for their potential to produce lignocellulolytic enzymes and degradation of ligno-cellulosic biomass.

Conflict of Interest None.

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