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Isolation and characterization of the culturable microbes associated with gut of adult dung beetle *Onitis philemon* (Fabricius)

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Abstract
Experiments were done to identify and characterize the gut microbes of the adult dung beetle *Onitis philemon* for their ability to degrade cellulose, pectin and lignin. Ten bacterial isolates were isolated from the gut of *O. philemon* and identified using biochemical and 16S rDNA analysis. Cellulose degraders were identified as *Acinetobacter baumannii* (OPNF5.3), *Citrobacter amalonaticus* (OPYH5.15), *Citrobacter freundii* (OPNH10.3) *Aeromonas caviae* (OPNH8.2) and *Acinetobacter* sp. (OPMH8.8). The cellulolytic index ranged from 3.25 to 5.0 indicating robust CMCase production. Three isolates namely *Aeromonas hydrophila* (OPNH5.10), *A. caviae* (OPNH8.2) and *C. freundii* (OPNH10.3) were able to hydrolyze pectin and the maximum pectinase activity was also seen in the isolate *C. freundii* OPNH10.3 which was 1.08IU/ml. *Citrobacter amalonaticus* (OPYH5.15) and *C. freundii* (OPNH10.3) were also positive for lignin peroxidase indicating that they could degrade lignin. *Citrobacter* species could be playing an important role in the digestion of complex organic matter thus aiding in provision of nutrition to the dung beetle. The significance of identifying these isolates lies in possible application in organic waste decomposition.

Keywords: *Onitis philemon*, gut bacteria, lignin peroxidase, enzyme assay

Introduction
Dung beetles (Coleoptera; Scarabaeidae) play very important role in the wellbeing of our planet. They improve nutrient recycling and soil structure (Brown *et al*., 2010) [1]. The habitats of dung beetles are diverse which includes, farmland, grasslands, desert and forest. They consume dung of herbivores and omnivores but prefer dung of herbivores (Losey and Vaughan, 2006; Nichols *et al*., 2008) [2, 3]. *Onitis philemon* is a tunneler and its main food source is cow dung. Dung is the undigested residue of plant matter which has passed through the animal's gut. Cellulose, lignin, pectin and other complex carbohydrates can remain undigested. These complex polysaccharides can be degraded by microorganisms by producing the concerned enzymes such as cellulase, pectinase and lignin modifying enzymes. Reports suggest that ruminants and insects have the capability to degrade complex polysaccharides through the use of symbiotic microorganisms with their own specific degrading enzymes (Inoue *et al*., 2005) [4]. Scarab beetles are reported to be able to effectively utilize various cellulose plant matter and animal waste as energy sources (Huang *et al*., 2010) [5]. Scarab beetle that feed on fresh or decaying plant materials (Egert *et al*., 2005) [6] feature fermentative guts with highly diverse bacterial communities (Colman *et al*., 2012) [7]. These bacterial groups exhibit cellulolytic, pectinolytic, lignolytic, lignocellulolytic, and hemicellulolytic activities (Andert *et al*., 2010 and Anand *et al*., 2010) [8, 9] and are responsible for conversion of polymeric food integrants to simpler forms that can be digested by the insect (Kane, 1997) [10]. Research on gut microflora of adult dung beetle has not been reported yet. As these beetles feed mainly on dung, it is possible that microbes able to degrade complex organic matter may be associated with the beetle gut. These microbes efficiently produce complex carbohydrates degrading enzymes hence can be used for the purpose of organic waste decomposition. Hence this study was conducted to characterize the culturable microflora associated with the gut of adult *Onitis philemon*.

Material and Methods
Dung beetles collection and dissection of insect gut
Dung beetle (*Onitis philemon*) was collected from a dairy farm located in the district of Bengaluru, Karnataka, India and kept in aerated container. Identity was confirmed by Dr. K. Veenakumari, Principal Scientist and taxonomist, ICAR-NBAIR, Bengaluru. The adult beetles were maintained live using cow dung as medium. The collected beetles were starved overnight,

so that gut remains clear of any food particle. The insect was surface sterilized twice with 70% ethanol for 1 min and washed with distilled water. Entire gut was aseptically removed in a UV laminar flow hood. The isolated gut was kept in 10mM phosphate buffer and minced with the help of sterile micro pestle (Vasanthakumar *et al*., 2006) [11].

Isolation and identification of isolates

The gut extract collected was serially diluted and spread plated on Nutrient agar (NA), yeast peptone dextrose adenine agar (YPDA), potato dextrose agar (PDA). Plates were incubated at 37ºC for 48hrs. All chemicals used for media preparation were acquired from HiMedia Laboratories Pvt. Ltd. The bacterial isolates were identified using morphological, biochemical and through 16S rDNA technique. The individual colonies were assessed in terms of nature, shape, pigmentation, and gram reaction and IMViC test.

16S rDNA analysis

The bacterial DNA was extracted using Hipura Himedia genomic DNA extraction kit. Universal primer fd1 and rp2 were used for 16S rDNA PCR. The PCR reaction conditions consisted of an initial denaturation step at 94°C for 3 mins, final denaturation at 94°C for 1mins, annealing at 45°C for 1 min, extension at 72°C for 2 min, and a final extension cycle at 72°C for 10 mins, 35 cycles. The generated sequences were compared with sequences available in GenBank by using the BLASTn program (http://www.ncbi.nih.gov) (Shayne *et al*., 2003) [12].The sequences were aligned using BioEdit alignment editor and phylogenetic tree was constructed using maximum likelihood method using Molecular Evolutionary Genetics Analysis version 6 (MEGA6) program based on Kimura-2 parameters with 1000 replicates of bootstrap values.

Screening of cellulose degrading bacteria

CMCase Plate assay

Cellulose-degrading ability of the gut bacteria was tested by placing bacterial suspension grown overnight in LB medium. The test was conducted on 1% CMC agar media with the following composition: KH_2PO_4 0.5 g, $MgSO_4$ 0.25 g, cellulose 10 g, agar 15 g, distilled water 1 L. Diameter of 4mm Well was made by using sterilized cork borer. The well was filled with the cultures grown in broth and the plates were incubated at 37°C for 48 h. The Plates were flooded with Gram's iodine solution; colonies showing zone of hydrolysis were selected as positive for cellulose degradation. Negative control was also maintained (Kasana *et al*., 2008) [13]. Hydrolysis capacity of bacterial isolates was calculated by taking the ratio of diameter of clear zone and bacterial colony diameter (Andri *et al.,* 2015) [14].

Enzyme assay of Cellulase

Bacterial cultures were inoculated into LB broth and kept for incubation at 37°C for 48hrs. The culture broth was centrifuged and supernatants were used as crude enzyme. Cellulase activity of crude enzyme was measured by the DNS (3,5-dinitrosalicylic acid) method (Miller, 1959) [15]. CMCase assay was determined by incubating 0.5 mL of Crude protein sample with 0.5 mL of 1% cellulose in 0.05 M sodium citrate buffer (pH 4.8) at 55°C for 30 min. Filter paper assay was determined by incubating 0.5 mL of crude protein sample with 1.0 mL of 0.05 M sodium citrate buffer (pH4.8) containing Whatman no.1 filter paper strip (1.0 × 6.0 cm). After incubation for an hour at 50°C, absorbance was read at 540 nm using spectrophotometer (Hitachi, U-2910). The total enzyme activity of cellulase and FPCase were interpreted in international unit (IU).

Screening of pectin degrading bacteria

Pectinase plate assay

Pectinolytic activity of isolates was tested by placing overnight grown bacterial suspension in LB medium. The plate assay was conducted on modified pectin agar medium (Asif *et al.,* 2012) [16] composed of (g/L) Pectin-10.0, Yeast extract-2.0g, $MgSO_4 \cdot 7H_2O$-0.5g, $MnSO_4 \cdot 5H_2O$-0.01g, K_2HPO_4-3g, KH_2PO_4-2g, Agar-20g. To this distilled water was added to make upto 1 liter. The bacterial suspension was added to the 4mm wells made on pectin agar plates. The plates were incubated at 37°C for 3-5 days. Plates were then flooded with iodine solution containing containing 0.25% iodine, 0.5% potassium iodide (Anam & Zakia 2012) [17]. Pectinolytic activity was observed as a clear zone surrounding the colony. The hydrolytic zones diameters were measured in millimeter and pectinase positive isolates selected.

Assay for pectinase

For quantification of pectinase, crude pectin was used as a substrate. Bacterial cultures were inoculated into LB broth and kept for incubation at 37°C for 48hrs. After incubation the culture broths were centrifuged and supernatants were used as crude enzyme. Pectinase activity of crude enzyme was measured by the DNS (3,5-dinitrosalicylic acid) method (Miller, 1959) [15]. The reaction mixture containing 0.5 mL of crude protein sample with 0.5 mL of 1% pectin in 0.05 M sodium citrate buffer (pH 4.8), incubated at 40°C for 10 min. To this 1ml of DNSA reagent was added and samples were boiled at 90°C for 10mins. To stop the reaction 1ml of Rochelle's salt solution was added. The absorbance was read at 540 nm using spectrophotometer (Hitachi, U-2910). The total enzyme activity of was interpreted in international unit (IU).

Statistical analysis

Hydrolysis capacity of bacterial isolates was calculated by taking the ratio of diameter of clear zone and bacterial colony diameter (Andri *et al.,* 2015) [14]. The data for zone of inhibitions of enzymes were expressed as Mean ± SE and were subjected to one way ANOVA using PROC ANOVA (SAS version 9.3; SAS institute). When ANOVA was significant, comparisons of relevant means were made using Tukey's post-hoc significance test at 5% significance level.

Screening of lignin degrading bacteria

Lignin peroxidase assay was done using pyrogallol as a substrate. 1ml of 1% of pyrogallol in 0.5% of H_2O_2 was mixed with 1ml of 48h grown culture in lignin peroxidase medium. Lignin peroxidase medium was composed of dextrose 4g/l, peptone 4g/l, yeast extract 4g/l, calcium carbonate 2g/l and veratryl alcohol 20mM. The tubes were kept for centrifugation at 200rpm at 37°C. Colour change from yellow to brown is indication of positive test. One control tube was maintained without bacterial culture (Elisashvili *et al.,* 2010) [18].

Results

Isolation and Identification of isolates

Upon plating the gut contents (Fig. 1) of *Onitis philemon* on different nutrient media, 10 bacteria were isolated as unique

and assigned strain numbers. Initial characterization showed that all were Gram negative with characteristic biochemical reaction (Table 1). All were rods, positive for methyl red and citrate utilization test expect isolate OPNH5.10 which showed negative result. Five isolates were positive for indole test and five were negative. Only one isolate OPNH5.10 was positive for Voges–Proskauer test. The 10 purified cultures were identified by 16S rDNA sequence analysis. The analysis identified the ten isolates as *Acinetobacter baumannii* (OPNF5.3), *Acinetobacter* sp. (OPMH8.8), *Morganella morganii* (OPNH5.6), *Aeromonas hydrophilla* (OPNH5.10), *A. caviae* (OPNH8.2), *Aeromonas* sp. (OPMH10.8), *Citrobacter amalonaticus* (OPYH5.15), *C. freundii* (OPNH10.3), *Providencia rettgeri* (OPMH8.10), and *Providencia* sp. (OPMH8.14) (Table 1). The identified bacteria showed 96–99% nucleotide homology with other bacterial strains available in the NCBI database and the sequences were submitted to NCBI (Table 2). The MEGA 6 programme was used for phylogenetic tree construction with 1000 bootstrap value (Fig. 2). This phylogram clearly depicts that there is a clustering of the *Aeromonas* sp. though the above indicated tree is a paraphyletic tree, it obeys and retained the similar species in close proximity clusters with the respective reference sequences. Interestingly this tree is out grouped with *Citrobacter freundii* which stood as a ancestor to all the above considering references, and further molecular and morphological relational studies shows a way in the detection of the evolutionary relationship with the out-group (Fig. 2).

Fig 1: Dissection of gut

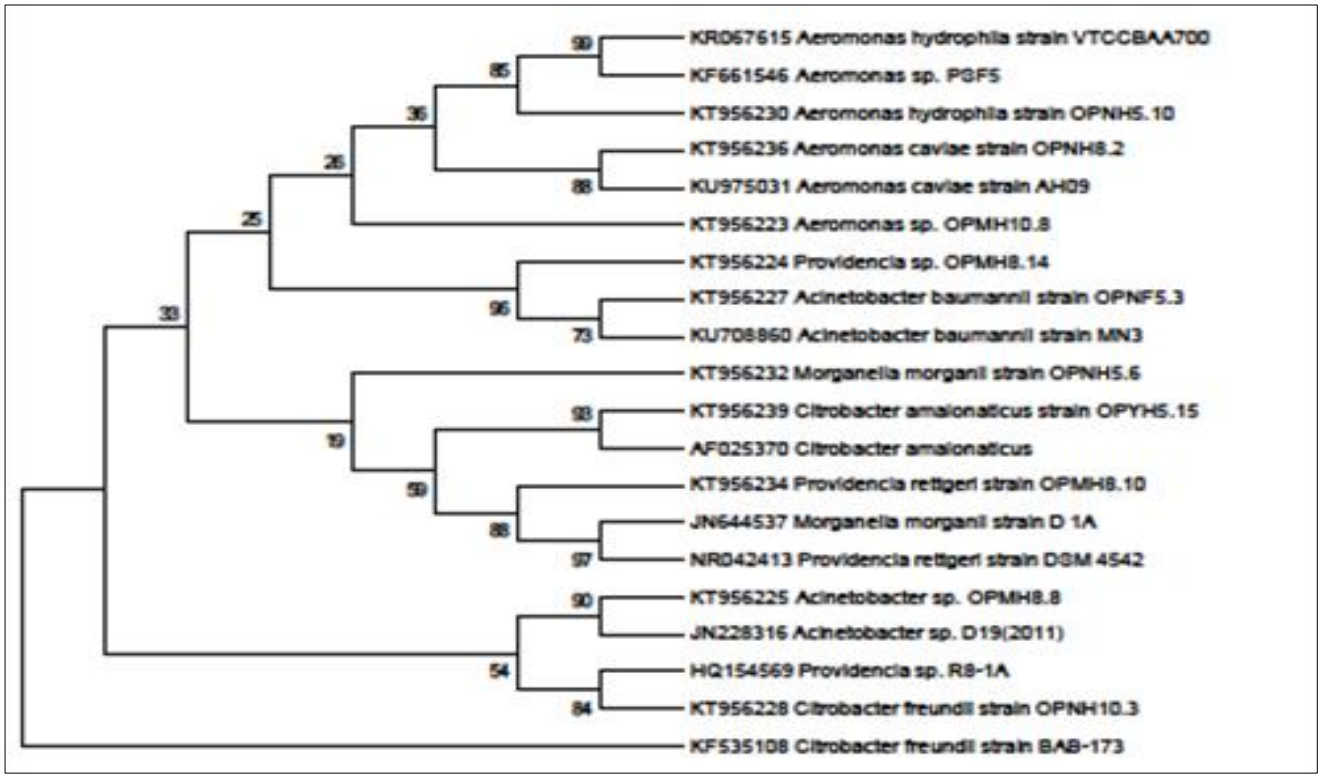

Fig 2: Molecular phylogenetic analysis (MEGA 6) of by maximum likelihood method using 16S rRNA gene sequences of NCBI GenBank. The numbers at branch points of the tree designate boot strap values

Table 1: Morphological characterization of bacterial isolates

Isolate	Morphological characterization						Biochemical characterization			
	Colony Shape	Pigment-ation	Margins	Elevation	Gram reaction	Bacteria Shape	Indole test	Methyl red test	VP test	Citrate utilization
OPNF5.3	Round	Translucent	Entire	Smooth	-	Rods	-	+	-	+
OPNH5.6	Mucoid	Opaque	Entire	Smooth	-	Rods	+	+	-	+
OPNH5.10	Round	Translucent	Entire	Convex	-	Rods	+	-	+	-
OPYH5.15	Mucoid	Translucent	Entire	Smooth	-	Rods	-	+	-	+
OPNH8.2	Circular	Transparent	Entire	Smooth	-	Rods	+	+	-	+
OPMH8.8	Circular	Translucent	Entire	Smooth	-	Rods	-	+	-	+
OPMH8.10	Circular	Translucent	Entire	Smooth	-	Rods	+	+	-	+
OPMH8.14	Circular	Translucent	Entire	Smooth	-	Rods	+	+	-	+
OPNH10.3	Circular	Translucent	Entire	Convex	-	Rods	-	+	-	+
OPMH10.8	Circular	Translucent	Entire	Flat	-	Rods	-	+	-	+

Table 2: BLAST-N results of 16S rDNA gene of isolates.

S. No.	Strain	Identification	Accession No.	Closest Strain In NCBI	Identity (%)
1	OPNF5.3	*Acinetobacter baumannii*	KT956227	*Acinetobacter baumannii* strain B8342	96
2	OPNH5.6	*Morganella morganii*	KT956232	*Morganella morganii* strain D_1A	99
3	OPNH5.10	*Aeromonas hydrophila*	KT956230	*Aeromonas hydrophila* strain VTCCBAA700	99
4	OPYH5.15	*Citrobacter amalonaticus*	KT956239	*Citrobacter amalonaticus*	99
5	OPNH8.2	*Aeromonas caviae*	KT956236	*Aeromonas caviae* strain AH09	99
6	OPMH8.8	*Acinetobacter* sp.	KT956225	*Acinetobacter* sp. D19(2011)	98
7	OPMH8.10	*Providencia rettgeri*	KT956234	*Providencia rettgeri* strain DSM 4542	97
8	OPMH8.14	*Providencia* sp.	KT956224	*Providencia* sp. R8-1A	99
9	OPNH10.3	*Citrobacter freundii*	KT956228	*Citrobacter freundii* strain BAB-173	96
10	OPMH10.8	*Aeromonas* sp.	KT956223	*Aeromonas* sp. PSF5	99

Table 3: Zone of clearance and their index for Cellulase and Pectinase enzyme of *Onitis philemon* gut bacteria.

Isolate	Zone of clearance diameter {mean(mm)± SE}	Well Diameter (mm)	Index
Cellulase Plate Assay			
Acinetobacter baumannii (OPNF5.3)	24.0±0.816497ab*	4.0	5.0
Citrobacter amalonaticus (OPYH5.15)	23.0±0.408248b*	4.0	4.75
Aeromonas caviae (OPNH8.2)	17.0±0.912871c*	4.0	3.25
Acinetobacter sp. (OPMH8.8)	21.0±1.290994ab*	4.0	4.25
Citrobacter freundii (OPNH10.3)	19.0±0.707107bc*	4.0	3.75
Pectinase Plate Assay			
Aeromonas hydrophila (OPNH5.10)	20.0±0.547723a#	4.0	4.0
Aeromonas caviae (OPNH8.2)	16.0±0.707107b#	4.0	3.0
Citrobacter freundii (OPNH10.3)	21.0±1a#	4.0	4.25

*Means of four replications and #Means of five replications. Means followed by the same letter in a column are not significantly different at $P < 0.05$, as determined by Tukey's test.

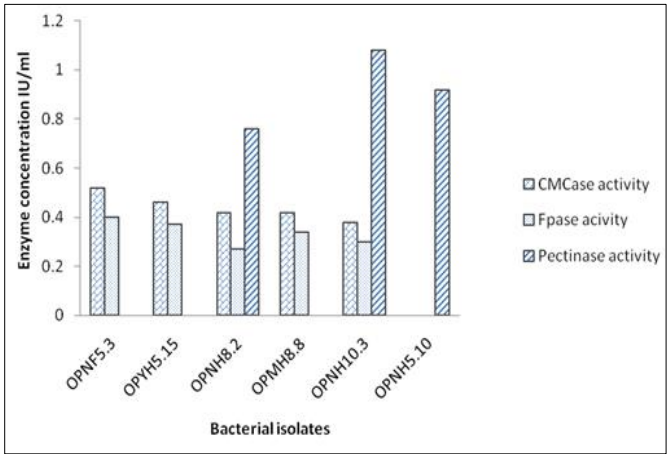

Fig 3: Enzyme activity of bacterial isolates from gut of dung beetle

Screening and quantification of cellulose degrading bacteria

The assay for cellulose degrading ability showed that five of the gut bacteria had cellulose degrading ability. These identified bacteria were *Acinetobacter baumannii* (OPNF5.3), *Citrobacter amalonaticus* (OPYH5.15), *C. freundii* (OPNH10.3) *Aeromonas caviae* (OPNH8.2) and *Acinetobacter* sp. (OPMH8.8). The zone of clearance was significantly ($P < 0.0001$) maximum in case of isolate *A. baumannii* (24.0mm) than rest of tested isolates in this study. The cellulolytic index ranged from 3.25 to 5.0 indicating robust CMCase production (Table 3). *Acinetobacter baumannii* had the highest cellulolytic index of 5.0, highest cellulase activity of 0.52 IU/ml and also highest FPase activity of 0.40 IU/ml. *C. amalonaticus* also exhibited high cellulose degrading ability with values of 4.75, 0.46 IU/ml and 0.37 IU/ml for cellulolytic index, cellulase activity and FPase activity respectively. *Aeromonas caviae* however showed lowest cellulolytic index of 3.25 (Fig. 3). These bacteria could be playing a significant role in the digestion of polysaccharide present in their food source (dung). Hence *A. baumannii*, *C. amalonaticus* have the potential for application in degradation of complex organic wastes.

Screening and quantification of pectin degradation bacteria

Out of 10 bacterial isolates tested only three isolates namely *Aeromonas hydrophila* (OPNH5.10), *A. caviae* (OPNH8.2) and *Citrobacter freundii* (OPNH10.3) were able to hydrolyze pectin (Table 3). The zone of clearance was significantly ($P < 0.0001$) maximum in case of *C. freundii* (21.0mm) and *A. caviae* (20.0mm). The maximum pectinase activity was also seen in *C. freundii* which was 1.08IU/ml (Fig. 3).

Assay for lignin degradation

Lignin peroxidase expression was tested by using pyragallol as a substrate. The isolates C*itrobacter amalonaticus* (OPYH5.15) and *C. freundii* (OPNH10.3) showed positive results with the appearance of brown color whereas no change in color was observed in case of control (Fig. 4).

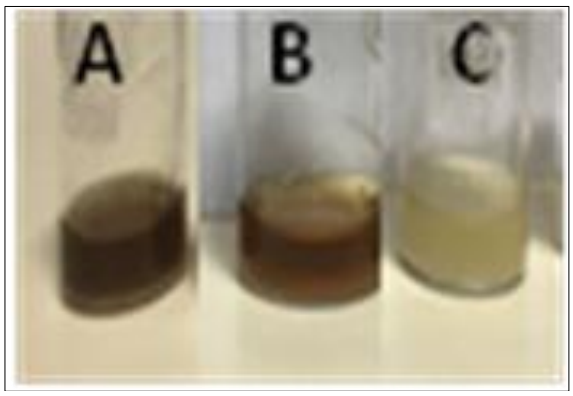

Fig 4: Lignin Peroxidase activity in the presence of pyragallol; A: *Citrobacter amalonaticus* (OPYH5.15), B: *Citrobacter freundii* (OPNH10.3), C: Control (without bacteria)

Discussion

Though studies on the type of microbes inhabiting the digestive tract of scarab beetles have been well documented (Egert *et al*., 2005; Cazemier *et al*., 2003; Zhang & Jackson 2008) [6, 19, 20]; the type of bacteria inhabiting the digestive tract of adult beetles is limited or not available. It is assumed that adults will pass on some of these bacteria to its progeny and some could be acquired from the environment. We wanted to see the type of bacteria that is present in the gut of adult dung beetle (*Onitis philemon*) and whether they could play important role in the decomposition of complex organic matter.

The present investigation revealed that the gut of the dung beetle *Onitis philemon* harboured culturable bacteria that had variations in their ability to degrade cellulose, lignin and pectin. Cellulose derived from plants is usually found in a mixture with hemicellulose, lignin, pectin and other substances and hence the organisms isolated from *O. philemon* will be useful for further applications. We could not find any earlier reports on the gut microflora of *O. philemon*. Characterization of the gut bacteria showed that *Acinetobacter baumannii* and *Citrobacter amalonaticus* were having efficient cellulose degrading ability. Studies coducted by Handique *et al*. (2017) [21] show that *Citrobacter* sp. is present in the gut of the white grub *Lepidiota mansueta* and exhibited efficient cellulose degrading ability with a cellulolytic index of 2.14 however in our studies *C. freundii* had a cellulolytic index of 5.0. Khianngam *et al*. (2014) [22] isolated cellulolytic bacteria from oil palm meal showing maximum enzyme activity of 0.233IU/ml and 0.17IU/ml, whereas the bacteria from *O. philemon* exhibited maximum cellulase enzyme activity of 0.52IU/ml and 0.46IU/ml. Hence the gut of *O. philemon* could be a good source for efficient cellulose degraders. *P. vulgaris*, *C. freundii*, *S. liquefaciens* and *Klebsiella* sp., were reported to be cellulose degrading bacteria and xyalnolytic bacteria from *Bombyx mori* (Anand *et al*., 2010) [9]. The digestive tract of *Poecilus chalcites* (ground beetle) harboured cellulose degrading bacteria like *C. freundii* and, *Pseudomonas* sp., (lehman *et al*., 2008) [23]. Cellulose degrading ability shown by *Acinetobacter baumannii* in our studies seems to be new information. Earlier studies have shown that that the gut of the scarab larva *Potosia cuprea* harboured microbes involved in cellulose degradation, proteolytic activities, and microbial fermentation (Lemke *et al*., 2003) [24].

Pectin is composed of a complex set of polysaccharides and abundant in the non-woody parts of terrestrial plants. Pectin lyase cleaves pectin in exo action pattern generating oligomers. In this study the gut bacteria of *O. philemon* showed that three bacteria were positive for pectin lyase and two of them *Aeromonas hydrophila* and *Citrobacter freundii* had good pectinolytic index. The pectin degrading bacteria namely *Erwinia* sp. was associated with gut of 5th instar larvae of *Bombyx mori* (Anand *et al*., 2010) [9]. The microflora associated with the gut of (bacteria, yeast as well fungi) insects have the ability to breakdown this polysaccharide. Reports are available on Longicorn beetle species wherein it was found that the bacteria associated with this beetle were able to produce pectinase efficiently (Park *et al*., 2007) [25].

Citrobacter amalonaticus (OPYH5.15) and *C. freundii* (OPNH10.3) were found showing positive result for lignin peroxidase activity. These two organisms also showed good cellulolytic index and *C. freundii* was also positive for pectin lyase indicating that *Citrobacter* spp. are important in degradation of complex organic matter. Actinomycetes like *Nocordia* and *Streptomyces* have been shown to produce extracellular peroxidases that can degrade both lignin and carbohydrate components of lignocellulose (Saha & Cotta 2007) [26]. Though our studies were qualitative it does implicate *Citrobacter* spp. as important for the gut of *O. philemon* and the study by Handique *et al*., (2017) [21] showed that *Citrobacter* spp. are the main cellulose degrading inhabitants of the Scarab beetle *Lepidiota mansueta*, but they did not test them for lignin degradation. Reports are available for bacteria as lignin degraders eg., *Pseudomonas* sp., *Klebsiella* sp., *Bacillus* sp., *Citrobacter* sp., (Zuharlida *et al*., 2014) [27]. Researchers also have been reported the presence of peroxidase in lignolytic bacteria (Shi *et al*., 2013) [28].

Conclusion

This study shows the first insight of association of cellulose, pectin and lignin degrading associated with the gut of adult dung beetle (*Onitis philemon*). *Citrobacter* species could be playing an important role in the digestion of complex organic matter thus aiding in provision of nutrition for the dung beetle. Whether these bacteria could be deployed in the management of waste matter needs further studies. We are currently looking into the use of insect gut bacteria for decomposition of farm and kitchen wastes.

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