Original Research

Journal Home page : www.jeb.co.in ★ E-mail : editor@jeb.co.in

Journal of Environmental Biology

JEB™ p-ISSN: 0254-8704
e-ISSN: 2394-0379
CODEN: JEBIDP

DOI : http://doi.org/10.22438/jeb/41/6/MRN-1420

Functional characterization of bacteria isolated from different gut compartments of white grub, *Anamola dimidiata*, larvae

K.R. Msango Soko[1], R.C. Bhattacharya[2], B. Ramakrishnan[3], K. Sharma[1] and S. Subramanian[1]*

[1]Division of Entomology, ICAR-Indian Agricultural Research Institute, New Delhi-110 012, India
[2]ICAR-National Institute for Plant Biotechnology, New Delhi-110 012, India
[3]Division of Microbiology, ICAR-Indian Agricultural Research Institute, New Delhi-110 012, India

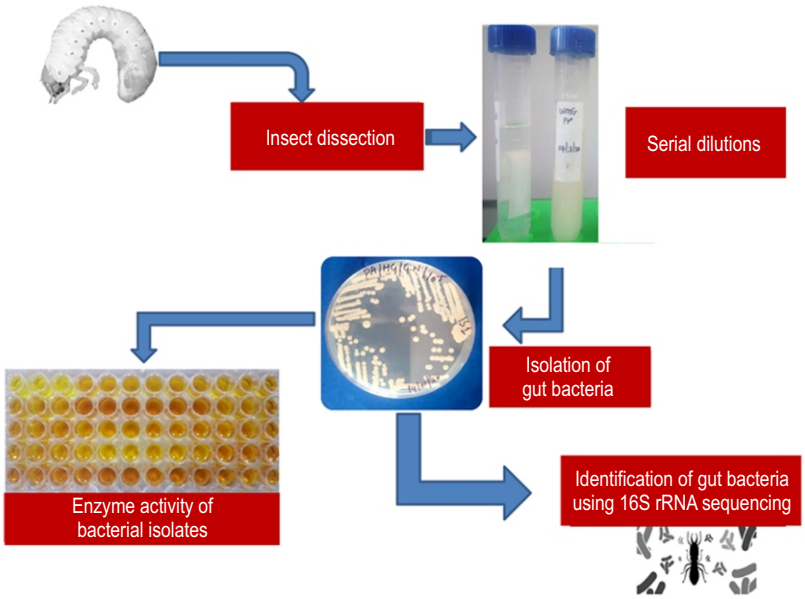
***Corresponding Author Email :** entosubra@yahoo.co.in

Paper received: 07.02.2020 *Revised received*: 21.05.2020 *Accepted*: 13.07.2020

Abstract

Aim: The aim of the present study was to isolate and characterize cellulolytic, lipolytic and nitrate reductase activities in the bacteria isolated from the gut of white grub, *Anamola dimidiata* larvae.

Methodology: Field collected third instar scarab larvae were dissected under aseptic conditions and inoculated on different bacteriological media to isolate gut bacteria. Identification of these isolates was carried out by amplifying and sequencing the 16S rRNA gene and comparing with their closest relatives in GenBank. Cellulolytic, lipolytic and nitrate reductase activities were assayed using Carbonmethyl cellulose (CMC), Rhodamine B and nitrate broth media.



Results: The majority of culturable bacteria in the gut of *A. dimidiata* belonged to two phyla: *Firmicutes* (62.5%) and *Proteobacteria* (37.5%). Forty aerobic and eleven anaerobic bacterial strains were isolated and tested for cellulolytic, lipolytic and nitrate reductase activity, and twenty seven and thirty one cellulolytic and lipolytic gut bacteria were identified, respectively, with 19 isolates exhibiting both activities whereas ten facultative anaerobic bacteria isolates were positive for nitrate reductase activity.

Interpretation: These bacterial isolates may be good sources for profiling novel isolates and enzymes for industrial use besides identifying new solutions for pest control.

Key words: Aerobic, *Anamola dimidiata,* Facultative anaerobes, Gut bacteria

How to cite : Msango Soko, K.R., R.C. Bhattacharya, B. Ramakrishnan, K. Sharma and S. Subramanian: Functional characterization of bacteria isolated from different gut compartments of white grub, *Anamola dimidiata*, larvae. *J. Environ. Biol.*, **41**, 1526-1535 (2020).

© Triveni Enterprises, Lucknow (India)

Introduction

The gut of insects is a complex environment that is inhabited by microbes whose localization and functional role vary considerably. Some insects posses special gut modifications such as paunches that influence endogenous microbial community structure (Jones *et al.,* 2019). Recent studies have shown that insects through their microbial associations have developed integrative strategies to contend challenges they encounter in their environments such as modulation of foraging behavior, enhancement of resistance to pathogens, digestion of recalcitrant food sources, vitamin provisioning and metabolism of xenobiotic substances (Akami *et al.,* 2019; Jones *et al.,* 2019). Among these are bacteria which colonize special niches within the gut and develop relationship with either other microbes or their hosts. With the current concerns of climate change related events such as pest outbreaks and global warming due to increased use of fossil fuels; insects with cellulose converting systems alongside with their associated evolutionary lineages with bacteria have been suggested as new targets for insect pest control as well as providing researchers with information on managing plant biomass for sustainable alternative energy sources (Wanatable and Tokuda, 2010; Handique *et al.,* 2017).

The family Scarabaeidae under the order Coleoptera with over 30,000 species are exclusively herbivorous or saprophagous with the ability to decay plant material, including roots and organic matter (Huang and Zhang, 2013; Huang *et al.,* 2012). The scarab beetles are economic pests of most agricultural crops in many parts of the world (cereals, groundnuts, pulses, sugarcane, plantation crops, turf, pastures and solanaceous crops) where the grubs are root feeders and their beetles feed on the leaves of host plants (Jackson and Klein 2006; Theurkar *et al.,* 2012; Bhattacharyya *et al.,* 2017). Scarabs are one of the major edaphic invertebrates with an estimated biomass of 5-54 larvae m^{-2} reaching up to 600 larvae m^{-2} in severe outbreaks (Majeed *et al.,* 2014). The feeding ability of different scarab beetle grub species appear to differ significantly and this in part is believed to be due to their symbiotic gut bacteria which may be shaping their plasticity (Huang and Zhang, 2013; Zhang *et al.,* 2018). The guts of these saprophagous macro invertebrates also play a critical role in transforming soil organic matter and in carbon and nitrogen cycle of terrestrial environments.

This in part is due to a complex microbial community in their intestinal tract which depolymerizes cellulosic or lignocellulosic components in their diet into simpler digestive products that can be assimilated by the host (Egert *et al.,* 2003; Lemke *et al.,* 2003). Many strategies are currently utilizing microbes based enzymes to degrade plant biomass for bioenergy production and scarab larvae have been suggested as a good model as they consume celluloses from multiple sources and have the ability to extract nutrients and energy from these sources with the help of endogenous enzymes complimented with proteinases and cellulolytic enzymes produced by symbiotic microbes. Cellulose digestion efficiency estimated up to 65% of scarab diet is attributed to the bacteria within the scarab larval intestinal tract which produce a combined action of enzymes such as endoglucanases, exoglucanase and β-glucosidase (Handique *et al.,* 2017). Due to a wide diversity of microbes in the gut of scarab larvae which are involved in lignocellulosic degradation through enzymatic and fermentative processes, scarabs stand to assist the biofuel industries to prospect for new sources of efficient enzymes of microbial origin (Huang *et al.,* 2010). Many strategies are currently utilizing microbes based enzymes to degrade plant biomass for bioenergy production and scarab larvae have been suggested as a good model as they consume celluloses from multiple sources and have the ability to extract nutrients and energy from these sources with the help of endogenous enzymes complimented with proteinases and cellulolytic enzymes produced by symbiotic microbes. The aim of the present study was to isolate and characterize cellulolytic, lipolytic and nitrate reductase activities in the bacteria obtained from the gut of white grub, *Anamola dimidiata* larvae.

Materials and Methods

Insect collection: The third instar *A. dimidiata* grubs were collected from a potato field at Modipuram, UP, India. The insects were transferred to our laboratory in aerated plastic jars (with potato slices) and maintained under a regime of 16:8 hr light and dark period with rearing temperature of 26 ± 1°C and 70% RH at the Division of Entomology, IARI, Pusa Campus; New Delhi, India until they were dissected. The larvae were starved for 24 hr to clear the gut prior to dissection.

Insect dissection and isolation of aerobic gut bacteria: The larvae were rinsed in double distilled water for 30 sec followed by 70%) ethanol for 60 sec and again rinsed in double distilled water for 30 sec to remove the disinfectant. The sterilized larvae were dissected using sterile microscissors under laminar flow to extract the gut. The extracted gut was separated into midgut (MG), anterior-hindgut (AHG), fermentation chamber (FC) and post-hindgut (PHG) and each compartment was briefly rinsed in sterilized 0.85% NaCl and placed in a sterile 1.5 ml eppendorf tube containing sterilized 0.85% NaCl and homogenized with a sterile homogenizer. The gut homogenates from various compartments were serially diluted and inoculated on autoclaved Nutrient Agar, Tryptone Soy Agar and *Pseudomonas* Isolation Agar (HiMedia Pvt. Ltd. Mumbai, India). The inoculated plates were incubated at 37°C for 24-48 hr. The bacterial colonies were differentiated based on their color, size, and morphology and a single representative isolate of each morphotype was purified by streaking on corresponding agar plates repeatedly until the purity of each culture was obtained. Enumeration of the isolates was performed by calculating the number of CFUs and the values of mean colony counts were used for calculating viable counts of cultivable bacteria and expressed as CFU in 1 ml of sample.

Isolation of anaerobic gut bacteria: To profile anaerobic gut bacteria from *A. dimidiata,* only the midgut and fermentation chamber of *A. dimidiata* larvae were used. Fully grown 3rd instar

larvae were pre-chilled for 1-2 min to immobilize them and also for easy handling. The larvae were then surface sterilized in 70% ethanol for 60 sec followed by rinsing in double distilled water before dissection. Individual insects were dissected in aseptic conditions under lamina flow using aseptic dissection scissors and forceps and the midgut and fermentation chamber were removed and immediately transferred to a 1.5 ml sterile eppendorf tube containing sterile 0.85% NaCl solution and sealed with parafirm to avoid exposure to oxygen. Three separate samples of each gut compartment were prepared as described above and the samples were kept in -20°C awaiting further downstream processes. Samples were homogenized in an anaerobic environment comprising an air tight chamber sterilized with alcohol and continuously flashed with CO_2. Serial dilutions were inoculated on Thioglycollate media (composed of Tryptone, 20 g l^{-1}, NaCl 2.5 g l^{-1}, D (+)-Glucose 5.5 g l^{-1}, L-Cystine 0.5 g g^{-1}l, Dipotassium Phosphate, Sodium Sulphite 0.2 g l^{-1}, 1.5 g l^{-1}, Methylene blue 0.002 g l^{-1}, Sodium Thioglycollate 0.6 g l^{-1} and Agar 15 g l^{-1}) and incubated under anaerobic condition at 28°C. The media was autoclaved at 121°C for 20 min. The number of colonies was recorded after 72 hr and used to calculate the initial inoculum size. The colonies were purified by streaking multiple times on corresponding media under anaerobic conditions until pure cultures were obtained.

DNA extraction and PCR amplification: Distinct representative purified colonies of bacteria were selected for identification based on 16S rRNA gene sequence analyses. Individual, purified bacterial isolates were grown on nutrient broth for 24 hr at 37°C. After 24 hr of growth, the broth cultures were centrifuged at 13,000 rpm to separate the pellet and supernatant. The supernatant was discarded and the pellet was used for DNA extraction using a modified cetyltrimethylammonium bromide (CTAB) method. The extracted DNA quality was checked on an agarose gel and quantified using a NanoDrop: 3300 Fluoro-Spectrometer (Thermo Scientific, Wilmington, DE, USA). The 16S rRNA of each isolate was amplified by PCR using BioLine Master Mix and eubacterial primers 27F-(10µM), (5'→ AGAGTTTGATCCTGGCTCAG→ 3') and 1492R-(10µM), (5'→ AAGGAGGTGATCCAGCCGCA→3') (Takara Bio India Pvt. Ltd). Each reaction contained approximately 50 ng DNA, 25 µl Master Mix (2X) and 0.5 mM of each primer. The PCR was carried out in a Bio-Rad C1000-thermal cycler (Bio-Rad Laboratories Inc, Berkeley, CA, USA) as follows: one cycle at 94°C for 5 min, 35 cycles at 94°C for 1 min, 52°C for 1 min and 72°C for 1 min 40 sec, followed by 72°C for 10 min and 4°C forever. PCR products were examined by electrophoresis in 1.2% agarose gel, and bands were visualized by staining with ethidium bromide.

The gels were run at 100 V for 1 h in TAE buffer (40 mM Tris-acetate, 1 mM ethylene-diamine-tetra-acetic acid (EDTA); pH 7.4). Gels were visualized under UV in the Gel Documentation system of Alpha ImagerTM gel imaging system (Alpha Innotech, San Leandro, CA, USA). Sequencing of 16S rRNA gene was outsourced from a private lab (AgriGenome Labs Pvt. Ltd, Kerala, India). Only high-quality sequences were checked with their closed relatives using the NCBI-BLAST algorithm and bacterial isolates were identified based on their similarity with existing sequences. The nucleotide sequences obtained were submitted to the GenBank database and assigned with accession numbers.

Qualitative screening for cellulolytic and lipolytic gut bacteria: Cellulolytic gut bacteria were screened by inoculating the isolates on Carboxyl Methyl Cellulose (CMC) agar plates following a protocol as described by Handique *et al.* (2017). Screening for the presence of lipolytic bacteria was carried out by qualitative plate assay using Rhodamine B media following a protocol as given by Feng *et al.* (2011). The cellulolytic and lipolytic index of the isolates was scored as follows: Enzyme activity (index) =Diameter of clear zone + colony- Diameter of colony (mm)/Diameter of colony (mm).

Cellulolytic activity assay: Cellulolytic activity was estimated by measuring the amount of reducing sugars released following the DNSA method of Miller (1959). Individual bacterial isolates with high CM Case activity were inoculated in cellulase production media containing 10 g CMC, 0.2 g magnesium sulphate, 0.75 g potassium nitrate, 0.5 g Dipottasium phosphate, 0.2 g iron sulphate, 0.04 g calcium chloride, 2 g yeast extract and 1g D-glucose [pH 7.0] (Lisdiyanti *et al.,* 2012). The bacterial cultures were incubated for 37°C for 36 hr. After incubation, the cell-free crude enzyme produced was collected by centrifugating the samples at 10000 rpm for 15 min at 4°C. The cell-free extract was kept at 4°C until next use as enzyme source.

Lipolytic assay: Lipase activity of bacterial isolates was estimated by using p-nitrophenyl butyrate (PNPB, 27 mM) as per the method described by Zibaeea *et al.* (2012) with slight modification as follows: each reaction mixture consisted of 50 µl of PNPB solution dissolved in 50 mM Tris-HCl buffer, pH 8.0. The reaction mixture was mixed with 15 µl of enzyme source and incubated at 37°C. After 30 min incubation period, 50 µl of distilled water was added to each tube (control and treatment) and the OD of the mixture was read at 410 nm in micro-plate reader (Eon Microplate Reader, BioTek Instruments India, Ltd) using the Gen5 ver. 2.4 software. Lipolytic activity was calculated by measuring the release of para-nitropheyly (pNP) using standard solutions of pNP (0.01 to 0.1 µg ml^{-1}). One unit of enzyme releases 1.0 µmol of p-nitropheyly per minute.

Nitrate reductase assay: As the reduction of nitrate is generally an anaerobic respiration in which an organism derives its oxygen from nitrate, we attempted to assay the anaerobically isolated gut bacterial isolates to establish if they may also be involved in nitrate reduction following a protocol for qualitative nitrate reductase assay as described at **Sigma-aldrich.com.** In brief, the anaerobic bacterial isolates were inoculated in nitrate broth (peptone 5 g l^{-1}, meat extract 3 g l^{-1}, potassium nitrate 1g l^{-1} - final pH 7.0. The samples were incubated at 37°C for 24 hr. After incubation, five drops of sulfanilic acid and α-Naphthylamine solution (in 5 N acetic acid) were added to the tubes containing

cultures and shaked thoroughly. A distinct appearance of red or pink color after few minutes indicated nitrate reduction. Zinc powder was added if the tubes did not turn pink after addition of the reagents mentioned above. A colorless medium after addition of zinc indicated nitrate reduction, while pink color was scored as negative.

Phylogenetic analysis: To assess the phylogenetic relationships between the bacterial isolates from *A. dimidiata,* 16S rRNA gene sequences were assembled and aligned using BioEdit Sequence Alignment Editor V. 7.0.5.3 (Hall 1999) and was analyzed using MEGA 7.0 (Tamura *et al.,* 2004). Phylogenetic trees were constructed by the neighbor-joining method with Kimura two-parameter correction (Kimura, 1980). To calculate the support for each clade, bootstrap analysis was performed with 1000 replications (Felsenstein, 1985).

Data analysis and statistics: Analyzable data (cellulolytic indices, lipolytic indices, among others) were subject to descriptive statistics, t-test and analysis of variance (ANOVA) using the SAS software. Tukey's honest significant difference (HSD) was used to evaluate differences between each factor at P=0.05.

Results and Discussion

The results of gut bacteria enumeration suggested that colonization of gut bacteria across the gut compartments appeared to be even as the results on colony forming units (CFUs) indicated no significant differences between the gut compartments on culturable aerobic (MG=8.01±1.19 AHG=8.82±0.58 FC=8.75±0.60, PHG= 8.66±0.62, P=0.8825) and anaerobic gut bacterial isolates (midgut=10.64 ± 1.30 Fermentation chamber =10.79 ± 1.17, t-value (4df) =0.09, P >t=0.9356) from *A. dimidiata.* Generic identification of isolates was carried out by 16S rRNA gene sequencing analyses. Similarity checks of the resulting 40 sequences for aerobic and 11 sequences for anaerobic bacterial isolates were carried out with closest relatives in NCBI and almost all isolates had similarity ≥ 97% with their closest relatives from NCBI database.

A single aerobic isolate, WG37-MG and one anaerobic isolate (WG-ANE-MG5) belonging to Proteobacteria had only similarity to the extent of 96% and 93%, respectively, with their closest relatives from GenBank, thus indicating that these two isolates may be unique ones. The majority of culturable bacterial isolates from *A. dimidiata* belonged to Firmicutes and Proteobacteria. The results further indicated that *A. dimidiata* is mainly dominated by Firmicutes (62.5 %) and Proteobacteria (37.5%). These results also suggest that the majority of culturable anaerobic gut bacteria belong to the families Firmicutes (55%) and Proteobacteria (45%). A study by Zhang and Jackson (2008) reported that a core community of gut bacteria is present in the fermentation chamber of *Costelytra zealandica,* with the majority of bacteria being located in the hindgut wall. A number of aerobic and anaerobic cellulolytic bacteria belonging to the family *Enterobacteriaceae* with endo-xylanase and beta-xylosidase have been lately isolated from the hindgut of *Holotrichia parallela,* another species of scarab beetles prevalent in China (Zhang *et al.,* 2018). The sequences were submitted to GenBank and assigned accession numbers as outlined in Tables 1 and 2.

Insects employ diverse mechanisms that enable them to survive in diverse ecological niches. Center to these are enzymatic proteins which can be produced by the insects complemented by microbial associations that are used as digestive helpers which gives the insects the plasticity to survive on diverse nutrient sources (Mika *et al.,* 2013). The identified gut bacterial isolates were screened for cellulolytic activity by using CMC agar plate assay. Highly significant differences (P<.0001) were found between the bacterial isolates on their ability to hydrolyze CMC (Table 3). The cellulolytic bacterial isolates are dominated by three major genera namely *Bacillus* (67%), *Pseudomonas* (22%) and *Enterobacter* (11%).

The genera *Bacillus* and *Pseudomonas* have been reported to contain species with an ability to produce cellulolytic enzymes (Kothari *et al.,* 2013; Kumar *et al.,* 2014) and it is apparent from the results of the present study. The previous reports on gut bacterial isolates from Coleopteran insects suggest that *Bacillaceae* dominate the cellulolytic gut bacterial symbionts in insects. A comparative analysis of four gut compartments indicate that the fermentation chamber and anterior hindgut regions harbour cellulolytic gut bacteria predominantly. Henrissat and Bairoch (1993) indicated that the enzymatic digestion of cellulose is a complex process requiring a diverse group of glycoside hydrolases called cellulases. Endoglucanases (EC-3.2.1.4) attack the cellulose molecule randomly and dissociate after making few cleaves. Exoglucanases (EC-3.2.1.91) remove subunits at both reducing and non-reducing ends of cellulose, releasing either cellobiose or glucose and *β*-glucosidase (EC-3.2.1.21) completely hydrolyze cellobiose to glucose. A number of insects such as *Hieroglyphus banian, D. caroloina* grasshopper, *Periplenata americana, Odontotermes obesus* and *Philosomia ricini* have been reported to possess endogenous cellulases, particularly, *Beta*-glucosidases (Taggar, 2015). The results of present study on cellulolytic assay reveal that the gut bacterial isolates from *Anamola dimidiata* possess endoglucanase, exoglucanase and beta-glucosidase activities. The cellulolytic bacterial isolates are significantly enriched in the hindgut, especially in the fermentation chamber, and this may be an evolutionarily acquired anatomical modification to harbour certain specific symbionts aiding in digestion of cellulose in scarabaeids. Several aerobic hemi-cellulolytic and endo-xylanase bacterial strains had earlier been isolated from the fermentation chamber of scarabaeid, *Holotrichia parallela* (Zhang *et al.,* 2018).

A single bacterial isolate *Bacillus* sp. WG42-FC isolated from the fermentation chamber appears to be unique as it possesses significant exoglucanase compared to the rest of the isolates and we presume that this isolate may be associated with exoglucanase production in aiding digestion of cellulose in *A. dimidiata* (Fig. 1). Lipases of microbial origin, particularly bacteria

Table 1: Culturable aerobic gut bacteria isolated from the gut of *A. dimidiata*

Strain ID	GenBank Accession No	Closest relative in GenBank	Similarity (%)
WG36	MN793065	*Pseudomonas aeruginosa* strain 16S-DB2	99
WG37	MN793066	*Pseudomonas aeruginosa* strain F18	96
WG38	MN793067	*Pseudomonas aeruginosa* strain FMDP002	100
WG16	MN794228	*Bacillus toyonensis* strain PK1-17B	99
WG17	MN744366	*Bacillus subtilis* strain JN005	99
WG18	MN794025	*Bacillus cereus* strain S5	97
WG02	MN794224	*Bacillus altitudinis* strain B7	99
WG03	MN744374	*Bacillus anthracis* strain B12-B41	99
WG04	MN794023	*Enterobacter sp.* strain AA150	99
WG05	MN744363	*Enterobacter hormaechei* strain IA3	100
WG24	MN795601	*Bacillus cereus* strain Cps1	100
WG25	MN795598	*Bacillus subtilis* strain EN4	98
WG27	MN794021	*Bacillus cereus* strain CPO 4.232	100
WG28	MN744373	*Bacillus cereus* strain SBMAX30	98
WG29	MN793061	*Pseudomonas aeruginosa* strain NBRITSS	99
WG30	MN793062	*Pseudomonas aeruginosa* strain 16S-DB6	100
WG31	MN793060	*Bacillus wiedmannii* strain IKb-SK-CIFE	99
WG32	MN793063	*Bacillus cereus* strain FJAT-46988	100
WG33	MN794022	*Pseudomonas aeruginosa* strain G RN2-1	99
WG39	MN795599	*Bacillus subtilis* strain VM 06	100
WG40	MN795600	*Pseudomonas psychrophila* strain KTA	99
WG41	MN793068	*Bacillus wiedmannii* strain WS4-21	98
WG42	MN795595	*Bacillus cereus* strain B24	100
WG43	MN793069	*Pseudomonas aeruginosa* strain D1-3	100
WG01	MN794223	*Bacillus cereus* strain DCRUST BT FtM-5	99
WG10	MN794229	*Bacillus velezensis* strain B1-6	100
WG11	MN794226	*Bacillus halotolerans* strain VSH 03	99
WG07	MN794025	*Pseudomonas sp.* strain TRB175	99
WG08	MN793158	*Bacillus cereus* strain AK6	99
WG09	MN744375	*Bacillus tequilensis*	100
WG12	MN744364	*Enterobacter hormaechei*	99
WG13	MN794227	*Bacillus subtilis* subsp. *spizizenii*	99
WG14	MN794230	*Bacillus subtilis* strain SES61	99
WG15	MN744365	*Pseudomonas fragi* strain 15-D5	99
WG20	MN795596	*Bacillus subtilis* strain BJ-DEBCR-31	100
WG21	MN744371	*Bacillus wiedmannii* strain FJAT-46982	99
WG22	MN744372	*Pseudomonas sihuiensis* strain QSRB1	100
WG06	MN794225	*Bacillus sp.* X28	98
WG34	MN794024	*Bacillus mobilis* strain PK4-2	100
WG35	MN793064	*Bacillus cereus* strain SCS58	100

Annotation= MG=Midgut; AHG=Anterior Hindgut; FC=Fermentation Chamber; PHG=Posterior Hindgut

Table 2: Anaerobic gut bacteria isolated from the midgut and fermentation chamber of *A. dimidiata*

Isolate ID	NCBI Accession Number	Closest relative from GenBank	Similarity (%)
WG-ANE-MG2	MN793166	*Enterobacter* sp. strain MFM	100
WG-ANE-MG3	MN793167	*Raoultella terrigena* strain PGP374	99
WG-ANE-MG4	MN793169	*Bacillus toyonensis* strain HBUAS56013	99
WG-ANE-MG5	MN793170	*Escherichia coli* strain 244	93
WG-ANE-FC1	MN793159	*Enterobacter* sp. strain YB48	99
WG-ANE-FC4	MN793160	*Bacillus subtilis* strain 3-17	100
WG-ANE-FC6	MN793161	*Bacillus tequilensis* strain JAAKPT	99
WG-ANE-FC7	MN793162	*Bacillus subtilis*	99
WG-ANE-FC8	MN793163	*Bacillus amyloliquefaciens* strain FJAT-46318	100
WG-ANE-FC9	MN793164	*Bacillus subtilis* strain CL2	100
WG-ANE-FC12	MN793165	*Enterobacter sp.* strain MFM	99

Online Copy

Table 3: Cellulolytic gut bacterial isolates from *A. dimidiata*

Strain ID	Source	Cellulolytic Index
WG16	MG	1.13 ± 0.00
WG17	MG	2.38 ± 0.15
WG18	MG	0.00 ± 0.00
WG36	MG	0.00 ± 0.00
WG37	MG	1.99 ± 0.38
WG38	MG	1.50 ± 0.07
WG02	AHG	1.92 ± 0.08
WG03	AHG	1.09 ± 0.05
WG04	AHG	1.59 ± 0.11
WG05	AHG	2.08 ± 0.37
WG24	AHG	0.87 ± 0.45
WG25	AHG	1.09 ± 0.05
WG27	AHG	0.00 ± 0.00
WG28	AHG	1.61 ± 0.12
WG29	AHG	0.00 ± 0.00
WG30	AHG	0.00 ± 0.00
WG31	AHG	1.70 ± 0.11
WG32	AHG	0.00 ± 0.00
WG33	AHG	1.50 ± 0.00
WG07	FC	1.82 ± 0.26
WG08	FC	0.00 ± 0.00
WG09	FC	1.48 ± 0.04
WG01	FC	2.04 ± 0.04
WG10	FC	0.00 ± 0.00
WG11	FC	1.98 ± 0.24
WG12	FC	2.43 ± 0.12
WG13	FC	1.76 ± 0.07
WG14	FC	1.34 ± 0.09
WG15	FC	1.73 ± 0.42
WG39	FC	0.00 ± 0.00
WG40	FC	0.00 ± 0.00
WG41	FC	0.91 ± 0.40
WG42	FC	1.75 ± 0.07
WG43	FC	0.00 ± 0.00
WG06	PHG	2.02 ± 0.20
WG20	PHG	3.52 ± 0.14
WG21	PHG	0.00 ± 0.00
WG22	PHG	2.03 ± 0.16
WG34	PHG	0.00 ± 0.00
WG35	PHG	2.79 ± 0.15
Mean		1.16
p-Value		<.0001
SE(d)		0.227
Tukey HSD at 5%		0.9237

Annotation: MG=Midgut; AHG= Anterior Hindgut; FC=Fermentation Chamber; PHG=Posterior Hindgut. Means are cellulolytic index ± S.E. of bacterial isolates

Table 4: Lipolytic index of gut bacteria isolated from *A. dimidiata*

Strain ID	Source	Lipolytic Index
WG16	MG	3.13 ± 0.07
WG17	MG	1.31 ± 0.10
WG18	MG	1.54 ± 0.04
WG36	MG	0.00 ± 0.00
WG37	MG	1.92 ± 0.04
WG38	MG	2.58 ± 0.15
WG02	AHG	1.88 ± 0.07
WG03	AHG	0.00 ± 0.00
WG04	AHG	0.00 ± 0.00
WG05	AHG	0.00 ± 0.00
WG24	AHG	3.13 ± 0.07
WG25	AHG	2.38 ± 0.19
WG27	AHG	2.46 ± 0.04
WG28	AHG	2.54 ± 0.22
WG29	AHG	1.54 ± 0.04
WG30	AHG	3.84 ± 0.15
WG31	AHG	3.50 ± 0.07
WG32	AHG	1.08 ± 0.50
WG33	AHG	3.00 ± 0.13
WG01	FC	0.00 ± 0.00
WG07	FC	1.92 ± 0.11
WG08	FC	2.04 ± 0.34
WG09	FC	1.79 ± 0.15
WG10	FC	1.37 ± 0.07
WG11	FC	1.42 ± 0.04
WG12	FC	0.00 ± 0.00
WG13	FC	1.75 ± 0.13
WG14	FC	0.00 ± 0.00
WG15	FC	0.00 ± 0.00
WG39	FC	1.71 ± 0.15
WG40	FC	2.54 ± 0.18
WG41	FC	2.88 ± 0.31
WG42	FC	2.50 ± 0.29
WG43	FC	2.17 ± 0.51
WG06	PHG	0.00 ± 0.00
WG20	PHG	1.58 ± 0.15
WG21	PHG	3.25 ± 0.07
WG22	PHG	1.54 ± 0.23
WG34	PHG	1.89 ± 0.13
WG35	PHG	2.54 ± 0.11
Mean		1.72
p-Value		<.0001
CV (%)		22.71
SE(d)		0.324
Tukey HSD at 5%		1.316

Annotation: MG=Midgut; AHG= Anterior Hindgut; FC=Fermentation Chamber; PHG=Posterior Hindgut. Means are lipolytic index ± S.E. of bacterial isolates

are used widely in biotechnological applications (Gupta *et al.,* 2003). In addition to cellulases, lipolytic enzymes (EC 3.1.1.3) are vital in insect lipid acquisition, storage and mobilization and are fundamental to many developmental processes (Santana *et al.,* 2017). Thirty-one gut bacterial isolates were found to have lipolytic activity (Table 4). The genus *Bacillus* dominated the group of lipolytic bacterial isolates with 71% followed by *Pseudomonas* with 29%. In this study, the degradation of *p*-nitrophenyl butyrate to release *p*-nitrophenol by lipase catalyzed hydrolysis was used to quantify lipase activity in selected bacterial isolates. The results of lipase activity of gut bacterial isolates from *A. dimidiata* are presented in Table 5. The measurement of lipase activity among the gut bacterial isolates indicated highly significant differences (<.0001) between the gut bacterial isolates on their lipolytic ability

Table 5: Lipolytic activity (µmol min^{-1}) of selected culturable gut bacteria of *A. dimidiata*

Isolate ID	Lipase activity (µmol min^{-1})
Control	0.00±0.00
WG-08	1.01±0.09
WG-16	2.35±0.04
WG-24	2.37±0.04
WG-25	1.93±0.07
WG-27	1.10±0.11
WG-28	2.36±0.02
WG-30	0.88±0.01
WG-31	0.99±0.07
WG-32	2.34±0.03
WG-35	2.32±0.00
WG-38	1.40±0.07
WG-40	2.34±0.03
WG-41	2.38±0.02
WG-42	2.34±0.01
WG-43	0.92±0.01
General mean	1.69
p-Value	<.0001
SE(d)	0.069
Tukey HSD at 5%	0.2775

Values are mean ± S.E.

with bacterial isolate *Bacillus* sp. WG 41-FC having the highest lipase activity and isolate *Bacillus* sp. WG08-FC having the lowest activity all isolated from the fermentation chamber. The majority of the screened bacterial isolates were found to have significantly high lipolytic activity suggesting that *A. dimidiata* may be depending more on these lipolytic bacteria to complement its lipid digestion and assimilation. Generally, all the four gut compartments had bacterial isolates with significantly higher lipase activity and their lipolytic activity was not statistically different from each other. Gut bacteria belonging to genera *Bacillus, Enterococcus, Enterobacter, Klebsiella* and *Pseudomonas* have been reported from other insects associated with lipolytic activities and also host defense against pathogens (Liu *et al.,* 2018). Rajeshkumar *et al.* (2013) reported lipolytic activities of *Bacillus tequilensis, Bacillus subtilis, Pseudomonas sp.* and *Bacillus flexus* isolated from soil samples.

Eleven anaerobic bacteria cultures isolated from the midgut and fermentation chamber of *A. dimidiata* were subjected to qualitative nitrate reductase assay to screen their ability to break down nitrate. The results indicated that all the anaerobic bacterial isolates possessed the ability to break down nitrate, except for isolate WG-ANE-FC6. Nitrate reductase is an important enzyme that catalyzes the conversion of nitrate to nitrite-ammonia in the N-cycle. The nitrate reductase isolates belonged to three genera: *Bacillus, Enterobacter* and *Raoultella.* The bacteria symbionts aid in fixing nitrogen into ammonia which in turn is utilized to synthesize vitamins and amino acids for the host insect (Brune and Dietrich, 2015). The bacterial groups especially Proteobacteria and Firmicutes are reported to fix atmospheric nitrogen; and the genus *Enterobacter* contributed to

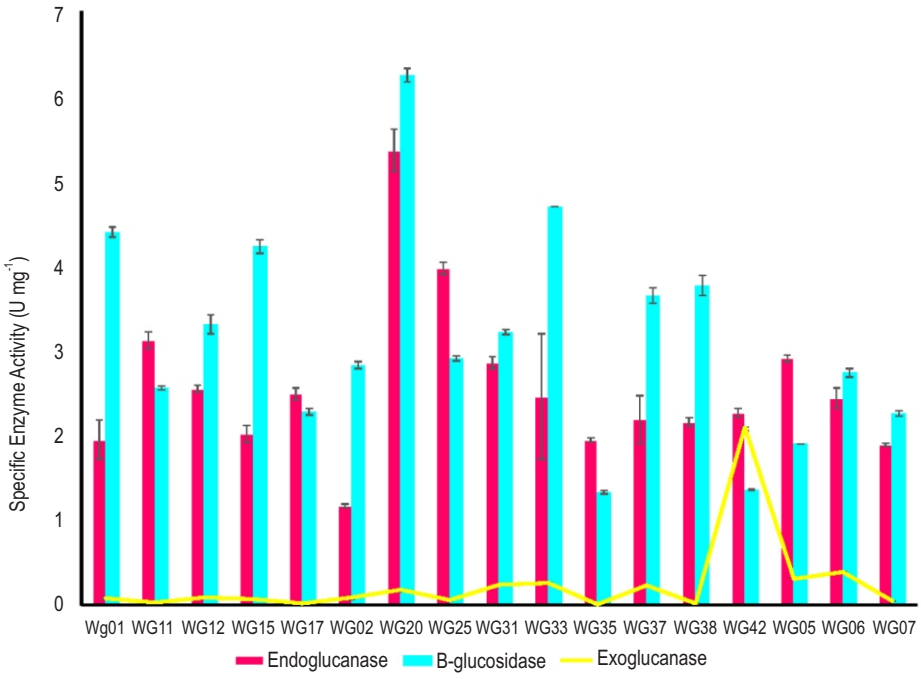


Fig. 1: Specific enzyme activity of gut bacterial isolates showing varying levels of endoglucanase, beta-glucosidase and exoglucanase activity.

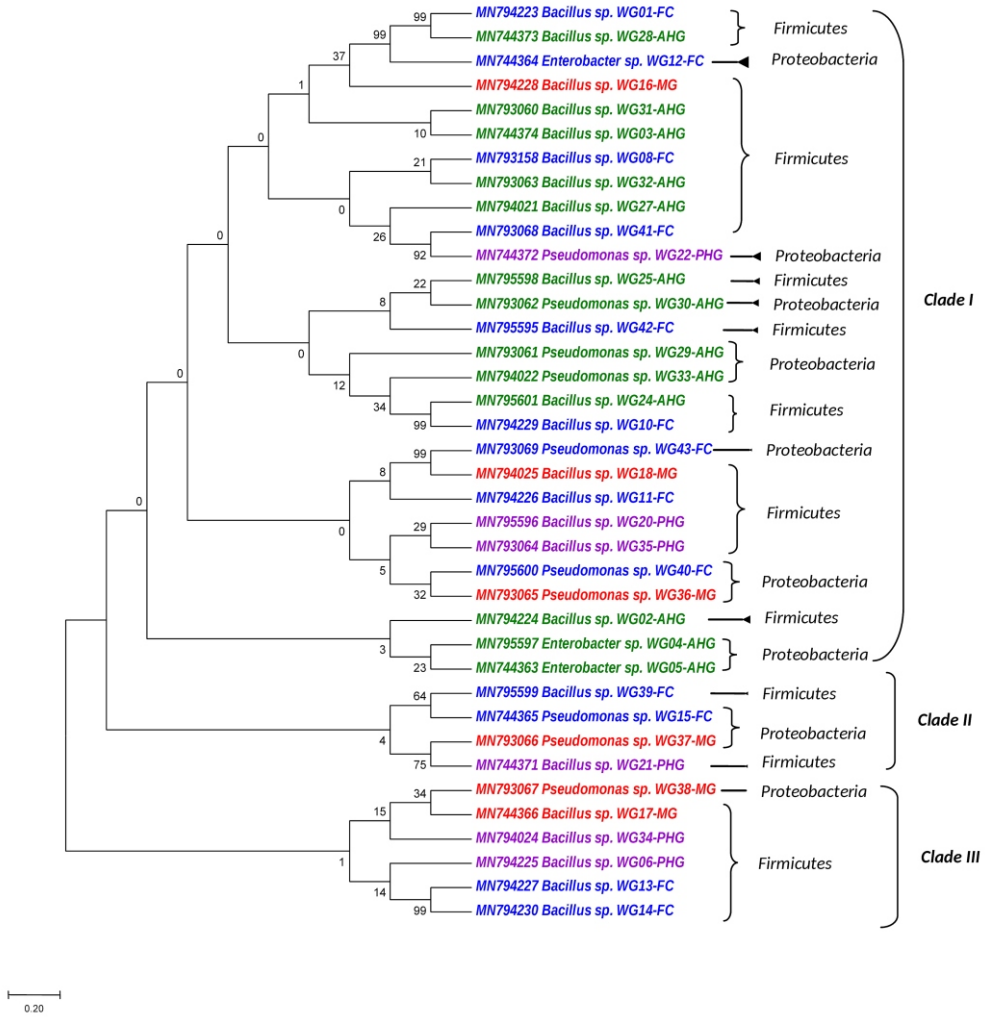


Fig. 2: Phylogenetic tree showing evolutionary relationships of gut bacteria isolated from *A. dimidiata*. Bacterial isolates highlighted with same color were identified from the same gut compartment.

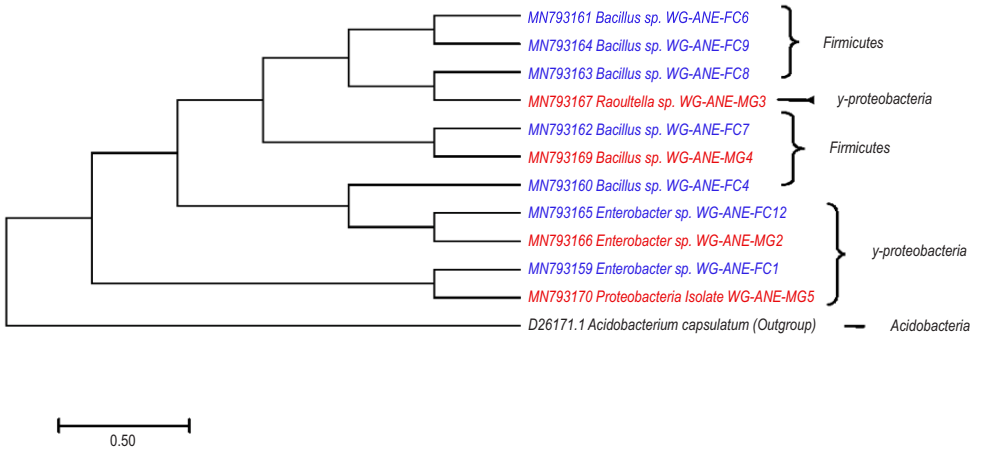


Fig. 3: Phylogenetic tree showing evolutionary relationships of anaerobic gut bacteria isolated from *A. dimidiata*. Bacterial isolates highlighted with same color were identified from the same gut compartment.

host nutrition by fixing atmospheric nitrogen in wood boring beetles (Hernández-García *et al.,* 2017). Zhang *et al.* (2018) indicated that the fermentation chamber is a typically anoxic environment containing obligate and facultative anaerobic bacteria believed to be critical in polysaccharide digestion, thus it is presumed that being an anaerobic environment, some of these anaerobic bacteria housed in the fermentation chamber may also be involved in ammonium oxidation (anammox).

Phylogenetic relationship analyses of culturable aerobic and facultative anaerobic gut bacteria 16S rRNA gene sequences from *A. dimidiata* (Fig. 2, 3) showed that Firmicutes as the dominant phyla with *Bacillus* as the dominant genus with twenty-six different species for aerobic bacteria isolates and six bacterial isolates for facultative anaerobic isolates. The proteobacteria formed the second dominant phyla comprising the genus *Pseudomonas* and *Enterobacter* with ten and four bacterial isolates for aerobic culturable gut bacteria whereas *Raoutella, Enterobacter* and a proteobacteria isolate WG-ANE-MG5 isolated from the midgut for anaerobic gut bacteria isolates. In addition to the two groups (Firmicutes and Proteobacteria) forming major clades, it is interesting to note that bacteria isolates, some even belonging to different genera, isolated from the same or adjacent gut compartments tended to cluster together suggesting a close relationship between these isolates compared to other isolates. However, the occurrence of *Bacillus* as the only dominant culturable gut bacteria in *A. dimidiata* suggests that this insect may have limited culturable gut bacterial diversity. This study provides information on important culturable gut bacteria of *A. dimidiata* that have cellulolytic, lipolytic and nitrate reductase activities which may be of significance in biotechnology and environmental remediation such as degradation of cellulosic substances from agricultural waste.

Acknowledgments

The authors gratefully acknowledge the help and support given by the ICAR-Indian Agricultural Research Institute, New Delhi and NAHEP-CAAST programme of Indian Council of Agricultural Research, New Delhi and Indian Council for Cultural Relations, Government of India for their support for this research programme. Special thanks should go to members of the Insect Molecular Genetics and Microbial Genetics laboratories of the Division of Entomology and Division of Microbiology, ICAR-IARI, for their assistance.

References

Akami, M., N.Y. Njintang, O.A. Gbaye, A.A. Andongma, M.A. Rashid, C.Y. Niu and E.N. Nukenine: Gut bacteria of the cowpea beetle mediate its resistance to dichlorvos and susceptibility to *Lippia adoensis* essential oil. *Sci. Rep.,* **9**, 6435 (2019).

Bhattacharyya, B., S. Bhagawati, H. Mishra, D. Gogoi, K. Pathak, S. Bhattacharjee and S. Borkotoki: Evaluation of some granular insecticides against white grub, *Lepidiota mansueta* B. in potato (*Solanum tuberosum* L.). *J. Entomol. Zool. Stud.*, **5**, 1441-1444 (2017).

Brune, A. and C. Dietrich: The gut microbiota of termites: digesting the diversity in the light of ecology and evolution. *Ann. Rev. Microbiol.*, **69**, 145-166 (2015).

Egert, M., B. Wagner, T. Lemke, A. Brune, and M.W. Friedrich: Microbial community structure in midgut and hindgut of the humus-feeding larva of *Pachnoda ephippiata* (Coleoptera: Scarabaeidae). *Appl. Environ. Microbiol.*, **69,** 6659-6668 (2003).

Feng, W., X.Q. Wang, W. Zhou, G.Y. Liu and Y.J. Wan: Isolation and characterization of lipase-producing bacteria in the intestine of the silkworm, *Bombyx mori*, reared on different forage. *J. Insect Sci.,* **11**,135 (2011).

Felsenstein, J.: Confidence limits on phylogenies: An approach using the bootstrap. *Evolution,* **39**, 783–791 (1985).

Gupta, R., N. Gupta and P. Rathi: Bacterial lipases: An overview of production, purification and biochemical properties. *Appl. Microbiol. Biotechnol.,* **64**, 763–781 (2004).

Hall, T. A.: BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. In: Nucleic Acids Symposium Series. Information Retrieval Ltd., *London,* **41**, 95-98 (1999).

Handique, G., A. Phukan, B. Bhattacharyya, A.A.L.H. Baruah, S.W. Rahman and R. Baruah: Characterization of cellulose degrading bacteria from the larval gut of the white grub beetle *Lepidiota mansueta* (Coleoptera: Scarabaeidae). *Arch. Insect Biochem.,* **94**, e21370 (2017).

Huang, S. and H. Zhang: The impact of environmental heterogeneity and life stage on the hindgut microbiota of *Holotrichia parallela* larvae (Coleoptera: Scarabaeidae). *PLoS ONE,* **8**, e57169 (2013).

Huang, S.W., H.Y. Zhang, S. Marshall and T.A. Jackson: The scarab gut: A potential bioreactor for bio-fuel production. *Insect Sci.,* **17**, 175-183 (2010).

Huang, S., P. Sheng and H. Zhang: Isolation and identification of cellulolytic bacteria from the gut of *Holotrichia parallela* larvae (Coleoptera: Scarabaeidae). *Int. J. Mol. Sci.,* **13**, 2563-2577 (2012).

Henrissat, B. and A. Bairoch: New families in the classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochem. J.,* **293**, 781-788 (1993).

Hernández-García, J.A., C.I. Briones-Roblerol, F.N. Rivera-Orduña and G. Zúñiga: Revealing the gut bacteriome of *Dendroctonus* bark beetles (Curculionidae: Scolytinae): Diversity, core members and co-evolutionary patterns. *Sci. Rep.,* **7**, 13864 (2017).

Jackson, T.A. and M.G. Klein: Scarabs as pests: A continuing problem. *Coleopterists Bulletin,* **60**, 102-119 (2006).

Jones, A.G., C.J. Mason, G.W. Felton and K. Hoover: Host plant and population source drive diversity of microbial gut communities in two polyphagous insects. *Sci. Rep.,* **9**, 2792 (2019).

Kothari, V.V., R.K. Kothari, C.R. Kothari, V.D. Bhatt, N.M. Nathani, P.G. Koringa, C.G. Joshi and B.R.M. Vyas: Genome sequence of salt-tolerant *Bacillus safensis* strain VK, isolated from saline desert area of Gujarat, India. *Genome. Announc.,* **1**, e00671-13 (2013).

Kumar, D, R. Parshad and V.K. Gupta: Application of a statistically enhanced, novel, organic solvent stable lipase from *Bacillus safensis* DVL-43. *Int. J. Biol. Macromol.*, **66**, 97-107 (2014).

Lemke, T., U. Stingl, M. Egert, M.W. Friedrich and A. Brune: Physico-chemical conditions and microbial activities in the highly alkaline gut of the humus-feeding larva of *Pachnoda ephippiata* (Coleoptera: Scarabaeidae). *Appl. Environ. Microbiol.*, **69,** 6650-6658 (2003).

Lisdiyanti, P., E. Suyanto, N.F. Gusmawati and W. Rahayu: Isolation and characterization of cellulase produced by cellulolytic bacteria from peat soil of Ogan Komering Ilir, South Sumatera. *Int. J. Environ. Bioen.,* **3**, 145-153 (2012).

Online Copy

Liu, R., W. Wang, X. Liu, Y. Lu, T. Xiang, W. Zhou and Y. Wan: Characterization of a lipase from the silkworm intestinal bacterium *Bacillus pumilus* with antiviral activity against *Bombyx mori* (Lepidoptera: Bombycidae) Nucleopolyhedrovirus *in-vitro*. *J. Insect Sci.,* **18**, 3 (2018).

Majeed, M.Z., E. Miambi, I. Barois, R. Randriamanantsoa, E. Blanchart and A. Brauman: Contribution of white grubs (Scarabaeidae: Coleoptera) to N_2O emissions from tropical soils. *Soil Biol. Biochem.,* **75**, 37-44 (2014).

Mika, N., H. Zorn and M. Rühl: Insect-derived enzymes: A treasure for industrial biotechnology and food biotechnology. In: *Yellow Biotechnology II*. Springer, Berlin, Heidelberg, pp. 1-17 (2013).

Miller, G.L.: Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem.,* **31**, 426-428 (1959).

Rajeshkumar, M.P., V.S. Mahendran and V. Balakrishnan: Isolation and identification of lipase producing organisms from diverse soil samples of Kolli hills. *Int. J. Curr. Microbiol. Appl. Sci.,* **2**, 205-210 (2013).

Santana, C., L. Barbosa, I. Júnior, T. Nascimento, C. Dornelas and L. Grillo: Lipase activity in the larval midgut of *Rhynchophorus palmarum*: Biochemical characterization and the effects of reducing agents. *Insects,* **8**, 100 (2017).

Taggar, M.S.: Insect cellulolytic enzymes: Novel sources for degradation of lignocellulosic biomass. *J. Appl. Nat. Sci.,* **7**, 625 – 630 (2005).

Tamura, K., M. Nei and S. Kumar: Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proc. Natl. Acad. Sci. USA,* **101**, 11030-11035 (2004).

Theurkar, S.V., S.B. Patil, M.K. Ghadage, Y.B. Zaware and S.S. Madan: Distribution and abundance of white grubs (Coleoptera: Scarabaeidae) in Khed Taluka, part of Northern Western Ghats, MS, India. *Int. Res. J. Biol. Sci.,* **1**, 1-6 (2012).

Watanabe, H. and G. Tokuda: Cellulolytic systems in insects. *Annu. Rev. Entomol.,* **55**, 609-632 (2010).

Zhang, Z.Y., Y. Yuan, M.W. Ali, T. Peng, W. Peng, M.F. Raza, Y. Zhao and H. Zhang: Cultivable anaerobic and aerobic bacterial communities in the fermentation chambers of *Holotrichia parallela* (Coleoptera: Scarabaeidae) larvae. *PloS ONE,* **13**, e0190663 (2018).

Zhang, H. and T.A. Jackson: Autochthonous bacterial flora indicated by PCR-DGGE of 16S rRNA gene fragments from the alimentary tract of *Costelytra zealandica* (Coleoptera: Scarabaeidae). *J. Appl. Microbiol.,* **5**, 1277-1285 (2008).

Zibaee, A., M. Fazeli-Dinan and C. License: Purification and characterization of a digestive lipase in *Naranga aenescens* Moore (Lepidoptera: Noctuidae). *SOAJ.,* **1**, 38-54 (2012).

On Line Copy