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Research Article

Cellulolytic Bacteria Associated with Gut of Longhorn Beetle, *Prionomma bigibbosum* (Coleoptera: Cerambycidae): an Electron Microscopic Study

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ABSTRACT

The present study aims to investigate the gastrointestinal microbiota in adult non-feeding Cerambycid beetle *Prionomma (Ancyloprotus) bigibbosum* White (1853) (Coleoptera: Cerambycidae: Prioninae: Prionini) and isolate cellulolytic bacterial strains. Scanning electron micrograph revealed the presence of abundant bacteria firmly attached to the hindgut. The gut flora was isolated and screened on Carboxymethylcellulose (CMC) agar medium using CMC as the sole carbon source. The cellulolytic activity was measured both qualitatively and quantitatively. Cellulolytic efficiency was assessed by the DNS method. Potential cellulose-degrading bacterial isolates were subjected to phenotypic and genotypic characterization. A Gram-positive, non-motile, Oxidase-positive coccoid isolate, designated as PBI9, was found to be an efficient cellulose-degrading strain. The 16S rRNA gene analysis revealed that the isolate was most closely related to *Mammaliicoccus fleurettii, M. stepanovicii and M. lentus* (99.24%, 99.17%, and 99.17% of similarity, respectively) and identified as *M. sciuri* (99.86% similarity) (NCBI Accession number MZ351443). This appears to be the first study undertaking SEM of gut microbiota of longhorn beetle, *P. bigibbosum*, and to report the *P. bigibbosum* gut as a new in place of novel source of cellulolytic bacteria.

Keywords: Cerambycid, Mammaliicoccus sciuri, PBI9, Prionomma bigibbosum, Scanning Electron Microscope

Introduction

The northeastern region of India is part of a mega biodiversity hotspot and is the habitat of diverse arthropod fauna. Phytophagous Cerambycid adults and larvae are rampant pests of crops and vegetations [1, 2]. Digestion of cellulosic materials of the woody tissues involves cellulase enzymes either secreted by the gut or obtained from their gut symbionts [3]. Interestingly, adults of some Cerambycid beetles under sub-family prioninae do not feed [4]. After emergence, the adults mate and lay eggs without any further feeding [5]. The gut symbionts of arthropods are reported to be involved in many vital functions such as defense towards pathogens and parasites [6], adaptation to environment [7], influence on insect-plant interaction [8], impact on population dimension [9], pesticide detoxification [10], and behavioral manipulation [11]. Therefore, it is interesting to study the gut microbiota of non-feeding adult Cerambycid beetle so as to detect the presence of symbiotic cellulolytic bacteria, which are expected to pass on horizontally between generations of cerambycids [12]. The contribution of gut microbiota in cellulose digestion has been confirmed previously [13], and both symbiont-dependent [14] as well as symbionts-independent [15] wood degradation has been reported in cerambycids. The presence and significance of endosymbionts are well established in different groups of arthropods [16]. In the present study, the gut of an adult beetle was exam-

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ined under the scanning electron microscope (SEM) to confirm the presence of autochthonous bacteria. This electron microscopic study of the gut of the longhorn beetle, *P. bigibbosum*, and reporting the gut as a novel source of cellulolytic bacteria have been done for the first time.

Material and Methods

Specimen collection and gut dissection

Adult longhorn beetles were collected from in and around North Eastern Hill University (NEHU) campus, Shillong, Meghalaya, India (25.5788° N, 91.8933°E) in the first week of June (peak season for adults to emerge between May and July every year) [1] and brought to the laboratory in live condition for dissection and further experiments. No chemicals were used for killing purposes in order to avoid chances of interference with gut microbiota. Before dissection, the insect specimens were kept in a deep freezer (−20°C) for 30 minutes for killing, were surface sterilized with 5.25% sodium hypochlorite solution, immersed in 70% ethanol, and thoroughly washed with distilled water. The dissection was done aseptically using sterile dissecting scissors and forceps under a laminar airflow hood on Petri dishes containing paraffin.

Culture media

The composition of the CMC agar medium used for isolation, screening, and qualitative analysis of cellulolytic bacteria was as follows: peptone 10.0 g, CMC 10.0 g, $MgSO_4.7H_2O$ 0.2 g, $NaCl_2$ 0.5 g, $CaCl_2$ 0.1 g, Agar 15.0 g per litre of distilled water, initial pH was adjusted at 6.5. For quantitative analysis of cellulolytic potential of bacteria, the isolates were inoculated in broth medium ($g.L^{-1}$) containing: 1.5 g KH_2PO_4, 2.5 g $Na_2HPO_4.7H_2O$, 0.3 g $MgSO_4.7H_2O$, 0.5 g $NaCl_2$, 0.1 g $CaCl_2$, 0.005 g $FeSO_4.H_2O$, 0.0016 g $MnSO_4$, 10.0 g CMC at pH 6.5. The media composition has been standardized in our lab.

Isolation of cellulolytic bacteria

The gut contents were used to detect the presence of cellulolytic bacteria in the gut fluid. The macerated gut content was mixed with distilled water in a micro-centrifuge tube, vortexed and serially diluted up to 10^{-7} dilutions, and plated directly onto a CMC agar plate as inoculum. The inoculated plates were incubated at 32 ^{0}C for 18-48 hours until colonies were visible [17]. Further, pure cultures were obtained by repeated streaking on CMC agar plates. The pure culture plates were codified and preserved at 4°C till further investigation. All the inoculations were done in triplicates.

Screening of cellulolytic microbes

Qualitative assays were performed for primary screening of potential cellulolytic bacteria. The CMC agar plates were inoculated and incubated at 32˚C for 18-48 hours. After an appropriate incubation period, the plates with visible colonies were flooded with 1% Congo-red solution (w/v) and allowed to stand for 15 minutes. The dye was poured off, and the plates were washed with 1 M $NaCl_2$ thoroughly and repeatedly [18]. The duplicate CMC-agar plates were flooded with Gram's Iodine solution and allowed to stand for 5 minutes to develop a zone of hydrolysis to check the cellulolytic potential of the isolates [19]. The bacterial colonies with a distinct and highest diameter of hydrolysis zone on both stained plates were picked up for further investigation.

Estimation of cellulolytic activity of bacterial isolates

The broth cultures were incubated in a shaker incubator at 37°C up to 120 hours at 150 rpm. At every 12 hours interval, the bacterial cultures were collected in a 15 ml centrifuge tube and centrifuged at 12000 rpm for 10 minutes at 4°C in the cooling centrifuge (REMI, India). The supernatant obtained after centrifugation served as a crude enzyme for further assays. The cellulase enzyme activity of the isolated bacterial strains was evaluated on two substrates viz. CMC and Whatman no. 1 filter paper. Enzyme activity was assayed using 3, 5–dinitrosalicylic acid (DNS) reagent [20] by estimating reducing sugars released from CMC (endoglucanase or CMCase assay) and Whatman no. 1 filter paper (exoglucanase or FPase assay). For CMCase assay 2% carboxymethyl cellulose (w/v) (2.0 g CMC dissolved in 100 ml 0.5 M sodium citrate buffer, pH 5.5) and for FPase assay 50 mg (1.0 cm × 6.0 cm) Whatman no. 1 filter paper (saturated in 1 ml 0.5 M sodium citrate buffer, pH 5.5) were used as substrates. For the CMCase assay, 250 µl of 2% CMC (w/v) and 250 µl of crude enzymes were added to the test tube and incubated at 50 ^{0}C for 30 min. For the FPase assay, saturated filter paper (50 mg) strips and 500 µl of the crude enzyme were added and incubated for 60 min at 50°C. To the incubated mixture, 3 ml DNS

reagent was added to stop the reaction, followed by heating in boiling water for 5 min to develop color. The test tubes were allowed to cool down, and 1 ml of Rochelle salt was added to each tube when still warm. Reducing sugar liberated during the reactions was measured as absorbance at 540 nm. One unit (IU) of enzyme activity was defined as the amount of enzyme that released 1 µmol of reducing sugar equivalent to glucose (mg min^{-1}) mg^{-1} protein during the reaction [21]. Total protein concentration was quantified by Lowry's method using bovine serum albumin (BSA) as a protein standard [22].

Scanning electron microscopic (SEM) study of insect gut

The gut tissues of the adult beetle were used for the SEM study. To prepare a sample for the SEM study the insect was surface sterilized before dissection, as described earlier. The gut was incised longitudinally to expose the mucosal surface and then cut into small pieces with the mucosal surface uppermost. Pieces of dissected tissue were immersed in 0.9% physiological saline and immediately transferred and fixed in 1% glutaraldehyde (HIMEDIA) in 0.1 M Sodium Phosphate buffer solution (pH 7.0) for 5 minutes, followed by repeated washing in distilled water for 5-7 minutes to remove mucous partially. Thereafter, the tissues were dehydrated in graded ethanol as follows: 70% (5 minutes), 80% (5 minutes), 85% (5 minutes), 90% (5 minutes), 95% (5 minutes), 100% (absolute ethanol) (5 minutes). Following graded dehydration, the dissected tissues were immersed in hexamethyldisilazane (HMDS) (SRL) for 5 minutes, air-dried, and mounted on stainless steel stubs with double sticky tapes. This study treated the tissues with HMDS for sample drying instead of critical point drying [23]. The samples were then coated in sputter ion coater with gold immediately and scanned under a Carl-Zeiss EVO 18 (special edition) Scanning Electron Microscope (Zeiss, Oberkochen, Germany) to obtain scanning electron micrographs.

Identification of cellulolytic microbes
Morphological and biochemical characterization

The potent cellulolytic isolates were subjected to morphological and biochemical investigations. For morphological investigation and physiological characteristics, the strains were cultivated on a Nutrient agar medium (SRL) at 32°C for 24-48 hours. The colony morphology was examined by using light microscopy (LEICA DMRX Q_{600}). Gram staining, Motility, Catalase, Oxidase, Indole production, Citrate Utilization, Nitrate reduction, Starch hydrolysis test, DNase test, Urease test, and H_2S production were performed for identification [24, 25]. The isolates were identified using Bergey's Manual of Systemic Bacteriology [26]. The strains were maintained in Nutrient agar (SRL) slants and stored at -20 ^{0}C for future use.

Figure 1. Adult Longhorn beetle *P. bigibbosum*

Molecular Identification

The Bacterial identification of isolate PBI9 based on molecular characterization of the 16S rRNA gene was outsourced from Microbial Type Cell Culture Collection and Gene Bank (MTCC), CSIR Institute of Microbial Technology, Chandigarh, India. The protocol was as follows: Genomic DNA was isolated from pure culture using the ZR Bacterial DNA MiniPrep kit (Make Zymo Research). 16S rRNA gene was PCR amplified using universal 27F (AGAGTTTGATCCTGGCTCAG) and 1492R (TACGGYTACCTTGTTACGACTT). PCR product was visualized on 1% Agarose gel. PCR amplicon was gel eluted and purified using QIAquick Gel Extraction Kit (Make Qiagen). Purified PCR product was sequenced using the Sanger DNA sequencing method. Obtained sequences were visualized and analyzed using Finch TV software ver. 1.4. Assembled nucleotide sequences of 16S rRNA gene were subjected to similarity search using BLAST tool in NCBI (http://www.ncbi.nlm.nih.gov) and EzBiocloud portal (http://www.ezbiocloud.net).

Results and Discussion

Specimen collection and gut dissection

The collected specimen was identified *as Prionomma* (Ancyloprotus) *bigibbosum* (White, 1853) (Cerambycidae: Prioninae: Prionini) with two characteristic bumps in the middle of the pronotum [27, 28] (Figure 1). Since the adult long

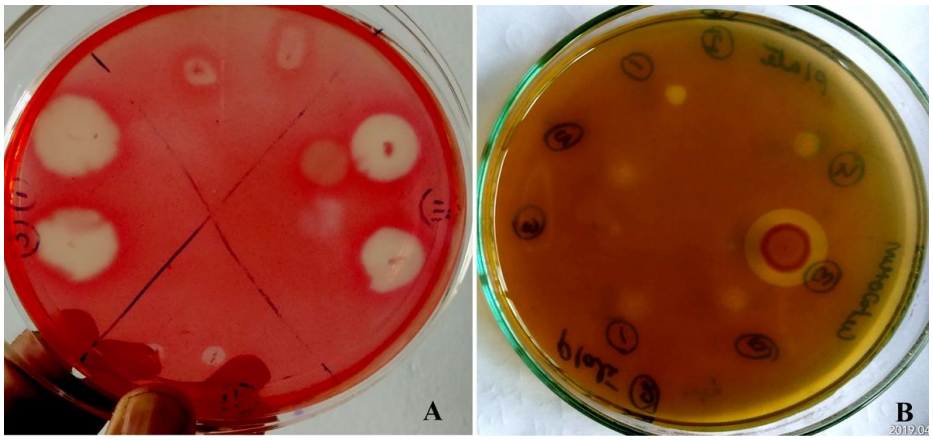

Figure 2. Qualitative assay for cellulase production by plate assay method. Colonies show a clear zone of hydrolysis when stained with (A) Congo red stain, (B) Gram's iodine stain.

horn beetles *P. bigibbosum* are non-feeders and only function in mating and egg-laying, the gut was found to be rudimentary, with no traces of any fresh cellulosic materials present as is evident in other actively feeding larval guts.

Isolation and screening of cellulolytic bacteria

To restrict the growth of non-cellulolytic bacteria present in the gut microenvironment, CMC, a soluble form of cellulose [29], was used as the single carbon source in both solid and broth culture media. Screening with a sole carbon source resulted in isolating 13 cellulolytic bacteria with distinct colony morphology. The strains were cultured in a CMC broth medium at different temperatures ranging from 25°C to 60°C and pH ranging from 4.0 to 10.0. On further screening, only one Gram-positive, Catalase, and Oxidase-positive coccus bacterial isolate showed considerable cellulolytic attributes on the CMC agar plate and was selected for further studies. During the qualitative assay, bacterial colonies grown on CMC agar medium formed a clear zone of hydrolysis around the colonies when the plates were stained with Congo Red and Gram's Iodine stains. The cellulose-degrading capacity of the isolates and the formation of the zone of hydrolysis is proportional to the binding capacity of the different stains with the degraded polymer [30] (Figure 2A and 2B). The strain PBI9 showed optimum activity during plate assay and was chosen for further quantitative assay.

Estimation of cellulolytic activity of bacterial isolates

During quantitative assays, the isolate PBI9 produced different amounts of glucose at different physiological parameters, which were referred to as standard glucose curves to determine the amount of glucose released by the bacterial cellulase enzymes. When quantifying endoglucanase or CMCase activity and exoglucanase or FPase activity, the highest enzymatic activity was recorded as 0.48 IU and 0.71 IU, respectively, post 120 hours of incubation (Figure 3). Enzyme activities were recorded at different temperatures, and pH revealed that at 32°C temperature and pH 6.5, both endocellulose and exocellulose were maximum. The bacterial isolate PBI9 is concluded to be a mesophilic, neutrophile bacteria. Since the optimum cellulase production was recorded post 120 hours of incubation, further standardization is needed for its better cellulase production in a lesser incubation time.

Morphological and biochemical characterization and molecular identification

The isolate PBI9 was found to be an anaerobic, Gram-positive, non-motile cocci bacterium. Colonies were creamy white, opaque in appearance with an irregular and undulate margin. The strain showed positive results for Catalase, Oxidase, Nitrate reduction, TSI test, and deoxyribonuclease (DNase) tests. The isolate showed negative results for citrate utilization, starch hydrolysis, H_2S production, and the Indole test. The results are summarized in Table 1. The 16S rRNA gene sequence of the isolate PBI9 was used for constructing the phylogenetic tree (Figure 4) by the neighbor-joining method [31] using MEGA X software [32]. The evolutionary distances were computed using the p-distance method [33]. The isolate was found

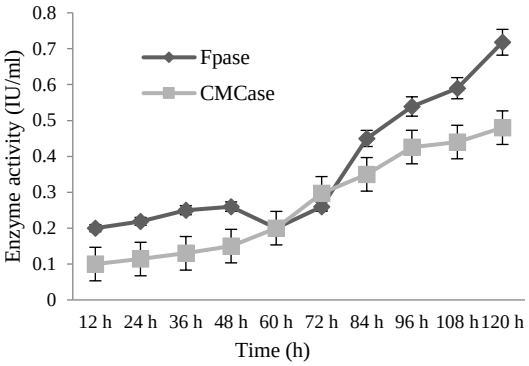

Figure 3. Endoglucanase (CMCase) and exoglucanase (FPase) activity of the isolated and characterized bacterial strain PBI9

Table 1. Physiological and biochemical characteristics of the isolated strain PBI9

Characteristics	Manifestations
Colony Morphology	+ve
Gram staining	+ ve
Motility	Non-motile
Oxidase	+ve
Catalase	+ve
Indole	-ve
Citrate	-ve
Nitrate reduction	+ve
H_2S Production	-ve
Starch hydrolysis	-ve
DNase test	+ve
Urease	-ve
Gelatinase	+ve
Lactose	+ve
Sucrose	+ve
Galactose	+ve
Sorbitol	+ve
Mannitol	+ve
Galactose	+ve

to be 99.86% similar to *Staphylococcus sciuri* strain DSM 20345 (T) and was identified as *Mammaliicoccus sciuri* (Basonym *S. sciuri*) [34] strain PBI9. The 16S rRNA gene sequence was submitted to the NCBI gene bank with GenBank Accession number: MZ_351443.1 [35].

Scanning Electron Microscopic (SEM) study of P. bigibbosum gut

In the present study for SEM imaging, the critical point drying step has been replaced with HDMS during sample preparation. SEM micrograph revealed the presence of abundant coccus bacteria firmly attached to the hindgut of the gastrointestinal tract of the longhorn beetle (Figure 5A, 5B, 5C, and 5D) even after repeated and thorough washing with physiological saline before fixation and mounting. The beetle being a non-feeding adult, it is apparent that the bacterial units in the electron micrograph constitute autochthonous microbiota, confirming our hypothesis [36]. In other studies, the autochthonous microbiota of the coleopteran gut was found to assist in the production of various hormones such as semiochemicals [37] and sex pheromone in *Dendroctonus terebrans* hindgut [38]. The number, distribution, and position of antennal sensilla are very crucial for semiochemicals and chemo-receptions in longhorn beetles. In earlier studies, SEM imaging was employed only to study antenna sensilla in different Cerambycid beetles such as *Allotraeus asiaticus*, *Callidiellum villosulum*, and *Aromia bungi* [39, 40]. In the present study, SEM was employed to examine insect gut in detail to confirm the presence of autochthonous bacteria.

Many reports support, explain, and justify the presence of autochthonous or allochthonous symbiotic bacteria in many insect groups. For example, stinkbugs harbor gut bacteria belonging to the genus Burkholderia that protect against Organophosphorus pesticides, which help in pesticide detoxification [41]. The endo-symbiotic bacteria belonging to the genus *Wolbachia*, *Arsenophonus*, *Sprioplasma*, *Cardinium* have manipulated host reproduction among arthropods by vertical transmission [9]. *Hamiltonella defensa* and *Serratia symbiotica*, the secondary endosymbionts, influenced the host's heat tolerance [42]. The insect and host plant interaction was reported to get influenced by the introduction of a secondary symbiont *Regiella insecticola*, in a vetch aphid [43]. Oliver *et al*. 2003 [44] showed that both *H. defensa* and *S. symbiotica* could increase aphid host resistance against parasitoid wasp.

M. sciuri group is known as animal pathogens, and they have been isolated from rodents, chickens, mammals, and in farm soil and water [45]. *M. sciuri* is reported as a causative pathogen of serious infections in humans, such as endocarditis, peritonitis, septic shock, and wound infections [46]. In the present study, *M. sciuri* has been reported as an autochthonous cellulolytic bacterial strain from *P. bigibbosum gut*. This seems to be the first report of a new isolation source of *M. sciuri* as well as with a new attribute, i.e., cellulolytic

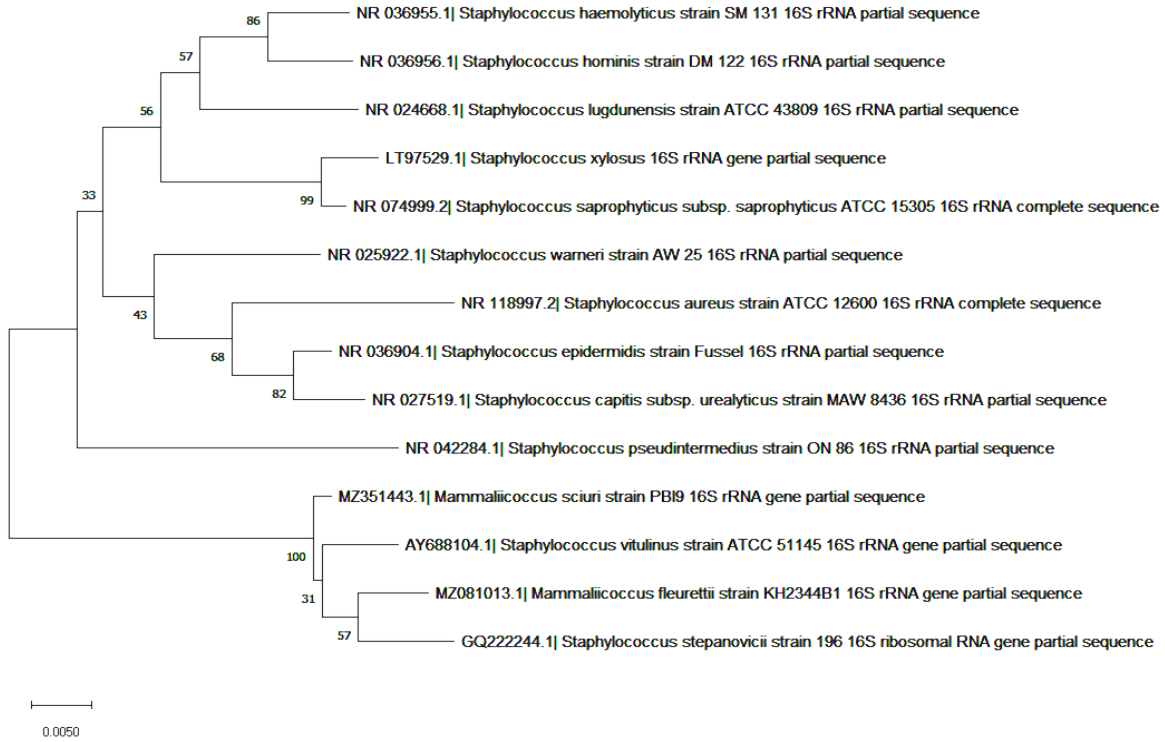

Figure 4. Phylogenetic tree based on 16S rRNA gene sequence showing the relationship of strain *M. sciuri* strain PBI9 with its closely related species. Tree was constructed by the neighbor-joining method. *S. stepanovicii* strain 196 was used as an out-group. Bootstrap values (%) based on 1000 replicates are given at nodes. Bar 0.005 represents substitution per site.

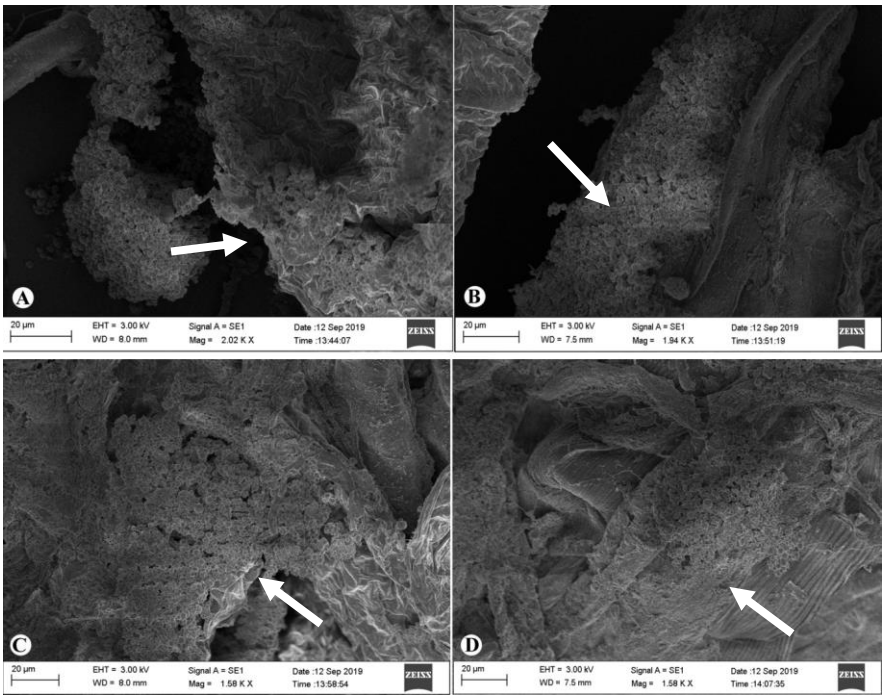

Figure 5. Scanning Electron Micrograph showing A. autochthonous coccoid bacterial cell firmly attached to lumen surface of the gut wall of *P. bigibbosum*. Scale 20µm (×2000); B. bacteria firmly attached to the gut wall of the hindgut. Scale, 20 µm (×1900); C. and D. Overview of the architecture of the bacteria attached to the gut wall of *P. bigibbosum*. Scale, 20 µm (×1500).

property. To delimit the bacterial count for the ease of identification of potent cellulolytic bacteria, the gut contents of all collected specimens were macerated together and plated onto the CMC agar plate directly. The presence of autochthonous cellulolytic gut microbiota in a non-feeding stage of a beetle might signify the horizontal transfer of gut microbiota. Although only one of the thirteen isolated bacterial species showed a considerable amount of cellulase activity, all the species screened were positive for using CMC as the sole carbon source.

Conclusion

SEM micrograph of the gut provided evidence for the presence of autochthonous bacteria in the gastrointestinal tract of *P. bigibbosum* and further biochemical assays verified the existence of the cellulase-producing microbiota within the micro-environment of the gut. The present study was the first one using a scanning electron microscope to demonstrate the gut microbiota of *P. bigibbosum* and it is suggested that these autochthonous microorganisms might have a beneficial functional potential that needs to be evaluated in future investigations. Furthermore, enzymes produced by the longhorn beetle gut microbiota might have a significant role in digestion, especially for substrates such as cellulose, which few animals can digest. Besides, some other critical functional roles must be assigned to these autochthonous cocci bacteria. The significance of the presence of cellulolytic bacteria in non-feeding adult beetle needs further investigation to appraise the role of these autochthonous cellulase enzyme-producing bacteria. Since the research work intends to look for available novel sources for isolation of potent cellulolytic bacteria, the choice of the *P. bigibbosum* gut as a source of bacteria isolation is well justified. To the best of our knowledge, this is the first study undertaking SEM of gut microbiota of longhorn beetle, *P. bigibbosum* and to report the gut as a novel source of cellulolytic bacteria.

The present study is part of the first author's doctoral research, which aims to detect and isolate cellulose-degrading bacterial isolates from insect guts.

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