Chapter-1

ISOLATION OF CELLULOLYTIC BACTERIA
1.1 Introduction

Cellulose is the major constituent of plant matter and thus represents the most abundant organic polymer on Earth. $10^{15}$ kg of cellulose is synthesized and degraded on Earth each year. Cellulose makes a large fraction of the plant dry weight, being typically in the range of 35-50%. It gives stability to the plants even in the absence of water and makes them more resistant. Lignocellulosic biomass makes about 50% of the total plant biomass in the world with an estimated annual production of 10-50 billion tons (Sanchez and Cardona, 2008). Cellulose is a remarkably stable homopolymer, consisting of a linear polymer of $\beta-1, 4$-linked glucose units. The individual cellulose chains contain from about 100 to more than 10,000 glucose units, packed tightly in parallel into microfibrils by extensive inter- and intra chain hydrogen bonding interactions, which account for the rigid structural stability of cellulose. In nature, cellulose is always associated with a variety of other polysaccharides, such as starch, pectin, lignin and a variety of hemicellulose (Brown et al., 1996).

In recent years, accumulation of waste cellulose is increasingly realized as an environmental problem and the utilization of the waste cellulose has become a welcome issue. It is the most widely used natural substance and has become one of the most important commercial raw materials (Lynd et al., 2002). Coir, pineapple and banana leaves have been used as natural cellulose fiber source for textiles and also for paper (Majumdar and Chanda, 2001). Rice and wheat straw have also been used to produce regenerated cellulose fibers as an alternative to wood for cellulose-based materials (Lim et al., 2001). Biomass can also be converted into carbon, hydrogen and oxygen to produce various chemicals, enzymes and proteins. Cellulose and hemicellulose can be used for producing single-cell proteins, cellulosolytic enzymes and for culturing mushrooms (Majumdar and Chanda, 2001). Fibers obtained from straw can be used to
produce all kinds of paper and paper boards, without using any wood pulp. Cellulose can be modified in many different ways to create new compounds that are useful in a variety of applications. The compound can be chemically altered to make the synthetic fiber called Rayon, which is used in clothing. It can also be adjusted to make cellophane (food wrap) which is used to cover food to prevent early spoilage (Moncreff, 1975). Increase in fuel costs and scarcity of petroleum sources led to the use of lignocellulosics to produce ethanol and other sugars by fermentation (Reddy and Yang, 2004).

The cellulose polymer is composed of crystalline and amorphous regions. Cellulose consists of D-glucose units, which condense through β (1, 4)-glycosidic bonds. Cellulose is a straight chain polymer: unlike starch, no coiling or branching occurs, and the molecule adopts an extended and rather stiff rod-like conformation, aided by the equatorial conformation of the glucose residues. The multiple hydroxyl groups on the glucose from one chain form hydrogen bonds with oxygen molecules on the same or on a neighbour chain, holding the chains firmly together side-by-side and forming microfibrils with high strength. This strength is important in cell walls, where the microfibrils are meshed into a carbohydrate matrix, conferring rigidity to plant cells. The average degree of polymerization of cellulose ranges between 1000-10,000 glucose units. Cellulose from wood pulp has typical chain lengths between 300 and 1700 units; cotton and other plant fibers as well as bacterial celluloses have chain lengths ranging from 800 to 10,000 units (Klemm et al., 2005).

The cellulose molecule is more resistant to hydrolysis because of the secondary and tertiary configuration of its chain and its close association with other protective polymeric structures such as lignin, starch, pectin, hemicellulose, proteins and mineral elements present within the plant cellwall. The molecular structure of cellulose, the structure of an elementary
fibril and a microfibril are important features from the standpoint of the hydrolytic degradation of cellulose (Fan et al., 1980a).

The primary occurrence of cellulose is the existing lignocellulose material in forest, with wood as the most important source. By catalyzing the decay of forest and agricultural wastes, the cellulase in combination with hemicellulases and ligninases recycle nutrients which would otherwise remain as inert waste for a considerably longer period of time. Cellulase refers to a family of enzymes which act in concert to hydrolyze cellulose. Cellulolysis is the process of breaking down cellulose into glucose units. The cellulolytic enzymes can be divided into three groups based on their enzymatic activities: 1) endoglucanases 2) exoglucanases and 3) β-glucosidase. Endoglucanases, also known as 1, 4-β-D-glucan-4-glucanohydrolases, attack the cellulose at arbitrary internal amorphous sites and cleave the polysaccharide chain by inserting a water molecule in the 1, 4-β bond. The results are oligosaccharides of various lengths with a reducing and a nonreducing end. The exoglucanases start at either the reducing or nonreducing end of these oligosaccharide chains and release either directly glucose or the cellobiose dimer. The glucose releasing enzymes are called glucanases and the cellobiose releasing enzymes are called cellobiohydrolases. The exoglucanases can also work autonomous and peel cellulose chains from microcrystalline cellulose. Finally the β-glucosidases (or β-glucoside glucohydrolases) hydrolyze the cellobiose dimers and the cellodextrins of various lengths to glucose (Lynd and Weimer, 2002; Himmel and Ding, 2007).

Cellulolytic microorganisms are found among extremely variegated taxonomic groups. Cellulolytic microorganisms can be found in all biota where cellulosic waste accumulates. They usually occur in mixed populations comprising cellulolytic and non-cellulolytic species, which often interact synergistically. These interactions lead to the complete degradation of cellulose,
which is ultimately converted into CO$_2$ and water under aerobic conditions and into CO$_2$, methane and water under anaerobic conditions (Smith and Bryant, 1979).

The cellulase is released into the substrate and the free enzymes start hydrolyzing the cellulose. The glucose to a length of maximum four glucose molecules are taken up by the microorganism and either used directly or cleaved further via intracellular hydrolases. Most of the facultative anaerobic bacteria that produce noncomplexed cellulase systems are most often used in the industrial production of cellulolytic enzymes, because the secreted enzymes can easily be harvested (Lynd and Weimer, 2002).

Cellulases are synthesized in nature by a number of fungi and bacteria. The cellulolytic microbes occupy a broad range of habitats. These microbes play a major role in converting the complex polysaccharides into simple sugars, which they assimilate. The cellulolytic microorganisms are ubiquitous in nature and they include protozoa, fungi and bacteria. The cellulose-decomposing bacteria include aerobic, anaerobic, mesophilic and thermophilic strains, inhabiting a great variety of environments, including the most extreme temperature, pressure and pH. Cellulolytic bacteria have been isolated from many different sources, including the rumen (Hungater, 1957) soils (Blackhall et al., 1985) compost (Madden, 1982) municipal solid waste (Stutzenberger et al., 1970) sewage sludge (Murray et al., 1984), hot springs soil (Sissons et al., 1987). They cellulolytic bacteria have also been isolated from the Indian flying fox (Pteropus giganteus) (Anand and Sripathi, 2004), silk worm larvae (Anand et al., 2010) silver cricket, Wood Borer, Bark Beetles (Delalibera et al., 2005), Rhagium inquisitor, Tetropium castaneum, Plagionotus arcuatus, Leptura rubra (Grunwald et al., 2010) Rhagium inquisitor (Zverlov et al., 2003) and Heterotermes indicola (Vasan et al., 2011).
Insects are among the most successful animals on Earth with regard to their biomass and biodiversity. It is estimated that up to 20% of all insects are obligately associated with symbiotic microorganisms. The role of symbiotic microbes in the production of cellulolytic enzymes has been widely recognized for insects feeding on lignocellulosic biomass (Morrison et al., 2009). In nature, many cellulolytic species exist in symbiotic relationships with secondary microorganisms (Ljungdahl and Eriksson, 1985).

Despite the economic and ecological importance of beetles in general and wood-boring beetles in particular, little is known about the diversity of their gut microorganisms. In addition, nothing is known about the potential role of gut microorganisms in cellulose digestion by wood-boring as well as phytophagous insects. Therefore, the gut of wood-boring and phytophagous insects represents an unexplored habitat for the discovery of new microbes and new insect microorganism interaction. The nutrient-poor composition of the sapwood and phloem substrates, it seems likely that microbes provide nutritional supplements that benefit their hosts. The present study was aimed at isolating, identifying and quantifying cellulolytic microbes from the gut of coconut pest *Oryctes rhinoceros* (rhino beetle), *Bombyx mori* (silk worm) and *Heterotermes indicola* (termite).
1.2 Materials and methods

1.2.1 Collection of insects

Fifth instar larvae of *B. mori*, that vigorously feed on mulberry leaves were obtained from Central Sericulture Research Institute, Samayanallur, South India. They were reared at room temperature (32 ±1°C) and humidity of 82-90% with mulberry leaves as diet. The adult beetle *O. rhinoceros* were collected from the coconut plantation in and around Tiruchirappalli. Tiruchirappalli is situated at the centre of the Tamil Nadu. It is located at an altitude of +78 MSL. Alive adult beetles were collected in plastic containers and taken to the laboratory for a complete microbial analysis.

Termites were obtained from the dead and dry woods in the Anna University, Tiruchirappalli campus. It is located at an altitude of + 78 meter MSL. The environmental conditions such as, temperature and humidity were 32± 2°C and 80 to 90% respectively. The termite’s specimens were identified using morphological characteristics (Department of Entomology, St. Joseph college of Arts and science, cuddalore, Tamil nadu, India).

1.2.2 Isolation of cellulolytic bacteria from the gut of phytophagous insects

*B. mori* larvae were fed on UV sterilized mature mulberry leaves. Five larvae were used for the isolation of bacteria. The entire digestive tract was aseptically removed by dissection. The isolated digestive tract was washed with 0.85% NaCl solution, chopped with a sterile blade, homogenized and the supernatant was taken and serially diluted to estimate total bacterial count and plated on Luria agar plates and also on 0.6% of Carboxy Methyl Cellulose (CMC) containing plates and incubated at 37°C for 24-48 hr. Cellulolytic activity of cellulose degrading bacteria growing on CMC medium was assayed subsequently by the degradation of Whatmann No. 1 filter paper in Berg’s broth.
The adult beetles of *O. rhinoceros* were dissected out under aseptic conditions after surface sterilization with 70% ethanol and the alimentary canal was dissected out and ground in physiological saline and was serially diluted up to $10^{10}$ dilution and plated onto Luria agar plates and 0.6% CMC containing agar plates. The plates were incubated at 37°C for 24-48 hr and the cellulolytic colonies were scored (Vasanthakumar *et al.*, 2006).

The termites, *H. indicola* were surface sterilized by submersion in 70% ethanol for 1 min and rinsed in sterile water before dissection. Insects were dissected aseptically using sterile fine scissors and fine-tipped forceps and the guts were removed in 0.01 M sterile phosphate buffered saline (PBS). The guts were washed in PBS and transferred to 1.5-ml microfuge tubes with 0.5 ml of PBS. The cells were sonicated for 30 sec, macerated with a sterile plastic pestle, and vortexed at medium speed for 10 sec to separate microbial cells from the gut wall. The supernatant was taken and serially diluted up to $10^{10}$ dilution to estimate total bacterial count and plated onto Luria agar plates and 0.6% CMC supplemented agar plates and incubated at 37°C for 24-48 hr (Wenzel *et al.*, 2002). Cellulolytic activity of cellulose degrading bacteria on CMC medium was assayed using degradation of Whatmann No. 1 filter paper in Berg’s broth (Anand *et al.*, 2010). The number of cultivable bacteria per gut was calculated from the average number of colonies present on primary isolation plates (Pelczar *et al.*, 1995).

Number of colony forming unit (CFU)/ ml = (number of colonies x dilution factor/volume plated) (Pelczar *et al.*, 1995). The bacterial colonies were further subcultured on Berg’s medium for studying their cellulolytic ability.

### 1.2.3 Screening and selection of cellulolytic bacteria

Screening and selection of cellulolytic bacteria was done as described by Wood (1980). 0.6% CMC supplemented with Luria agar medium was prepared. Wells of 5 mm diameter were
cut on the agar. The newly isolated bacterial colonies were individually cultured in the Luria broth and the cells were harvested and loaded onto each well cut on the agar medium. The plates were incubated for 24-48 hr. After incubation, the plates were flooded with 0.2% (w/v) Congo red solution for 15 min, and then destained by washing the plate with 1 M sodium chloride solution for several times.

### 1.2.4 Determination of exoglucanase (total cellulase) activity

Total cellulase activity of newly isolated cellulolytic bacterial strains was determined as described by Mandels et al., (1976). An aliquot of 0.5 ml of cell-free culture supernatant from each bacterial culture was taken in a clean test tube and 1 ml of Sodium citrate buffer (pH 5.8) (0.336 g of citric acid and 2.470 g of trisodium citrate was dissolved in 100ml of distilled water). At the temperature of 50°C, one strip of Whatmann no. 1 filter paper 1.0 x 6.0 cm (~ 50mg) was added to each tube and incubated for 1 hr. Tubes were vortexed till the filter paper settled at the bottom of the tube. After incubation, 3.0 ml of Dinitrosalicylic acid (DNS) was added to each tube and mixed well. The glucose standards were prepared by dissolving 0.2- 5.0 mg of glucose per ml and the enzyme blank was prepared by mixing 1.0 ml citrate buffer and 0.5 ml enzyme. Spectro zero was prepared by adding 1.5 ml sodium citrate buffer with 3.0 ml DNS. The sample mixtures, glucose standards, enzyme blank and the spectro zero were boiled together for exactly 5 min in a vigorously boiling water bath containing sufficient water.

After boiling, the tubes were transferred to a cold water bath and 20 ml of distilled water was added, mixed by completely inverting the tube several times. The color formed is measured against the spectro zero at 540 nm. Cellulase activity was expressed in filter paper unit (FPU) per ml of undiluted culture filtrate. One FPU is defined as the quantity (in mg) of reducing sugar liberated in one hr under the standard assay conditions. Reducing sugar produced in one hour
was calculated by comparing $A_{540}$ with that of standard curve. Exoglucanase unit was calculated using the formula, FPU/ml units ml$^{-1} = $ mg glucose released x 0.185 was calculated (Ghose, 1987).

1.2.5 Determination of endoglucanase activity

The endoglucanase activity was determined by the colorimetric method as described by Miller, (1959) using the DNS reagent. The principle of this method based on the determination of the colour developed after the reaction between the reducing sugars liberated from cellulose by DNS reagent. Bacterial culture was taken in 2 ml microfuge tubes and centrifuged at 13,000 rpm for 5 min. Sample was prepared by mixing 250 µl of culture supernatant, 0.9 ml of (1 %; w/v) CMC solution in 50 mM sodium phosphate buffer (pH 7.0) and 250 µl of distilled water. The mixture was incubated at 40°C in a water bath for 30 min. Similarly the glucose standards (0.2-5 mg of glucose per ml) enzyme blanks, substrate blanks and the spectro zero were prepared. After incubation, 1.5 ml DNS-reagent and the tubes were placed in a boiling water bath for 5 min and allowed to cool. The O.D. of the samples was immediately measured at 540 nm. One enzyme unit was equivalent to 1 µmol of glucose equivalents released per min (Miller, 1959). Endoglucanase unit was calculated using the formula, Carboxy Methyl Cellulase /ml units ml$^{-1} = $ mg glucose released x 0.37 was calculated (Ghose, 1987).

1.2.6 Determination of cellobiase activity

Cellobiase activity was assayed following a method described by Rajoka et al., (1997) with slight modifications. The total assay mixture of 3 ml was prepared by mixing 1 ml of 15 mM cellobiose, 1.0 ml of 50 mM sodium phosphate buffer (pH 7.0) and 1.0 ml of culture supernatant. The reaction was initiated by the addition of activated enzyme (enzyme incubated for 10 min at 50°C) and the mixture was further incubated at 40°C for 10 min. The reaction was
stopped by the addition of 3 ml of sodium carbonate (2% w/v) solution. Cellobiase activity was determined by measuring the glucose release by standard glucose oxidase method (GOD). One unit of activity is defined as the number of micromoles of the product (para-nitrophenol or glucose) released per minute per ml of enzyme preparation (Rajoka et al., 1998). Cellobiase unit was calculated using the formula, Cellobiase (CB) /ml units ml⁻¹ = mg glucose released x 0.0926 was calculated (Ghose, 1987).

1.2.7 Effect of pH and temperature on cellulase production

Cellulase activity was measured using the method of Mendels et al. (1969). The optimal pH for cellulase enzyme production by the cellulolytic bacteria was determined by preparing the basal Berg’s medium supplemented with Whatmann filter paper as carbon source. The culture medium were prepared under various pH using 50 mM acetate buffer (for pH 3 and 4), 50 mM Tris-base buffer (for pH 6) and 50 mM sodium phosphate buffer (for pH 8-10). To determine the optimum pH, the substrate and crude enzymes (dilution 1:50 in various buffers) were separately preincubated for 10 min at 50°C and the enzyme reactions were allowed at 50°C for 30 min.

To determine the optimum temperature, one ml of the crude enzyme was diluted in filter paper in citrate buffer (pH 5.8) and preincubated at various temperatures (30 to 60°C) for 10 min. After preincubation, reaction mixture containing 250 μl of the diluted enzymes and 250 μl of the substrate were incubated for 30 min at the designed temperatures from 30°C to 60°C.

The above reactions were stopped by adding 500 μl of DNS to the solution. The mixtures were then heated in boiling water for 5 min, cooled down to room temperature and finally 2.5 ml of distilled water was added. The amount of reducing sugar liberated was determined by measuring the absorbance at 540 nm. Blank was the reaction mixture without the enzyme and the control was prepared by adding the crude enzyme after the DNS reagent. Calibration curve was
prepared using 0.2-1.0 mg/ml glucose. Cellulase specific activity was defined as the amount of enzyme liberating 1 mg of glucose min\(^{-1}\) mg protein\(^{-1}\) under the specified reaction conditions (Miller, 1959).

1.2.8 Identification of isolated cellulolytic bacteria

Isolated cellulolytic bacteria from the gut of phytophagous insects were identified morphologically by examining the bacteria using visual investigation using a light microscope. Motility tests were performed using the hanging-drop technique and motility medium plates (1% nutrient broth, 5.3% gelatin, 0.3% agar, 0.1% KNO\(_3\), pH 7.2) and the plates were incubated overnight at 30°C and characterized by standard biochemical tests (Sneath et al., 1986) and 16S rRNA typing.

1.2.9 Biochemical tests

The utilization of sugars under aerobic and anaerobic conditions was tested by the mellow test through bacterial minimum biochemical reaction tubes (Himedia, Mumbai) (Dong and Cai, 2001). The tests were conducted for sucrose, lactose, glucose, maltose, D-mannitol raffinose, arabinose, D-xylose inulin, oxidase, Catalase, indole production, methyl red, hydrogen sulphide production, Voges-proskauer, citrate, lysine decarboxylase, gelatin hydrolysis, Esculin hydrolysis, acetate utilization, Nitrate reduction and ortho-Nitrophenyl-β-galactoside (ONPG).

1.2.10 16S rRNA typing

The 16S rRNA typing for the isolated cellulolytic bacteria was done through the commercial service (MWG, Bangalore) with the following methodology. Universal primers, 27f (5’-AGAGTTTGATCMTGGCTCAG-3’) and 1492s (5’-ACCTTGGTACGACTT-3’) were used for 16S rRNA typing (Gurtler and Stanisich, 1996). The 16S rRNA fragment was amplified using BigDye terminator V3.1 cycle sequencing Kit in MWG thermocycler with the following
program: 30 s at 95°C, 30 s at 55°C, and 4 min at 72°C for 35 cycles. The PCR reactions were terminated at 72°C for 7 min and, thereafter, cooled at 4°C. The sequencing reaction mixer was prepared by mixing 1µl of BigDye v3.1, 2µl of 5x sequencing buffer, 1µl of 50% Di Methyl Sulfoxide, 4 Pico moles of primer (2µl) and genomic DNA. The constituted reaction was denatured at 95°C for 5 min. Cycling began with denaturing at 95°C for 30 sec, annealing at 52°C for 30 sec and extension for 4 min at 60°C and cycle repeated for a total of 30 cycles in a MWG thermocycler. To ascertain the specificity of the PCR amplification, negative control (PCR mix without DNA template) and positive control (PCR mix with species DNA template) were included. Amplification was confirmed by electrophoresis analysing 5 µl PCR reaction mixtures on a 1% agarose gel. The PCR product was purified on sephadex plate (Edge Biosystems) to remove unbound labelled and unlabelled nucelotides and salts. The purified reaction mixture was loaded on to the 96 capillary ABI 3700 DNA analyzer and electrophoresis was carried out for 4 hr and the 16S rRNA sequence was read. The sequences of 16S rRNA of the cellulolytic bacteria were submitted to National Centre for Biotechnology information (NCBI), GenBank.

1.2.11 Phylogenetic analysis

The 16S rRNA sequences were aligned and paralleled with the sequence of corresponding bacterium in NCBI, GenBank by Blast software. The homologous characteristics were analysed and phylogenetic tree was constructed by Mega 2 software.
1.3 Results

1.3.1 Bacterial isolates from the gut of phytophagous insects

Culturable bacterial strains were isolated from the gut of *B. mori*, *O. rhinoceros* and *H. Indicola*, in order to identify and study the cellulase producing strains. Most probable numbers (MPN) of cellulolytic bacteria were determined by inoculating in the tubes containing filter paper. Cellulolytic and total viable MPN were determined with CMC broth (0.6%; w/v) and total viable count (TVC) broth respectively (Table 1). Utilization of cellulose was detected visually by noting a decrease in the settled volume of the substrate.

1.3.2 Density of bacteria in the gut of *Bombyx mori*

*B. mori* larvae are widely used for silk production. Some of the digestive enzymes that degrade the carbohydrates might be produced by gut microflora. Totally, $6.11 \pm 0.12 \times 10^{11}$ CFU/ml isolates were obtained from the digestive tract of *B. mori*. Among the isolated strains 60.87 ±0.51 x10<sup>10</sup> CFU/ml were facultative anaerobes and 2.70 ± 0.21 x 10<sup>9</sup> CFU/ml were strict anaerobes. 4.078 ± 0.011 x 10<sup>5</sup> CFU/ml of cellulolytic bacteria were screened and selected on CMC plates by congored overlay method.

1.3.3 Density of bacteria in the gut of *Orcytes rhinoceros*

*O. rhinoceros* is a rhinoceros beetle is a major pest of coconut and oil palms. Totally, $3.63 \pm 0.43x 10^{11}$ CFU/ml isolates were obtained from the digestive tract of *O. rhinoceros*. Among the isolated strains 36.201 ± 0.51 x 10<sup>10</sup> CFU/ml were facultative anaerobes and 1.35 ±0.21 x 10<sup>9</sup> CFU/ml were strict anaerobes. 2.162 ± 0.02 x 10<sup>5</sup>CFU/ml of cellulolytic bacteria were screened and selected on CMC plates by congored overlay method.
Table 1 Number of cellulolytic bacteria isolated from the entire digestive tract of phytophagous insects (n=3; ±SD)

<table>
<thead>
<tr>
<th>Phytophagous Insects</th>
<th>Anaerobe</th>
<th>Total No. of colonies (LB agar) (CFU/ml)</th>
<th>Cellulolytic colonies (on Cellulose supplemented Agar) (CFU/ml)</th>
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<tr>
<td><em>Bombyx mori</em> (Silk worm)</td>
<td>Facultative Anaerobes</td>
<td>60.87 ±0.51 x10^{10}</td>
<td>4.078 ± 0.011 x 10^{5}</td>
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<td>Obligate anaerobes</td>
<td>2.70 ± 0.21 x 10^{9}</td>
<td>Nil</td>
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<td><em>Oryctes rhinoceros</em> (Coconut Beetle)</td>
<td>Facultative Anaerobes</td>
<td>36.201 ± 0.51 x 10^{10}</td>
<td>2.162 ± 0.02 x 10^{5}</td>
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<td></td>
<td>Obligate anaerobes</td>
<td>1.35 ± 0.21 x 10^{9}</td>
<td>Nil</td>
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<tr>
<td><em>Heterotermes indicola</em> (Termite)</td>
<td>Facultative Anaerobes</td>
<td>20.71 ± 0.5 x 10^{10}</td>
<td>3.009 ± 0.01 x 10^{5}</td>
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<tr>
<td></td>
<td>Obligate anaerobes</td>
<td>1.91 ± 0.23 x 10^{9}</td>
<td>Nil</td>
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</table>
1.3.4 Density of bacteria in the gut of *Heterotermes indicola*

*H. indicola* is a termite which feeds on dry food has many symbionts in its gut for digestion. Totally, $2.08 \pm 0.12 \times 10^{11}$ CFU/ml isolates were obtained from the digestive tract of *H. indicola*. Among the isolated strains $20.71 \pm 0.5 \times 10^{10}$ CFU/ml were facultative anaerobes and $1.91 \pm 0.23 \times 10^{9}$ CFU/ml were strict anaerobes. $3.009 \pm 0.01 \times 10^{5}$ CFU/ml of cellulolytic bacteria were screened and selected on CMC plates by congored overlay method. Results were subjected to ANOVA (SPS software) analysis which showed that there is statistical significance between the cellulolytic bacteria isolated from each insect ($P \geq 0.05$).

1.3.5 Identification of isolated cellulolytic bacteria

Totally twenty cellulolytic bacterial strains were isolated from the gut of insects, *B. mori*, *O. rhinoceros* and *H. Indicola*. Among twenty strains, five strains showed clear zone after staining with Congo red (Fig. 1) and these five strains were identified through biochemical tests (Table 2) and 16S rRNA sequence typing. Thus the isolated bacteria were identified as *Enterobacter cloacae*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Pseudomonas fluorescens* and *Proteus mirabilis*. The 16S rRNA sequence of the above five bacterial strains were submitted to gene bank and their accession numbers are as follows: *E. cloacae* (FJ799063) (Fig. 2), *P. aeruginosa* (GU213909) (Fig. 3), *K. pneumoniae* (HQ231794) (Fig. 4), *P. fluorescens* (HQ231795) (Fig. 5) and *P. mirabilis* (HQ231796) (Fig. 6). The sequence similarities of the isolated strains were compared with the available databases in NCBI and the percentage of similarities were tabulated (Table 3). The 16S rRNA gene sequence of the isolates were deposited in NCBI and aligned with reference to 16S rRNA sequences of the European Molecular Biological Laboratory (EMBL), GenBank (GB, Germany) and the Data Base of Japan (DBJ) using the BLAST algorithm (Altschul *et al.*, 1997) available at NCBI in internet.
Table 2 Biochemical characteristics of isolated cellulolytic bacteria

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<td>Enterobacter cloace JV</td>
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<td>Pseudomonas aeruginosa JV</td>
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<td>Pseudomonas fluorescens JV</td>
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+ = Positive, - = Negative, R = Rod shape

Table 3 Sequence similarity analysis of isolated bacterial strains

<table>
<thead>
<tr>
<th>Insect source</th>
<th>Isolated cellulolytic strains and their NCBI accession number</th>
<th>Closely related microorganisms and their NCBI Accession no.</th>
<th>Sequence Similarity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heterotermes indicola (Termite)</td>
<td>Enterobacter cloace JV- FJ799063</td>
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<td></td>
<td>Pseudomonas aeruginosa JV- GU213909</td>
<td>Pseudomonas sp. GZ1-EF551040.1</td>
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<td>Klebsiella pneumoniae JV HQ231794</td>
<td>Klebsiella pneumoniae RSN19-HM751200.1</td>
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<td>Oryctes rhinoceros (Coconut Beetle)</td>
<td>Pseudomonas fluorescens JV HQ231795</td>
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<td>Bombyx mori (Silk worm)</td>
<td>Proteus mirabilis JV-HQ231796</td>
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</tr>
</tbody>
</table>
(Sacchi et al., 2005). Sequence data were aligned and the evolutionary relationship of the sequence information was studied by phylogenetic analyses.

The results of the phylogenetic analysis are presented in Figs. (7 to 11). The *E. cloacae* isolated from termite gut was phylogenetically closely related to *Enterobacter* sp. DQ11 (100% sequence similarity), *Enterobacter cloacae* subsp. *dissolvens*, *Pantoea agglomerans*, *Cronobacter sakazakii*, *Klebsiella* sp, *Leclercia* sp (99%). Two cellulolytic strains isolated from the gut of *O. rhinoceros* were *P. aeruginosa JV* and *K. pneumoniae JV*. *P. aeruginosa JV* showed sequence 99% similarity with *Pseudomonas* sp. GZ1, *Pseudomonas* sp. L29, *Pseudomonas* sp. NR2(2010) phylogenetically and *Klebsiella pneumoniae JV* showed 98% sequence similarity with *Bacterium* DX120E, *Klebsiella variicola At-22*, *Enterobacteriaceae bacterium PB29*. *Proteus mirabilis JV* and *P. fluorescens JV* were isolated from the digestive tract of *B. mori*. *Proteus mirabilis JV* showed sequence similarity with *Proteus mirabilis* strain FUA1237 (98%), *Proteus vulgaris* (97%), *Proteus mirabilis* strain MA (96%) and *P. fluorescens JV* showed 98% similarity with *Pseudomonas fluorescens* strain YUST-DW18 (98%).

**1.3.6 Effect of pH**

The effect of pH on cellulase enzyme production by the isolated bacterial strains was determined using DNS method. The highest cellulase activity was found between pH 6-7. The cellulase activity was observed between pH 4.0 and pH 9.0. Among the isolated bacterial strains, the maximum cellulolytic activity was showed by *P. aeruginosa JV* at pH 6 (Table 4 and Fig.12).
Fig. 1 Zone of cellulose utilization indicated by the hydrolysis of CMC because of cellulase production by the newly isolated bacterial strains grown on Luria agar supplemented with 0.6% CMC
The 16s rRNA sequence of *E. cloacae* was studied and compared with other available rRNA sequences of bacteria available at NCBI. The 16S rRNA sequence of *E. cloacae* was deposited at NCBI with accession no. FJ799063.
The 16s rRNA sequence of *P. aeruginosa* was studied and compared with other available rRNA sequences of bacteria available at NCBI. The 16S rRNA sequence of *P. aeruginosa* was deposited at NCBI with accession no. GU213909.
The 16s rRNA sequence *K. pneumoniae* was studied and compared with other available rRNA sequences of bacteria available at NCBI. The 16S rRNA sequence of *K. pneumoniae* was deposited at NCBI with accession no. HQ231794.
The 16s rRNA sequence *P. fluorescens* was studied and compared with other available rRNA sequences of bacteria available at NCBI. The 16S rRNA sequence of *P. fluorescens* was deposited at NCBI with accession no. HQ231795.
The 16s rRNA sequence *P. mirabilis* was studied and compared with other available rRNA sequences of bacteria available at NCBI. The 16S rRNA sequence of *P. mirabilis* was deposited at NCBI with accession no. HQ231796.
Fig. 7 Phylogenetic tree derived from the 16s rRNA sequence of *Enterobacter cloacae JV*
Fig. 8 Phylogenetic tree derived from the 16S rRNA sequence of *Pseudomonas aeruginosa* JV.
Fig. 9 Phylogenetic tree derived from the 16S rRNA sequence of *Klebsiella pneumoniae* JV
Fig. 10 Phylogenetic tree derived from the 16S rRNA sequence of *Pseudomonas fluorescens JV*
Fig. 11 Phylogenetic tree derived from the 16S rRNA sequence of *Proteus mirabilis IV*
Table 4 Effect of pH on the cellulase activity of the isolated cellulolytic bacteria (n=3; Mean±SD)

<table>
<thead>
<tr>
<th>Cellulolytic bacteria</th>
<th>pH 4</th>
<th>pH 5</th>
<th>pH 6</th>
<th>pH 7</th>
<th>pH 8</th>
<th>pH 9</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. aeruginosa JV</em></td>
<td>0.072±0.01</td>
<td>0.112±0.01</td>
<td>0.151±0.01</td>
<td>0.131±0.00</td>
<td>0.101±0.01</td>
<td>0.091±0.00</td>
</tr>
<tr>
<td><em>E. cloacae JV</em></td>
<td>0.069±0.01</td>
<td>0.102±0.01</td>
<td>0.127±0.01</td>
<td>0.141±0.01</td>
<td>0.112±0.00</td>
<td>0.087±0.00</td>
</tr>
<tr>
<td><em>P. fluorescens JV</em></td>
<td>0.057±0.01</td>
<td>0.099±0.01</td>
<td>0.135±0.00</td>
<td>0.127±0.00</td>
<td>0.118±0.01</td>
<td>0.087±0.01</td>
</tr>
<tr>
<td><em>K. pneumoniae JV</em></td>
<td>0.049±0.01</td>
<td>0.082±0.00</td>
<td>0.111±0.01</td>
<td>0.125±0.00</td>
<td>0.111±0.00</td>
<td>0.079±0.01</td>
</tr>
<tr>
<td><em>P. mirabilis JV</em></td>
<td>0.041±0.00</td>
<td>0.076±0.01</td>
<td>0.109±0.01</td>
<td>0.121±0.01</td>
<td>0.091±0.00</td>
<td>0.068±0.00</td>
</tr>
</tbody>
</table>

Fig. 12 Effect of pH on the cellulase activity of the isolated cellulolytic bacteria

1.3.7 Effect of temperature
Optimum temperature for the cellulase activity was determined by measuring the released glucose molecules by cellulase enzyme through DNS method at 30, 35, 40, 45, 50, 55 and 60°C. The isolated colonies showed activities between 35-45°C and the activity slowed down below 35°C and above 45°C. The maximum enzyme activity was observed in *P. aeruginosa* at 45°C (Table 5 and Fig. 13).

### 1.3.8 Total cellulase enzyme activity of isolated bacterial strains

#### 1.3.8.1 Determination of endoglucanase activity

Five cellulolytic strains produced endoglucanase using CMC as substrate. A comparison of enzyme activities among the strains revealed that the endoglucanase activity of *E. cloacae JV* (maximum of 4.97 CMC/ml) was greater than *P. mirabilis* which showed a limited endoglucanase activity of 3.01 CMC/ml. The cellulolytic bacteria isolated from the gut of termite and *O. rhinoceros* showed highest values whereas the cellulolytic isolate from the *B. mori* gut revealed low activity (Table 6 and Fig. 14).

#### 1.3.8.2 Determination of exoglucanase activity

Exoglucanase activity for the isolated cellulolytic strains was analyzed using Whatmann filter paper No.1 as substrate. The production of exoglucanase by *P. aeruginosa* isolated from *O. rhinoceros* has a maximum of 0.207 FPU /ml and *P. mirabilis* from *B. mori* showed a minimum activity of 0.148 FPU/ml (Table 6 and Fig. 14).

#### 1.3.8.3 Determination of cellobiase activity

Cellobiase activity for the isolated cellulolytic strains was determined using cellobiose as substrate. Cellobiase activity of all the isolated strains was closely related and the enzyme activity was ranging from 1.1-2.1 CB/ml (Table 6 and Fig. 14).

Table 5 Effect of temperature on the cellulase activity of the isolated cellulolytic bacteria (n=3; Mean ± SD)
## Table 6: Total cellulase enzyme activity of isolated cellulolytic bacteria

<table>
<thead>
<tr>
<th>Cellulolytic bacteria</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30°C</td>
</tr>
<tr>
<td><em>P. aeruginosa JV</em></td>
<td>0.091±0.002</td>
</tr>
<tr>
<td><em>E. cloacae JV</em></td>
<td>0.109±0.001</td>
</tr>
<tr>
<td><em>P. fluorescens JV</em></td>
<td>0.077±0.008</td>
</tr>
<tr>
<td><em>K. pneumoniae JV</em></td>
<td>0.109±0.009</td>
</tr>
<tr>
<td><em>P. mirabilis JV</em></td>
<td>0.111±0.007</td>
</tr>
</tbody>
</table>

Fig. 13 Effect of temperature on the total cellulase activity of the isolated cellulolytic bacteria

![Graph showing the effect of temperature on the total cellulase activity of the isolated cellulolytic bacteria](image)

Table 6 Total cellulase enzyme activity of isolated cellulolytic bacteria
### Bacterial Strains

<table>
<thead>
<tr>
<th>Bacterial Strains</th>
<th>Exoglucanase activity (FPU/ml)</th>
<th>Endoglucanase activity (CMC/ml)</th>
<th>Cellobiase activity (CB/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Enterobacter cloacae JV</em></td>
<td>0.197±0.12</td>
<td>4.97±0.13</td>
<td>2.1±0.16</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa JV</em></td>
<td>0.207±0.11</td>
<td>3.01±0.17</td>
<td>1.9±0.19</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae JV</em></td>
<td>0.154±0.13</td>
<td>4.22±0.16</td>
<td>1.6±0.12</td>
</tr>
<tr>
<td><em>Pseudomonas fluoroscens JV</em></td>
<td>0.179±0.09</td>
<td>2.32±0.23</td>
<td>1.6±0.09</td>
</tr>
<tr>
<td><em>Proteus mirabilis JV</em></td>
<td>0.148±0.23</td>
<td>3.09±0.15</td>
<td>1.1±0.18</td>
</tr>
</tbody>
</table>

Fig. 14 Total cellulase enzyme activity of isolated cellulolytic bacteria

**1.4 Discussion**
Totally twenty cellulolytic bacterial strains from the insect gut were screened by congored overlay method. Congo-red interacts with (1, 4) - β – D- glucans and (1, 3) β D-glucans and a clearing zone around the colony on the agar medium indicates the hydrolysis of cellulose. The bacteria secrete cellulase enzyme degrade cellulose into cellobiose, and then break down cellobiose to form glucose and finally metabolize glucose to organic acids (Lactic acid, acetic acid etc.). The organic acids thus formed lower the pH of medium. The pH difference affected the color of the medium and the formation of the yellow clear zone around the colony would finally appear indicating the cellulase enzyme production.

Among the twenty bacterial isolates only five bacteria were found to possess high cellulase enzyme activities. These bacterial colonies were selected based on DNS method. In this method free carboxyl groups were released by the oxidation of aldehyde groups of glucose molecule, which are formed by the action of cellulase enzyme. The cellulolytic strains isolated from *B. mori*, *O. rhinoceros* and *H. indicola* were identified as *E. cloacae*, *K. pneumoniae*, *P. mirabilis*, *P. fluorescens* and *P. aeruginosa* respectively. Insect gut microbiota is considered as a complex ecosystem containing over a hundred bacterial species including facultative anaerobes such as *Bacillus* sp. and *Salmonella* sp.(Khiyami and Alyamani, 2008).

*Enterococcus* sp. and *Xanthomonas* sp are already reported as facultative anaerobes (Varma *et al.*, 1994). The role of facultative anaerobes in the insect gut is reportedly to scavenge oxygen, which has permeated the exoskeleton into the gut (Madigan *et al.*, 2002). Presence of aerobic and facultative anaerobic bacteria from the larval gut demonstrated a potentially deep penetration of oxygen into the gut and an essential role of oxygen in the mineralization of aromatic compounds. The present study showed that, the majority of the insect intestinal microfloras are facultative anaerobes. The gut bacteria help the insects in the digestion of
cellulosic food was already known (Domingo et al., 1998). They also prevent the growth of pathogenic microorganisms (Kodama and Nakasuji, 1971) by inducing antibacterial peptide secretion and thus protect the host insects from the parasitoid attack (Oliver et al., 2003).

Silkworm *B. mori* is an economically important insect used in the production of commercial silk. The preferred food of silk worm is mulberry leaves. Silkworms are solely reared on mulberry leaves diet. First instar larvae are fed for tender leaves rich in protein and water content, but poor in carbohydrate content. As leaves grow, the protein and water content decrease, and the carbohydrate content increases (Aruga, 1994). Immediately after hatching, the larvae begin to consume 30,000 times more than their own body weight, of mulberry leaves and grow rapidly with the capability of utilizing various polysaccharides (Fenemore and Prakash, 1992). The bacterial strains isolated from the fifth instar larvae were found to have the ability to digest cellulose content of mulberry leaves. This suggests that these bacteria may secrete enzymes important for cellulose digestion. Gram positive *Bacillus circulans* and Gram negative *P. vulgaris, K. pneumoniae, E. coli, Citrobacter freundii, S. liquefaciens, Enterobacter* sp., *P. fluorescens, P. aeruginosa, Aeromonas* sp., and *Erwinia* sp. were isolated from the gut of *B. mori* (Anand et al., 2010).

*O. rhinoceros*, a Coleopteran insect feeds on young unopened coconut leaves as a sole diet. The coconut leaves are reportedly having higher carbohydrate content. The isolated bacteria isolated from the gut of the beetle were found to have symbiotic association with the beetle. Coleopteran insects were found to harbour highly diverse gut microflora dominated by sugar fermenting bacteria (Egert et al., 2005). The isolated bacterial strains such as, *P. aeruginosa* and *Bacillus* sp. from *O. rhinoceros* were found to degrade filter paper, a cellulosic substrate. These
bacterial strains were already reported to have association with insects helping them in cellulose digestion (Schafer et al., 1996).

*H. indicola* is a serious pest of drywood. It consumes wood fibre as sole diet, which contain cellulose. Cellulose is a rich energy source (as demonstrated by the amount of energy released when wood is burned), but remains difficult to be digested. Termites rely primarily on symbiotic protozoa (metamonads) such as *Trichonympha*, and other microbes in their gut to digest the cellulose and absorb the end products for their utilization as a carbon source. Termites are among the most important lignocellulose-digesting insects and harbour a variety of symbiotic microorganisms in their hindgut, including bacteria, Archaea and Eukarya (Konig, 2006) that live in the gut environment (Brune, 2007; Leadbetter, 2007). Symbiotic bacteria found in the gut of termites are known to produce some of the necessary digestive enzymes. The relationship is one of the finest examples of symbiotic relationship among animals. The symbiotic relationship of termite and microbes is permanent because of their necessity from each other to survive. The exploitation of cellulolytic bacterial symbionts in the gut has been reported in many termites (Termitidae) to digest cellulose (Breznak, 1982). *Bacillus cereus* strain Razmin A, *Enterobacter aerogenes* strain Razmin B, *Enterobacter cloacae* strain Razmin C, *Chryseobacterium kwangyangense* strain Cb and *Acinetobacter* strain Raminalimon bacteria was isolated and identified from the local termite *Coptotermes curvignathus* (Ramin et al., 2008). Gram-positive bacteria isolated from termites were identified through 16S rRNA typing and mainly assigned to the genera *Bacillus, Streptococcus* and *Staphylococcus*, but most of the isolates from termites were reportedly Gram-negative bacteria (Krasilnikov and Satdykov, 1970; Thayer, 1978). Interestingly, in both termites and coleopteran beetles, there is a clear compartmentalization of
the gut regions with distinct acidic and alkaline pH, each harboring specific groups of microorganisms (Lemke et al., 2003).

In *Eublaberus posticus* (cockroach) between $2.0 \times 10^5$ and $1.0 \times 10^7$ aerobic and between $6.0 \times 10^6$ and $2.0 \times 10^8$ anaerobic cellulolytic bacteria were found per ml gut content. The cellulolytic bacteria were assigned to the genera *Clostridium, Eubacterium, Serratia, Citrobacter* and *Klebsiella* (Cruden and Markovetz, 1979). A strictly anaerobic cellulolytic *Clostridium* was previously isolated from *Nasutitermes lujae* (Hethener et al. 1992). Carboxymethyl cellulose and cellobiose utilizing bacteria such as *Enterobacter aerogenes, E. cloacae* and *Citrobacter agropyrri* (Corneybactetrium) were isolated from the *Copotermes curuvignathus*. Cellulolytic bacterial strains such as; *Serratia marcescenes* and *Enterobacter aerogenes* have been isolated from Formosan termite *Coptotermes formosanus* (Adams and Boopathy, 2005). *P. vulgaris, P. mirabilis, Citrobacter freundii, Serratia liquefaciens* and *Klebsiella oxytoca*. These bacteria help in the digestion of cellulose and xylan in the diet of the bat, *Pteropus giganteus* (Anand and Sripathi, 2004). Two fungi isolated from the gut of *Saperda vestita* adults had strong cellulose degradation activity. Sequences of the ITS1, 5.8S, and ITS2 rRNA regions indicated that these fungi are highly similar to *Fusarium culmorum* and *Penicillium crustosum*, respectively (Delalibera et al., 2005).

The present study showed that *B. mori, O. rhinoceros* and *H. indicola* carry an appreciable quantum of microbial flora which included the bacterial strains such as, *P. mirabilis, P. aeruginosa, K. pneumoniae, P. fluorescens* and *E. cloaceae* identified through 16S rRNA typing. The use of 16S rRNA gene sequencing to study the relatedness of prokaryotic species is well established and has led to the increased availability of 16S rRNA databases. The convergence of these technical and computational advances has also enhanced the application of
16S rRNA gene sequence analysis to bacterial identification (Patel, 2001; Amann et al., 1995). It was recently reported that subtle sequence differences in the 16S rRNA gene could be used for species identification. The use of 16S rRNA gene sequences to study bacterial phylogeny and taxonomy has been used as the most common housekeeping genetic marker for a number of reasons. The reasons include its presence in almost all bacteria, often existing as a multigene family, or operons; the function of the 16S rRNA gene over time has not been changed, suggesting that random sequence changes are a more accurate measure of time (evolution) and the 16S rRNA gene (1,500 bp) is large enough for bioinformatics purposes (Ashelford et al., 2005). The 16S rRNA analysis for characterization of bacteria is excellent. The discriminatory power of this technique depends on genetic diversity of the class of isolates being examined (Blanc et al., 1993). Although no marker fulfills all of the requirements for epidemiological-ecological comparison of bacterial strain, 16S rRNA offers a clear advantage over other molecular methods because it is based on the rRNA sequences which are highly conserved among eubacteria. In addition, identification diversity and the ecological spread of environmental bacteria are difficult using other conventional methods.

All the isolated cellulolytic bacteria utilized both the forms of cellulose (amorphous and crystalline) and also all of the cellulolytic bacteria were identified as gram negative. The filter paper assay (FPA) is the key method for the analysis of total cellulase activity. The filter paper assay was performed with 1 × 1 cm strip of Whatman no. 1 filter paper, as the standard substrate because it is readily available, inexpensive and well accepted (Coward-Kelly et al. 2003). P. aeruginosa and E. cloacae have high cellulolytic activity and were able to grow in slightly alkaline pH (8) which corresponds to the insect gut pH, suggesting that they might play a role in the digestion of cellulose. The pH of the gut segment has a positive role in influencing the action
of any enzymes secreted into or carried with food into that segment. Carbohydrate digestive enzymes usually work best at near to the neutral pH or under slightly alkaline conditions (Nation, 2002). The cellulolytic bacterial counts increase as the insect grows from one larval stage to another and the bacterial strains were indeed helpful in the development of the insects by allowing them to utilize cellulose present in their diet as a carbon source (Stewart et al., 1988).