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Isolation and Identification of Cellulolytic Bacteria from the Gut of Three Phytophagus Insect Species

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ABSTRACT

The cellulolytic bacteria from the gut of three different phytophagous insects were studied to isolate novel cellulolytic organism for biofuel industry. Among the threse, gut of P. quatuordecimpunctata *larvae contained both highest no of total bacterial count (6.8x10^7 CFU/gut) and cellulolytic bacteria (5.42x10^3 CFU/gut). Fifteen different isolates were obtained from the gut of* O. velox, A. miliaris *and* P. quatuordecimpunctata. *All the isolates produced clear zone in CMC medium staining with Congo red. The isolates included Gram positive* Enterococcus, Microbacterium *and Gram negative* Aeromonas, Erwinia, Serretia, Flavobacterium, Acenitobacter, Klebsiella, Yersinia, Xenorhabdus, Psedomonas *and* Photorhabdus. *Out of the fifteen isolated and identified bacterial species, twelve bacterial species were novel being reported for first time as having cellulase activity.*

Key words: Gut bactéria, cellulolytic, *O. velox, A. miliaris, P. quatuordecimpunctata*

INTRODUCTION

The demand for fuel is increasing with the advancement of civilization. But dependence solely on fossil fuel is causing the depletion of its stock rapidly. Moreover, fossil fuel is not environment friendly. As a result, alternative source of fuel or renewable energy is the demand of present day. The production of bioethanol has been tried in different countries from food kernel and other starchy materials (Schubert 2006; Lin and Tanaka 2006) which causes increased food price. Lignocellulosic plant biomass that is abundant in nature has been considered an attractive alternative source of ethanolic biofuel that mitigate global worming, reduce competition for food and ensure economic sustainability (Lynd et al. 1991; Lynd et al. 2008). Cellulose of plants can be converted to constituent glucose by cellulase system and this glucose could be fermented to ethanol (Wyman 1999; Hamelinck et al. 2005).

Conversion of lignocellulosic materials into glucose by enzymatic method is a costly process (Wyman 1999; Wyman 2007). It has been suggested that reducing cellulase enzyme by half decreases the processing cost up to 13% (Lynd et al. 2008). Current limitations of enzymatic degradation of lignocellulosic biomass are mostly related to enzymatic stability, low specific activity and susceptibility to inhibitory agents or byproducts (Mousdale 2008; Kristensen et al. 2009). To make the process cost effective, efficient cellulase are being haunted (Oppert et al. 2010). Insects are very efficient in converting the plant materials into glucose with their highly efficient gut systems that can truly be considered

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as highly efficient natural bioreactors (Sun and Scharf 2010).

Cellulolytic activity has been reported in the gut fluids in different insect species belonging to ten insect orders (Watanabe and Tokuda 2001; Willis et al. 2010). The cellulase activity of these insects is historically attributed to symbiotic gut microorganisms. Herbivorous insects often rely on microbes in their guts to digest plant materials such as cellulose and lignin (Weibing et al. 2013). Hence, the guts of insects could be a potential source of microorganisms with novel cellulolytic activities for biofuel production.

The study on gut microbes of insects with cellulolytic activities is limited. The aims of this investigation was to isolate and identify novel cellulolytic bacteria for industrial use from three different phytophagous insect guts namely *O. velox* belonging to order orthoptera and *P. quatuordecimpunctata* and *A. miliaris* both belonging to order coleoptera.

MATERIALS AND METHODS

Insects and Gut Fluids Collection

O. velox at nymphal stage and larvae of *A. miliaris* and *P. quatuordecimpunctata* were collected from the leaves of infested host plants during April-June at midday from Fatickchari upozilla, Chittagong, Bangladesh. Until dissection, insects were kept on the plant host tissues. Five of each type of insect (nymph/larvae) were selected for dissection when they were actively feeding. After immobilizing on ice for around half an hour, the insects were surface sterilized by submersion in 70% ethanol for 1 min and rinsed in sterile water before dissection. Insects were dissected in 0.9% NaCl using dissection scissors and fine-tipped forceps to remove the intact entire guts. The guts of same insects were transferred to 1.5 mL centrifuge tubes containing 0.5 mL 0.9% NaCl, homogenized and vortexed with medium speed for 2 min to separate the microbial cells from the gut wall. All gut extractions were performed in a sterile ventilation hood on the same day of collection.

Enumeration of Total Microbes and Cellulolytic Microbes

The microbes present in the guts were enumerated by serial dilution of macerated guts of three insects with sterile water in two solid media using 10 µL of preparation. One medium (lysogenic broth agar plate) contained 10 g peptone, 5 g yeast extract, 5 g NaCl, 20 g agar and l L distilled water (this medium was used for total microbial count) (Bertani 2003). The other medium (CMC agar plate) contained 2.5 g $NaNO_3$, 0.2 g $MgSO_4$, 0.2 g NaCl, 0.1 g $CaCl_2.6H_2O$, 20 g agar, 1 g CMC and 1 L distilled water (this medium was used for the count of cellulolytic microbes). The lysogenic broth agar plates and CMC plates were incubated at 37^0C for overnight and until colonies were visible, respectively. The total viable count of cultivatable total microbes and cellulolytic microbes were expressed as the number of colony forming unit (CFU/gut).

Isolation of Cellulolytic Microbes through Filter Paper Digestion

The macerated guts of each insect were inoculated in 5 mL of basal salt media (2.5 g $NaNO_3$, 0.2g $MgSO_4$, 0.2 g NaCl, 0.1 g $CaCl_2.6H_2O$ in a liter water) containing filter paper (5 pieces of Whatman filter paper no.1, each of 5 mg and area 0.49 cm^2) for isolating the cellulolytic microorganisms. A control was prepared with basal salt media and 5 pieces of Whatman filter paper but no gut extract for checking the degradation/disappearance of filter paper. The tubes were incubated aerobically and shaken at 180 rpm at 37^oC in a shaker incubator. After the filter paper was visibly degraded, indicating the presence of cellulases, or after 4-6 wk (whichever came first), serial dilutions of the cultures (10 µL of each) were transferred to solid medium (CMC agar plate). As a control, a single agar plate from each culture preparation was opened in the UV laminar flow hood for 15 min. This was done to check the contamination from within the hood. All the plates were incubated at 37^oC until colonies were visible.

Screening of Microbes for Cellulolytic Potential

Bacterial colonies seemed to be different based on the colony size, shape and color found in the CMC plate for total count of cellulolytic bacteria or for the isolation of bacteria capable of filter paper digestion were transferred to two sets of CMC plates by tooth pick -one for identification of isolates and other for examining their cellulolytic potential using the Congo-Red overlay method (Teather and Wood 1982). For the Congo-Red method, plates were flooded with 0.1% Congo red (Sigma-Aldrich) for 10-15 min before de-staining with 1M NaCl solution for 15-20 min for several

times or until the clear zones around the colonies were visualized. Colonies showing discoloration of Congo red were taken as positive cellulose-degrading microbial colonies.

Identification of Potential Cellulolytic Microbes
Microbes, which produced clear zones, were identified based on the cultural, morphological and biochemical characteristics and using Bergey's Manual of Systemic Bacteriology (Buchanan and Gibbons 1974; Holt et al. 1984).

RESULTS AND DISCUSSION

Total bacterial count and count of cellulolytic bacteria
The total bacterial count and count of cellulolytic bacteria in three insect guts are shown in Table 1. The total bacterial count and total cellulolytic bacterial count were highest in *P. quatuordecimpunctata*. In *O. velox* gut, 0.01% of total bacteria were cellulolytic whereas it was 0.002 and 0.008% for *A.miliaris* and *P. quatuordecimpunctata*, respectively. The total no of bacteria in the insect gut larvae varied. Grasshopper *Zonocerus variegates* contained bacterial count 13-90x10^5 (Ademolu and Idowu 2011) whereas total bacterial count of *B. mori* was 6.08±3.08x10^{11} (Anand et al. 2010). The cellulolytic bacterial count varied from insect to insect. Bacterial count with CMCase activity in the hindgut of *H. parallela* was 1.14±0.13 x10^8 (Huang et al. 2012) whereas population density of cellulolytic bacteria in *S. vistita* ranged from 2.4x10^5 to 3.6 x10^6 CFU/gut (Delalibera et al. 2005). In this study, the bacterial count in different gut segments (foregut, midgut and hindgut) of larvae was not quantified. In many insect larvae, most of the cellulolytic bacteria are present in the hindgut (Huang et al. 2012) and the exogenous cellulolytic enzymes are localized to the insect hindgut (Oppert et al. 2010).

Table 1 - Total bacterial count and cellulolytic bacterial count of three insect guts.

Name of insect	Total bacterial count (CFU/gut)	Total cellulolytic bactéria(CFU/gut)
O. velox	3.1x10^7	3.2x10^3
A.miliaris	1.65x10^7	3.7x10^2
P. quatuordecimpunctata	6.8x10^7	5.42x10^3

Identification of cellulolytic microbes
Microbial colonies grown on CMC plate directly from the insect guts or after enrichment in filter paper were selected for the identification based on their size, shape, color and other visual characteristics as well as investigated by the physical and biochemical methods (Table 2) for identification. Out of the nineteen colonies, fifteen isolates were identified that represented twelve different genera. The isolates from *O. velox* were *Photorhabdus luminescens, Enterococcus faecalis, Enterococcus durans, Flavobacterium odoratum, Serretia marcescens* and *Serretia entomophila*. Isolates identified from *P. quatuordecimpunctata* were *Erwinia ananus,* A*eromonas salmonicida, Enterococcus casseliflavus* and *Acinetobacter calcoaceticus* whereas five different species *Klebsiella oxytoca, Microbacterium imperiale, Yersinia pestis, Xenorhabdus poinari* and *Pseudomonas saccharophila* were identified from *A. miliaris* (Table 3). Although different bacterial species were identified from different insect gut in this study but it did not indicate that they did not contain any common bacterial species. Actually, the purpose of this study was to isolate different cellulolytic bacteria from different insect guts. Hence, the isolates were selected on their variability in three insect guts as a whole not to encompass total bacterial community in every individual insect gut. The bacterial isolates were clustered into seven different phyla (Table 3).
Out of fifteen isolates, seven namely *Enterococcus faecalis, Serretia marcescens, Acenatobacter calcoaceticus, Erwinia ananus, Aeromonas salmonicida, Klebsiella oxytoca* and *Pseudomonas saccharophila* were capable of growing on filter paper. All the fifteen isolates of three insect guts produced zone of clearance when grown on CMC plates and stained with Congo red (Fig. 1). The isolates were incubated for different time period to produce the zone of clearance. Hence, the hydrolytic value of the isolates was not compared.
Some of the species found in this study were previously reported as cellulase producers. *S. marcescens* isolated from soil exhibited cellulase activity (Sethi et al. 2013). *E. casseliflavus/ gallinarum* species isolated from buffalo's and horse's cecum and buffalo's colon were able to degrade lignin, xylan and cellulose, respectively (Wahyudi et al. 2010). *Y. pestis*, pathogenic to human and other animals, has also been reported as cellulase producer (uniport data bank).

Table 2 - Physiological and biochemical characteristics of isolated bacterial strains.

Characteristic Features	Isolate's ID														
	OV1	OV2	OV3	OV4	OV5	OV6	PQ1	PQ2	PQ3	PQ4	AM1	AM2	AM3	AM4	AM5
Gram stain	–	+	+	–	–	–	–	–	+	–	–	+	–	–	–
Morphology	R	C	C	R	R	R	R	R	C	R	R	R	R	R	R
Spores	–	–	–	–					–						
Motility	+	–	–	–	+	+	+	–	+	+	–	+	–	+	+
Pigmentation	Y	–	–	–	–	–	Y	–	Y	–	Ro	–	–	Br	Ro
Oxygen relation	F	F	F	A	F	F	F	F	F	A	F	A	F	F	A
Catalase	+	–	–	+	ND	ND	+	+	–	+	+	+	+	–	+
Oxidase	–	ND	ND	+	ND	ND	–	+	ND	–	–	ND	–	–	+
Urease	–	ND	ND	+	–	–	–	–	ND	ND	+	ND	–	–	ND
Methyl red test	–	ND	ND	ND	–	+	ND	ND	ND	ND	ND	ND	+	–	ND
VP test	–	ND	+	ND	+	+	ND	+	+	ND	+	ND	–	–	ND
Indole test	+	ND	ND	–	–	–	+	ND	ND	ND	+	ND	–	–	ND
Nitrate reduction	–	–	+	–	+	+	–	+	+	ND	+	–	+	–	ND
H_2S production	–	ND	–	ND	–	–	ND	ND	–	ND	–	–	–	–	ND
Hydrolysis of Starch	ND	ND	ND	–	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	+
Gelatin	ND	ND	ND	+	+	+	+	+	ND	–	–	ND	ND	ND	–
Citrate utilization	–	ND	ND	ND	+	+	ND	ND	ND	+	+	ND	–	–	ND
Acid from glucose	+	–	ND	+	+	+	+	+	ND	+	+	–	–	+	–
Gas from glucose	ND	ND	ND	ND	ND	ND	ND	+	ND	+	+	ND	ND	ND	ND
Acid from															
Arabinose	+	ND	ND	+	–	–	+	ND	ND	+	+	+	+	–	+
Lactose	+	+	+	+	–	–	+	+	+	+	+	–		–	–
Maltose	+	ND	ND	ND	+	ND	+	+	ND	+	+	–	+	–	–
Mannitol	ND	ND	–	+	+	ND	+	+	+	+	+	–	+	–	–
Raffinose	+	–	–	ND	ND	ND	+	+	+	+	+	–	–	–	–
Rhamnose	+	ND	ND	+	–	–	+	ND	ND	+	+	ND	–	–	ND
Xylose	+	–	–	+	–	–	+	+	+	+	+	+	+	+	+
Sucrose	+	+	–	+	+	–	+	+	+	+	+	+	–	+	+

OV - *O.* velox, PQ - *P. quatuordecimpunctata*, AM - *A. Miliaris*, R - Rod shaped, C - Coccoid, Y - Yellow, Br - Brownish, Ro - Red orange, F - Facultative anaerobic, A - Aerobic, + Positive and – Negative, ND - Not done

Table 3 - Different bacterial isolates from three phytophagous insect guts.

Family/Phylum	Name of the isolate	Isolate's ID
Enterobacteriaceae	*Photorhabdus luminescens*	OV1
	Xenorhabdus poinari	AM4
	Erwinia ananus	PQ1
	Klebsiella oxytoca	AM1
	Yersinia pestis	AM3
	Serretia marcescens	OV5
	Serretia entomophila	OV6
Pseudomonadaceae	*Pseudomonas saccharophila*	AM5
Enterococcaceae	*Enterococcus faecalis*	OV2
	Enterococcus durans	OV3
	Enterococcus casseliflavus	PQ3
Vibrionaceae	*Aeromonas salmonicida*	PQ2
Micrococcaceae	*Microbacterium imperiale*	AM2
Neisseriaceae	*Acinetobacter calcoaceticus*	PQ4
Flavobacteriaceae	*Flavobacterium odoratum*	OV4

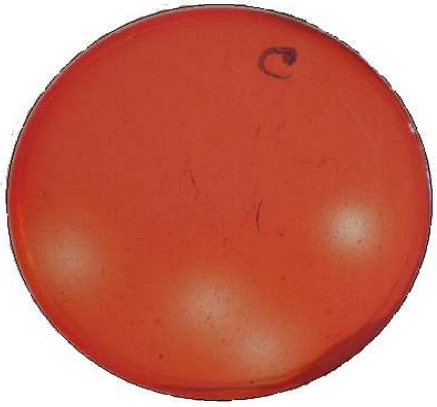

Figure 1 - Zone of clearance shown by the isolates in CMC plate

Although other species of bacteria isolated from the insect guts in this study were not reported as cellulase producers but many of their genera have been reported as cellulase producers. Several bacteria isolated from the soil have been reported as cellulose producer such as *F. johnsoniae* (Lednicka et al. 2000), *Pseudomonas mendocina* (Lednicka et al. 2000) and also from the gut of scrab *H. parallela* (*P. nitroreducens*) (Huang et al. 2012). *Enterococcus* sp was found in both insects, *O. velox* and *P. quatuordecimpunctata*. Members of the genus *Enterococcus* are among the most common gut bacteria detected in larval guts across a diversity of insect orders (Martin and Mundt 1972), including Diptera (Ahmad et al. 2006; Toth et al. 2006; Cox and Gilmore 2007) and Lepidoptera (Inglis et al. 2000; Broderick et al. 2004). Along with other genera, the bacterial community associated with larvae and adults of *D. velon* gut are dominated by members of the genera *Acinetobacter*, *Erwinia*, *Serratia* (Morales-Jiménez et al. 2009). Cellulase activity was also reported in *Acinetobacter anitratus* (Ekperigin 2007). *Acinetobacter lwoffi* and *Microbacterium paraoxydans* were found in the gut of *Ostrinia nubilalis* (*Lepidoptera*) and genus *Acinetobacter* sp and *Klebsiella* sp were found in the gut of *Leptinotarsa decemlineata* (*Coleoptera*). All the isolates showed cellulolytic activity (Velanova et al. 2012). Cellulolytic activity was also shown by *Microbacterium* present in the gut of *H. parallela* (Huang et al. 2012).

CONCLUSION

To the best of our knowledge, except *Serratia marcescens*, *Enterococcus casseliflavus* and *Yersinia pestis*, all other twelve species *Photorhabdus luminescens, Enterococcus faecalis, Enterococcus durans, Flavobacterium odoratum, Serretia entomophila, Erwinia ananus, Aeromonas salmonicida, Acinetobacter calcoaceticus, Klebsiella oxytoca, Microbacterium imperiale, Xenorhabdus poinari* and *Pseudomonas saccharophila* isolated from the guts of three insect specimen are reported first time as cellulase producers. They may be source of novel cellulase enzymes. However, quantitative analysis and characterization of the cellulase produced by these species will justify their use in biofuel and other cellulase based industries.

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