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ISOLATION OF CELLULOSE-DEGRADING *CELLULOSIMICROBIUM* FROM LARVAL GUT OF *PROTAETIA BREVITARSIS SEULENSIS*

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SUMMARY

Larvae live in soil have been well known for containing abundant microbiota in their gut. This study isolated six bacterial genera from the gut of larvae *Protaetia brevitarsis seulensis* including *Bacillus*, *Cellulosimicrobium*, *Microbacterium*, *Streptomyces*, *Krasilnikoviella* and *Isoptericola* based on specific media. Among these six genera, *Cellulosimicrobium* was collected for further analysis of cellulose-degrading features because of the most abundance and less studies up to now. Based on the 16S rDNA gene, the *Cellulosimicrobium* isolates were classified to *C. cellulans, C. aquatile, C. funkei, C. protaetiae* that were respectively isolated from four specific media such as modified ISP-2, MRS, modified anaerobic medium, modified fermentation medium. The analysis of their genome proved the presence of genes encoding for chitinases, alkyl resorcinol, and glucosidase in four strains. These cellulose degrading enzymes were useful for textile processing, paper recycling, production of nutritional supplements, food industry, production of alcohol from lignocellulosic materials, and beneficial microorganisms in denitrification and N-cycling in forest ecosystem as well as wastewater process.

Keywords: *Cellulosimicrobium, gut microbiota, gut larvae, Protaetia brevitarsis seulensis, cellulose-degrading bacteria*

INTRODUCTION

Early studies characterized the gut microbial communities by using classical techniques, but little information was known about their biological role (Brooks 1963). Brooks stated that further isolating and characterizing the insect gut biome is

meaningless unless they correlate with the host's ability to control the biome or the biome's influence on host physiology (Edward, Steinhaus, 1963). The difficulty in determining the composition of the microbiome by scientists has still emphasized the right in Brooks' view. With this reason, the next studies should focus on the functional role of the microbiome.

The diverse microbiome in larval gut is an important resource of species for the development of biotechnological products and processes (Ankrah, Douglas, 2018). The industrial useful enzymes, applied for many purposes such as for textile processing, paper recycling, production of nutritional supplements, food industry, production of alcohol from lignocellulosic materials (Berasategui *et al.*, 2016), antibacterial compounds for pest management and medical applications (Xia *et al.*, 2017) and strains of beneficial microorganisms in denitrification and N-cycling in forest ecosystem as well as wastewater process (Groffman, Tiedje, 1989; Philippot, Hallin, 2005; Michael Madigan *et al.*, 2015), were found. In this study, the insect larvae of *Protaetia brevitarsis seulensis* have been used for isolation of cellulose-degrading bacteria in particular the genus *Cellulosimicrobium*. The genes encoding for cellulose-hydrolyzing enzymes were confirmed by genome sequencing with the support of bioinformatic tools and denitrifying capacity of bacterial strains were predicted.

MATERIALS AND METHODS

Insect collection and processing

Larvae of *Protaetia brevitarsis seulensis* were collected in Jeongeup, South Korea. First of all, the larvae were disinfected with alcohol 70% for 1 min, then rinsed in a solution NaCl 0.85% (w/v). After that, their guts of 1 cm long were separated and cut into small pieces. These pieces were processed for rinsing in a solution NaCl 0.85% (w/v) and grinded by a tissue grinder. The serially diluted solution was spread on 4 different media in Table 1 for enumeration and isolation of microorganisms and for total DNA extraction. The colonies formed on plates were purified to fresh media to obtain pure isolates. The colonies with particular morphology were selected. A yellow-pigmented, circular, and convex colony was taxonomically classified. Then the isolated strain was stored in 20% glycerol at -80^{o}C.

Bioinformatic analysis of denitrification capacity

The whole genome sequencing was performed by Macrogen (Seoul, Republic of Korea). A library of 20-kb SMRTbell was constructed from the high molecular-weight genomic DNA (15 μg). This library was sequenced by using the PacBio RS II version 4.0 single-molecule realtime (SMRT), sequencing technology (Pacific Bioscicence), yielding 378-fold average genome coverage. The complete genome sequence of strains comprised two contigs with a sequencing depth of 178×; the largest contig was 4631595 bp with a circular structure, and the second linear contig was 152586 bp long. Sequences of isolates were annotated by the Prokka pipeline identified 4140 genes, including 4001 genes coding proteins, nine rRNA genes (5S, three; 16S, three; 23S, three), and 51 tRNA genes (http://www.vicbioinformatics.com/software.prokka.shtml). Gene annotation was also performed by NCBI Prokaryotic Genome Annotation Pipeline through Genome submission portal (http://www.ncbi.nlm.nih.gov/genome/annotation_prok/).

Table 1. Particular media for isolation of cellulose degrading bacteria from larvae guts, based on methodologies of Ho *et al.* (Han *et al.,* 2022).

Medium	Composition
Modified ISP-2	Glucose 0.2 g/L, yeast extract 0.2 g/L, malt extract 0.3 g/L, nalidixic acid 25 mg/L, $K_2Cr_2O_7$ 6 mg/L, distilled water up to 1000 mL, pH to 7, agar 20 g/L
MRS	Proteose peptone 10 g/L, beef extract 10 g/L, yeast extract 5 g/L, dextrose 20 g/L, polysorbate-80 1 g/L, ammonium citrate 2 g/L, sodium acetate 5 g/L, magnesium sulfate 0.1 g/L, manganese sulfate 0.05 g/L, dipotassium phosphate 2 g/L
Modified anaerobic medium	Anaerobic seawater (DSMZ 504) 10% (v/v), glucose 0.2 g/L, tryptone 0.2 g/L, yeast extract 0.1 g/L, sodium nitrate 3.0 g/L, casamino acids 0.2 g/L, dextrose 0.2 g/L, soluble starch 0.2 g/L, sodium pyruvat 0.2 g/L, pH 7, L-cysteinium chloride 0.5 g/L, DW 1000 mL
Modified fermentation medium	Anaerobe seawater (DSMZ 504) 10% (v/v), glucose 0.2 g/L, polypepton 0.25 g/L, yeast extract 0.25 g/L, KF 58 mg/L, pH 7, L-cysteinium chloride 0.5 g/L, DW 1000 mL

Catalase activity was detected by the production of oxygen bubbles with 3% (v/v) H_2O_2 adjusted to fresh cells. The oxidase reaction was tested using 1% (w/v) tetramethyl-*p*-phenylenediamine. Any motility of fresh live cells suspended in marine broth was observed by a light microscope. The hydrolysis of casein, cellulose, gelatin, starch, Tweens 20, 40 and 80, xanthine, xylan, reduction of nitrate was tested by methods described in Ho et al., 2022 (Han *et al.*, 2022). Through bioinfomatic analysis, the existence of cellulose-degrading genes within the genomes were determined using the online versions of BLASTx and tBLASTn (https://www.ncbi.nlm.nih.gov/).

RESULTS AND DISCUSSION

Isolation of cellulose degrading bacteria from larvae

The guts of *Protaetia brevitarsis seulensis* larvae differ in their pH (pH 7.04 ± 0.23) and were cultured with condition in Table 2.

A total of 80 bacterial isolates, numbered B01 to B80, were obtained from 135 colonies from each plating test. Of these 80 islolates, 32 were identified as Bacillus, 28 as *Cellulosimicrobium*, 2 as *Microbacterium*, 4 as *Streptomyces*, 14 as *Streptomyces*, and 3 as *Isoptericola*, respectively. Among these, isolates of the genus *Cellulosimicrobium* were collected for further analysis.

Cellulosimicrobium, formerly known as the species *Oerskovia*, are gram-positive bacilli in the order Actinomycetales. They rarely cause infections in humans. Early studies illustrated the important role of this genus in cellulolytic bacteria of soybean (Opazo *et al.*, 2012), in phytohormone-synthesizing microbes (Simranjeet Singh *et al.*, 2021), in denitrification of wastewater treatment (Sultanpuram *et al.*, 2015). However, few recently researches proved that pathogenic species of *C. cellulans* caused unusual infection (Rivero *et al.*. 2019).

Table 2. Culture conditions of bacteria isolated from *Protaetia brevitarsis seulensis* larvae.

Medium	Cultivation condition	Result	Abbreviation of sample
Modified ISP-2	25℃, aerobic	Aerobic Actinobacteria	BI
MRS	25℃, anaerobic	Lactic acid bacteria	BL
Modified anaerobic medium	25℃, anaerobic	Anaerobic respiration	BA
Modified fermentation medium	25℃, anaerobic	Fermentation	BF

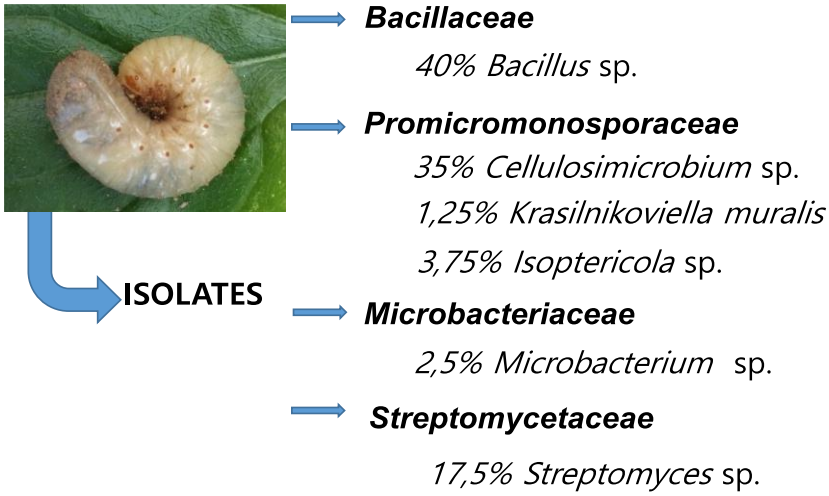

Figure 1. Composition of bacterial strains on specific media.

Table 3. Isolated strains of *Cellulosimicrobium.*

Samples	Name of taxon	Similarity (%)	Number accession
BA2, BA6-9, BA11-12, BA20-23, BA25-26, BA29, BI11, BI20, BI22-23,BF22	*Cellulosimicrobium cellulans*	99.5	CAOI01000359 - 365 BA20 - 23: OQ921765 - 770; OQ926621-24
BA19, BI4, BI5, BI31-32, BI39	*Cellulosimicrobium aquatile*	99.5	OQ921767,
BA3	*Cellulosimicrobium funkei*	99.6	AY501364
BI34	*Cellulosimicrobium protaetiae*	Novel strain in Ho *et al.*, 2022	MK966391

Results of isolation and species identification of *Cellulosimicrobium* isolates with 4 different media were shown in Table 3. Isolates were identified as either *Cellulosimicrobium cellulans, or C. aquatile, or C. funkei, and or C. protaetiae*. The results also indicated that these no isolates of *Cellulosimicrobium* found on MRS media, but the majority of this genus was to *C. cellulans*. Meanwhile, *C. funkei* was only found on the anaerobic respiration.

In 2007, Yoon *et al.* isolated from soil some strains belonging on the genus of *Cellulosimicrobium* with the gene sequence similarity of 16S rRNA between strain DS-61T and the type strains of *C. cellulans* and *C. funkei* were 97.4 and 97.6%, respectively (Yoon *et al.*, 2007). Many different novel strains of *Cellulosimicrobium* have been, since then, found. For instance, *C. arenosum* sp. nov. was isolated from marine sediment sand (Oh *et al.*, 2018). This strain possessed MK-9 (H4) as the predominant menaquinone and anteiso-C15:0 as the major cellular fatty acids. Next, *C. fucosivorans* sp. nov. was proposed a name of SE3T as the type strain linked to carotenoid production (Aviles and Kyndt 2021).

Examining cellulose-degrading capacity

Cell-wall degrading enzymes such as chitinase, cellulase and glucosidase (Fleuri, Kawaguti, and Sato 2009) of *Cellulosimicrobium* strains were investigated (Table 4).

Table 4. Biochemical characteristics of *Cellulosimicrobium* strains.

Characteristic	*C. cellulans*	*C. aquatile*	*C. funkei*	*C. protaetiae*
Motility	-	-	+	-
Oxidase	-	+	-	+
α-Chymotrypsin	+	w	-	-
β-Glucuronidase	-	+	-	-
N-Acetyl-β-glucosaminidase	+	-	-	-
Gelatin hydrolysis	-	+	+	+
Nitrate reduction	+	+	-	+

+, Positive; −, negative; w, weakly positive

In this study, BLAST results of chitinases genes indicated the predominant identity (90%) with genes coding of WP_082141409.1, WP_052877838.1 in case of *C. funkei*. Meanwhile, *C. cellulans* was to WP_141390946.1, WP_043656558.1, which were applied for producing chitinases. On the other hand, the analysis of AntiSMASH recorded biosynthesis gene clusters of these four strains, in which strain of *C. protaetiae* and *C. aquatile* consisted of gene clusters with some secondary metabolite regions including cluster of alkyl resorcinol. These clusters were basically referenced on studies

of Baerson *et al.,* 2010 (Baerson *et al.,* 2010) and Funabashi *et al.,* 2008 (Funabashi, Funa, Horinouchi 2008).

Moreover, the genome annotation of the denitrification pathway in these strains were shed light up. Gene annotations were proved by two cellulase and six glucosidase enzymes, including α-glucosidase, β-glucosidase, oligo-1,6-glucosidase, alpha- amylase under accession number on Genbank QJW36206, QJW38720, and glucosidase QJW37470, QJW36279, QJW36190, QJW38247, QJW37853, QJW37814 and alpha-amylase QJW35995. *P. brevitarsis* have been successfully grown in an artificial breeding environment and their morphology and growth characteristics have been examined. The fatty acid, amino acid composition, antioxidative component through biochemical analysis were carried out in previous studies, but gene annotations were less performed. Our results recorded a novel strain and other well-known *Cellulosimicrobium* strains. Their cellulose – degrading featureswere discovered %G+C content of 73.89% in BI34. This revealed the denitrification pathway of this strain.

CONCLUSION

In this study, the larvae gut of *P. brevitarsis* was used for isolation of the cellulose – degrading bacteria. Using four different media for isolation, isolates from six genus including *Bacillus*, *Cellulosimicrobium, Microbacterium*, *Streptomyces*, *Krasilnikoviella* and *Isoptericola* were obtained. Exception for *Bacillus* which were well known with cellulose – degrading features, *Cellulosimicrobium* were chosen for further analysis in this study because of their abundant proportion in isolates. The analysis of their genome proved the chitinases, alkyl resorcinol, and glucosidase enzymes in four strains of this genus. This study shed light up other features in cellulose-deregrading characteristics to apply in agriculture areas.

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