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Original Paper

Isolation and characterization of *Rhodococcus qingshengii*, a cellulolytic bacteria from *Cnaphalocrocis medinalis* (Lepidoptera: Crambidae) gut

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Abstract

Herbivorous Lepidopteran insects depend on microbial cellulases for cellulose digestion. The present study aimed to investigate the herbivorous Lepidoptera *Cnaphalocrocis medinalis* gut for isolation of cellulose degrading bacteria. A Gram positive rod, non-motile, mesophilic, neutrophile bacterial strain TCI11 was isolated from *C. medinalis* (Lepidoptera: Crambidae) 5th instar larvae gut. The isolate was found to be a potent cellulose degrader, which was isolated on carboxymethylcellulose agar medium using carboxymethylcellulose as a sole carbon source and screened by Congo red and Gram's iodine dye staining method. The cellulose degrading strain was characterized on the basis of Gram staining, morphological and biochemical properties. Based on 16S rRNA gene analysis, the strain was found to be most closely related to *Rhodococcus baikonurensis, Rhodococcus erythropolis, Rhodococcus globerulus* (99.77, 99.35 and 99.06% similarity, respectively) and was identified as *Rhodococcus qingshengii. R. qingshengii* is being reported for the first time as a potent cellulose degrader. The source of isolation of this particular bacterial strain is also novel.

Key words: carboxymethylcellulose, cellulose degrading bacteria, *Cnaphalocrocis medinalis, Rhodococcus qingshengii*.
Abbreviations: CDB, cellulose degrading bacteria; CMC, carboxymethylcellulose; CR, Congo Red; FP, filter paper; GI, Gram's iodine; MTCC, Microbial Type Cell Culture Collection and Gene Bank; NCBI, National Centre for Biotechnology Information.

Introduction

Microbial enzymes hydrolyzing cellulosic material play a pivotal role in the global carbon cycle (Wilson 2011). Complete cellulose hydrolysis is mediated by the synergistic action of three components of cellulases: (i) endoglucanase (EC 3.2.1.4), (ii) exoglucanase (EC 3.2.1.91) and (iii) β-glucosidase (EC 3.2.1.21) (Shewale 1982). Herbivorous insects are largely dependent on both intrinsic and extrinsic cellulase enzymes for cellulolysis (Martin 1983). There are four anticipated mechanisms for cellulolysis in insects. The first mechanism involves the exploitation of protozoan symbionts in the gut for cellulolysis (Cleveland 1924). The second mechanism involves exploitation of cellulolytic bacterial symbionts in the insect gut (Breznak 1982). The third mechanism explains the use of ingested fungal cellulases responsible for cellulolysis (Martin, Martin 1978). The fourth mechanism integrates the production of all cellulolytic enzymatic subunits necessary for cellulose digestion by insects (Martin 1991). Although cellulolysis is reported as a generally uncommon and disadvantageous trait for insects (Martin 1991), cellulose digestion has been demonstrated in gut fluids of some insects (Martin 1983) belonging to different orders such as Thysanura (Lasker, Giese 1956), Orthoptera (Cleveland et al. 1934), Isoptera (Martin, Martin 1978), and Coleoptera (Bayon, Mathelin 1980), Hymenoptera (Kukor, Martin 1983) indicating the existence of an intrinsic cellulase system in insect species.

Although the presence of gut microbiota throughout the holometabolous life is well established in many Lepidopteran insects, details of the cellulase system in Lepidopteran insects are little studied (Chen et al. 2016). The Order Lepidoptera is the most diverse, distinct and recognizable group of Class Insecta, which comprises exclusively herbivorous members (Solis, Pogue 1999). Moreover, it has been suggested that the occurrence of cellulose digestion in foliage feeders is very rare (Martin 1991). Therefor, the aim of the study was to screen and isolate cellulolytic bacteria from larvae gut of a herbivorous Lepidopteran, which is foliage feeder and solely dependent on cellulosic plant material as an energy source. In the present study, gut microbiota of *Cnaphalocrocis medinalis* (Lepidoptera: Crambidae), rice leaf roller, a pest of paddy, was chosen as a source for cellulolytic bacteria.

The present study examined the gut microflora of herbivorous Lepidoptera larvae of *C. medinalis* for isolation

and characterization of potent cellulolytic bacteria. *Rhodococcus qingshengii* was previously described as a carbendazim degrader (Xu et al. 2007) and a psychrotolerant diazotrophs bioinoculant on chickpea (Joshi et al. 2019). In this research work we reported *R. qingshengii* as a potent cellulose degrading bacteria (CDB) for the first time, based on its ability to utilize carboxymethylcellulose (CMC) as a sole carbon source for bacterial growth and degradation of CMC and Whatman filter paper by producing cellulase enzymes.

Materials and methods

Insect gut fluid collection

Five fifth instar *Cnaphalocrocis medinalis* larvae were collected live from a paddy field near North-Eastern Hill University campus, Shillong, Meghalaya, India (25.5788° N, 91.8933° E) India in July and brought to the laboratory for dissection and further experiments.

The larvae were freshly collected from field and killed immediately by storing in a deep freezer. Use of any chemicals was avoided to kill the specimen in order to avoid chances of interference with gut microbiota. Prior to dissection, the insect was surface sterilized with 5.25% sodium hypochlorite solution and immersed in 70% ethanol and thoroughly washed with distilled water. The dissection was done aseptically using sterile dissecting scissors and forceps in a laminar air flow hood. The gut content was used to study of the presence CDB in gut fluid.

Isolation of cellulolytic microorganisms

The macerated gut contents of all larvae were mixed with distilled water in a microcentrifuge tube, vortexed and serially diluted up to 10^{-7} dilution and plated directly onto a CMC agar plate as inoculum. The inoculated plates were incubated at 32 °C for 18 to 48 h until colonies were visible (Dantur et al. 2015). Further, pure cultures were obtained by repeated streaking on CMC agar plates. The pure culture plates were coded and preserved at 40 °C till further investigation.

Screening of cellulolytic microorganisms

Qualitative assays were performed employing two different staining techniques for primary screening of potential cellulolytic bacteria. The CMC agar plates were inoculated and incubated at 32 °C for 18 to 48 h. After an appropriate incubation period the plates were flooded with 1% Congo red solution (w/v) and allowed to stand for 15 min. The dye was poured off and the plates were washed with 1 M $NaCl_2$ thoroughly and repeatedly (Teather, Wood 1982). Duplicate CMC-agar plates were flooded with Gram's iodine solution and allowed to stand for 5 min for development of a zone of hydrolysis to assess the cellulolytic potential of the isolates (Gohel et al. 2014). The bacterial colonies with distinct and highest diameter of the zone of hydrolysis on both stained plates were selected for further investigations.

Screening of cellulolytic activity of bacterial isolates

The composition of CMC agar medium used for isolation, screening and qualitative analysis of cellulolytic bacteria was as follows: peptone 10 g, CMC 10 g, $MgSO_4$ $7H_2O$ 0.2 g, NaCl 0.5 g, $CaCl_2$ 0.1 g, agar 15 g per L distilled water and pH was adjusted at 6.5.

For quantitative analysis of the cellulolytic potential of bacteria, the isolates were inoculated in broth medium containing per 1 L: 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, 0.1g $CaCl_2$, 0.005 g $FeSO_4$ H_2O, 0.0016 g $MnSO_4$, 10 g CMC at pH 6.5. The broth cultures were incubated in a shaker incubator at 37 °C up to 120 h at 150 rpm. At every 12 h interval the bacterial cultures were collected in 15 mL centrifuge tube and centrifuged at 12000 rpm for 10 min at 4 °C in a cooling centrifuge (REMI, India). The supernatant obtained after centrifugation served as crude enzyme for further assays.

Optimization of culture conditions

To optimize culture conditions for maximum cellulase production, bacterial culture was grown in a wide range of different conditions such as temperature, pH and incubation period, as follows. To determine the effective temperature for cellulase production by the CDB, fermentation was carried out at 21, 29, 37, 45, 53 and 61 °C. Some microorganisms produce maximally during their exponential phase, whereas others in their stationary growth phase. Thus, the fermentation was carried out from 24 to 120 h, and the production rate was measured at 12 h intervals. The most suitable pH of the fermentation medium was determined by adjusting the pH of the culture medium to 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0. All experiments were performed in triplicate.

Measurement of cellulase enzyme activity

The enzyme activity on two substrates – CMC and filter paper – was studied to estimate the cellulolytic potential of the isolate. Enzyme activity was assayed using 3,5-dinitrosalicylic acid reagent (Miller 1959) by estimating amount of reducing sugars released from CMC (endoglucanase assay) and Whatman No. 1 filter paper (exoglucanase assay). For endoglucanase assay, 2% cabroxymethyl cellulose (w/v) (2 g CMC dissolved in 100 mL 0.5 M sodium citrate buffer, pH 5.5) and for exoglucanase assay 50 mg (1.0 × 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 endoglucanase assay, 250 µL of 2% CMC (w/v) and 250 µL of crude enzymes were added in a test tube and incubated at 50 °C for 30 min. For exoglucanase assay, to test tubes saturated filter paper (50 mg) strips and 500 µL of crude enzyme was added and incubated for 60 min at 50 °C. To the incubated mixture, 3 mL 3,5-dinitrosalicylic acid reagent was added to stop the reaction and heated in boiling water for 5 min to develop colour. The test tubes were allowed to cool down and 1 mL of Rochelle salt were added to each tube when still

warm. Reducing sugar liberated during the reactions was measured as absorbance at 540 nm. One unit 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 (Ghose 1987).

Total protein concentration was quantified by the Lowry's method using bovine serum albumin as a protein standard (Lowry et al. 1951).

Morphological and biochemical characterization of cellulolytic microorganisms

Potent cellulolytic isolates were subjected to morphological and biochemical investigations. For morphological and physiological description, strains were cultivated on Nutrient agar at 32 °C for 24 to 48 h. The morphology of cells was examined by using light microscopy (Leica DMRX Q600). Gram's staining, motility, catalase, oxidase, MR-VP test, indole production, citrate utilization, nitrate reduction, starch hydrolysis test, DNase test, urease test, H_2S production were performed for identification as described previously (Cowan, Steel 1965; Lanyi 1987). The isolates were identified by consulting Bergey's Manual of Systemic Bacteriology (Butchanan, Gibbons 1974).

Molecular identification of cellulolytic microorganisms

For bacterial identification based on molecular characterization, 16S rRNA was outsourced from the Microbial Type Cell Culture Collection and Gene Bank, CSIR Institute of Microbial Technology, Chandigarh, India. The protocol was as follows: Genomic DNA was isolated from pure culture using a ZR Bacterial DNA MiniPrep kit (Make Zymo Research). The 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 a 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 the BLAST tool in the NCBI (http://www.ncbi.nlm.nih.gov) and EzBiocloud portal (http://www.ezbiocloud.net).

The 16S rRNA gene sequence of the isolate was used for constructing a phylogenetic tree by the neighbor-joining method (Saitou, Nei 1987) using MEGA X software (Kumar et al. 2018). The evolutionary distances were computed using the p-distance method (Nei, Kumar 2000).

Results

Microbiological study of *C. medinalis* gastrointestinal tract extract revealed the presence of 13 different culturable cellulolytic bacteria based on different colony morphology. All isolated strains were cellulose degraders, but only one strain (TCI11) showed considerable cellulolytic activity and was selected for further study.

Bacterial isolates were screened based on formation of a clear zone around the bacterial colony, indicating their cellulose hydrolyzing capacity in the given medium. The qualitative assessment of cellulolytic potential revealed Gram's iodine stain to be more efficient over conventional the Congo red staining technique, with a faster reaction time and more prominent hydrolytic zone in a shorter reaction time (Fig. 1). The cellulolytic potential of any isolate was found to be independent of the cellulolytic index ascertained based on qualitative assay.

Since a pool of bacterial populations was expected to be present in the insect gut micro environment, to delimit the growth of non-cellulolytic bacteria present in the gut micro environment, CMC was used as the single carbon source for both solid and broth culture media. Screening with a sole carbon source resulted in isolation of 13 cellulolytic bacteria with distinct morphological colonies.

The strains were cultured in CMC broth medium at different temperatures. Enzyme activity recorded at

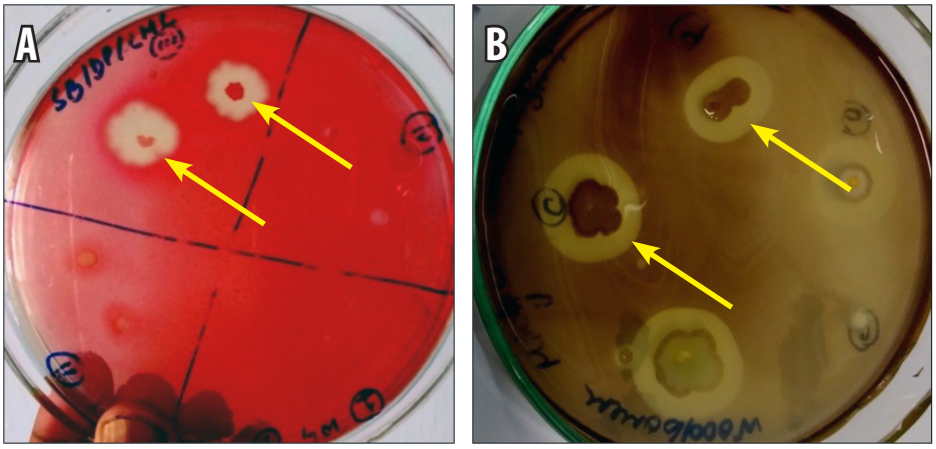

Fig. 1. Screening of cellulase production by plate assay method. A, Congo red stain; B. Gram's iodine stain. Colonies showing clear zone of hydrolysis are indicated by arrows.

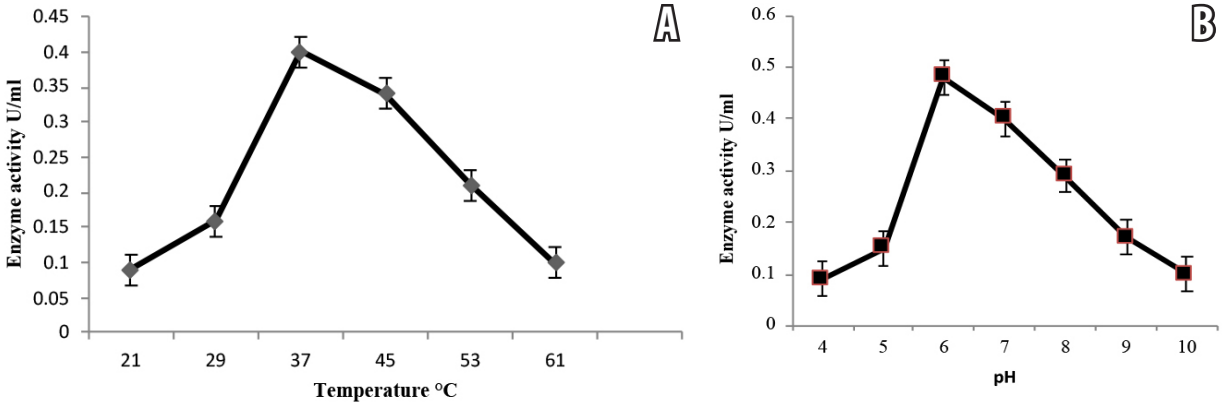

Fig. 2. Dependence of cellulase activity on temperature (A) and medium pH (B) of the isolated and characterized bacterial strain TCI11.

different temperatures revealed that *R. qingshengii* TCI11 yielded maximum cellulase production at 37 °C. Negative effects were observed on the production by increase or decrease in the temperature. *R. qingshengii* TCI11 was allowed to grow in media at different initial pH keeping all other culture conditions unaltered. The optimum initial pH was 6.0 and further increase in initial pH resulted in decrease in enzyme activity, concluding that the enzyme optimum pH is slightly acidic (Fig. 2).

The enzyme activity was measured in a period up to 120 h of bacterial growth at 24 h intervals. The maximum activity was recorded at 72 h, and declined significantly post 96 hours of incubation (Fig. 3).

Different levels of glucose concentrations were produced by bacterial isolates during quantitative analysis, which were compared to the glucose standard curve to obtain enzyme concentration. The highest protein accumulation in the extracellular medium during growth on CMC broth medium was 55 μg mL^{-1} post 72 h of incubation. The accumulation of protein declined subsequently. The highest endoglucanase and exoglucanase enzymatic activity was recorded as 0.233 and 0.956 units, respectively. Optimum activity was recorded after three days of incubation and subsequently declined significantly.

The selected isolate was an aerobic, Gram positive, short rod and non-motile bacterium. Colonies were light yellow, opaque, and raised with an irregular and undulate margin. The strain showed a positive result for catalase, Methyl Red and Voges-Proskauer, citrate utilization, nitrate reduction, starch hydrolysis, and deoxyribonuclease (DNase) tests. The isolate was found to be incapable of fermenting sugars like lactose, sucrose and glucose and of hydrogen sulfide production indicating a negative TSI test. Oxidase and indole test results were also negative (Table 1).

The 16S rRNA gene sequence of the isolate was used for constructing a phylogenetic tree by neighbor-joining method (Fig. 4). Comparison of the evolutionary distances was made using the *p*-distance method (Nei and Kumar, 2000). The isolate was found to be 100% similar with *R. qingshengii* strain JCM 15477 (T) and was named as *R. qingshengii*i TCI11. The 16S rRNA gene sequence was submitted to the NCBI gene bank with GenBank Accession: MZ_303718.1.

Discussion

The study of actinomycetes genus *Rhodococcus* (phylum Actinobacteria) is of great interest due to their great metabolic ability and transformation of environmental pollutants (Bell et al. 1998). Due to the small size of

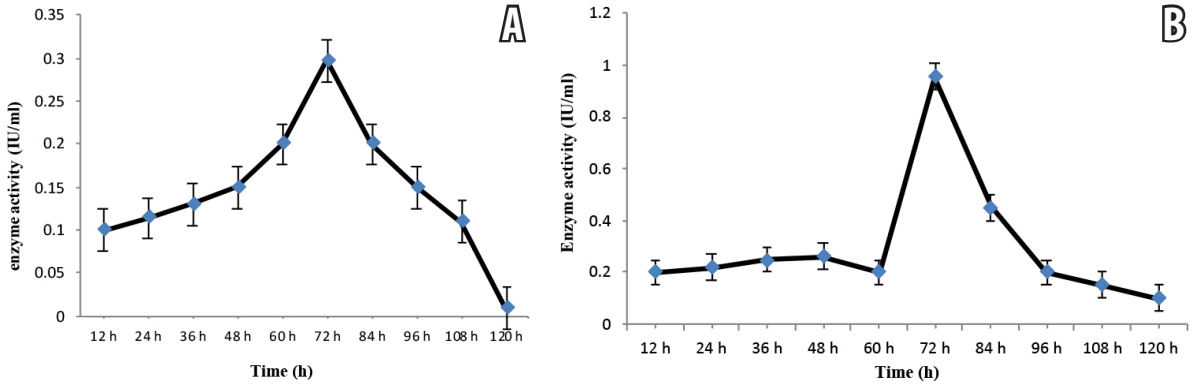

Fig. 3. Changes of endoglucanase (A) and exoglucanase (B) activity of the isolated and characterized bacterial strain TCI11 with time.

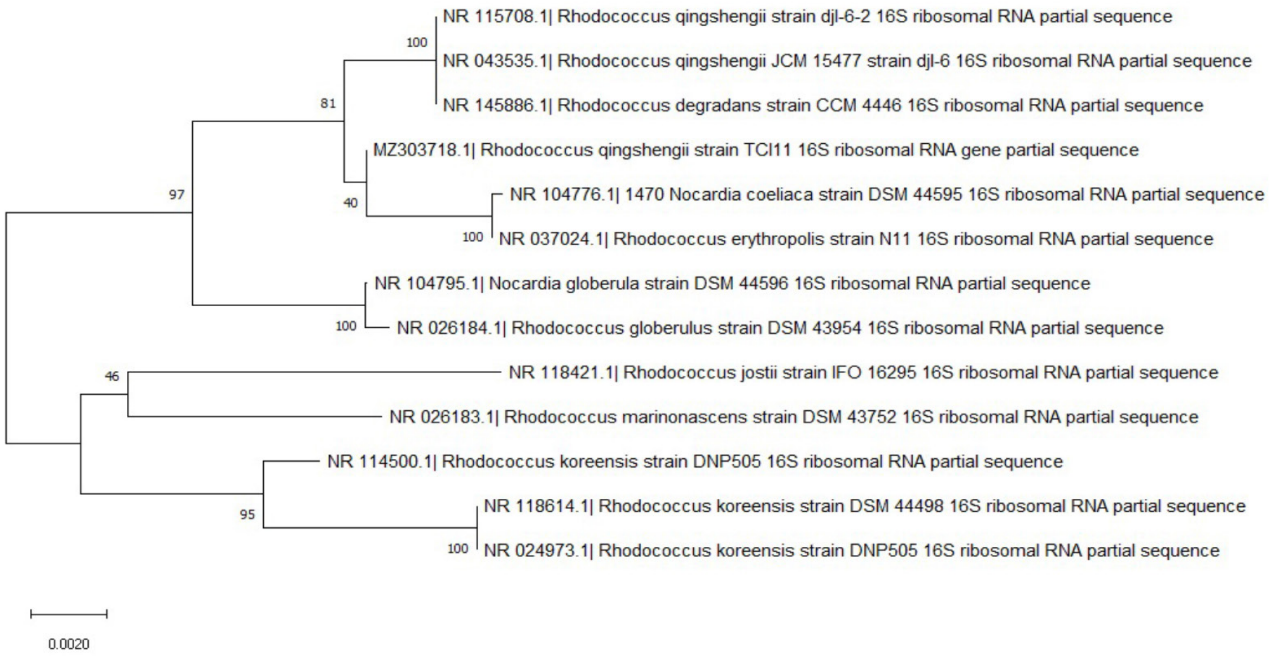

Fig. 4. Phylogenetic tree based on 16S rRNA gene sequences showing the relationship of strain R. qingshegii TCI11 with its closely related species. Tree was constructed by the neighbor-joining method. Bootstrap values (%) based on 1000 replicates are given at nodes. Bar 0.002 represents substitutions per site.

5th instar larvae (measuring 20 to 25 mm in length), multiple specimens were collected and dissected to collect gut contents. To avoid the growth of non-cellulolytic bacteria, the direct method for estimation of cellulolytic bacteria colony counts (Gylswyk 1969) was employed in this study. Thirteen types of cellulose degrading bacteria were isolated but only one isolate was found to be a potent cellulose degrader. The isolate was identified as *R. qingshengii*. Isolation of CDB from the insect confirmed the presence of an exogenous cellulase system in the insect. The strain showed positive results for both endonuclease and exonuclease activity in broth medium. The study demonstrated that the *C. medinalis* larvae harbor cellulolytic bacteria in their gut.

Table 2. Physiological and biochemical caharacteristics of the isolated strain TCI11

Characteristic	Manifestation
Gram stain	+
Morphology	Short rod
Motility	Non-motile
Colony morphology	Light yellow coloured colony with undulate margin
Catalase test	+
Oxidase test	–
Methyl red test	+
Voges-Proskauer test	+
Citrate utilization test	+
Nitrate reduction test	+
Triple sugar iron test	–
Indole test	–
Starch hydrolysis test	+
DNase test	+

The species isolated and characterized in the present study, *R. qinshengii,* previously has been characterized as a carbendazim degader, psychrotolerant diazotroph bioinoculant, and cellulose degrader (Xu et al. 2007; Joshi et al. 2019). The three most closely related species to *R. qinshengii* are *Rhodococcus baikonurensis* (a boron-tolerant bacteria) (Yoon et al. 2010), *Rhodococcus erythropolis* (a carbendazim degrader) (Zhang et al. 2013) and *Rhodococcus globerulus* (with desulphurization activity) (Yang, Marison 2005). Together with the present results, this information validates the significance of studying the genus *Rhodococcus* for their role in degradation and bioremediation of industrial and environmental pollutants (Bell et al. 1998).

The study demonstrated that the gut has a great potential to be a source of novel cellulolytic microorganisms and enzymes useful for future biofuel production. To the best of our knowledge, this is the first record of *R. qingshengii* being a potent cellulase degrader isolated from an herbivore Lepidopteran gut. For better fermentation conditions, culture conditions need to be optimized in the future.

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Technology, for timely characterization and identification of the isolate TCI11.

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