Cellulolytic Bacteria Screened from Qinling (China) for Biomass Degradation and Cellulases First Cloned from Bacillus methylotrophicus

Lingling Ma
Northwest Agriculture and Forestry University

Yingying Lu
Northwest Agriculture and Forestry University

Hong Yan
Northwest Agriculture and Forestry University

Xin Wang
Northwest Agriculture and Forestry University

Yanglei Yi
Northwest Agriculture and Forestry University

Yuanyuan Shan
Northwest Agriculture and Forestry University

Bianfang Liu
Northwest Agriculture and Forestry University

Yuan Zhou
Northwest Agriculture and Forestry University

Xin Lv (xinlu@nwsuaf.edu.cn)

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Abstract

Background

Cellulosic biomass degradation still needed more studies while bioenergy is becoming mainly energy in future and more evaluate bacteria isolation laid a foundation of further study. Qinling Mountains have unique biodiversity, acting as promising source of cellulose-degrading bacteria exhibiting noteworthy properties. The aim of this work was to find potential cellulolytic bacteria in depredating multiform carbon source cellulose substrate.

Results

In this study, 55 potential cellulolytic bacteria screened out and were identified. Based on the results of the investigation of cellulase activities and reducing sugar content via different carbon substrate effect, Bacillus methylotrophicus 1EJ7, Bacillus subtilis 1AJ3 and Bacillus subtilis 3BJ4 were further taken to hydrolyze wheat straw, corn stover and switchgrass, suggesting that B. methylotrophicus 1EJ7 was the most preponderant bacterium, obtaining highest sugar content (95mg/100mL) in switchgrass, wheat straw and corn stover. Scanning electron microscopy (SEM) and X-ray diffraction results of wheat straw surface and crystallinity indicated the hydrolyzation. By ascertaining the target sequence of cellulase for the cloning and expression in an economical and convenient manner, the genes of $\beta$-glucosidase (243 aa) and endoglucanase (499 aa) of B. methylotrophicus 1EJ7. Recombinant $\beta$-glucosidase from GH16 family and enzyme activity was 1670.15±18.94 U/mL. Endoglucanase consist of GH5 family catalytic domain and a carbohydrate-binding module belongs to CBM3 family and enzyme activity was 0.130±0.002 U/mL.

Conclusions

Screened, identified the cellulolytic bacteria from rotten wood of Qinling Mountains and explored their ability in degrading different carbon source cellulose substrate, including purified and natural carbon sources. Bacillus were the predominant species among the isolated strains, and Bacillus methylotrophicus 1EJ7 performant well on cellulose degradation. In the meantime, the $\beta$-glucosidase and endoglucanase were successfully cloned and expressed from Bacillus methylotrophicus for the first time. The strain and the recombinant enzyme have potential application in industrial production.

Background

Cellulosic biomass (composed of cellulose, lignin and hemicellulose) is one of the most abundant renewable resources. It is considered a potential and promising raw material for future energy production as well [1]. Cellulose is considered the critical component that can be converted into various value-added products: e.g. ethanol, 5-hydroxymethylfurfural (HMF), levulinic acid, butanol, alkanes, hexane, succinic acid, ethyl lactate, and other chemicals. In these procedures, cellulose should be firstly hydrolyzed to
glucose, where after, various bio- or chemical processes can be carried out. Therefore, the degradation of cellulotic material has aroused huge attention to enrich reducing sugars to the greatest extent.

Many methods, including acid-activated montmorillonite catalysts, steam explosion, acid and alkaline, enzymatic hydrolysis and microbiological methods, have been developed to hydrolyze cellulose. From the perspective of environmental friendliness and energy saving, the enzymatic hydrolysis and microbiological method are prioritized to be practically applied, both of which are associated with microorganisms, such as fungi and bacteria [2]. It is true that fungi exhibit a strong ability to secret considerable extracellular enzymes including multi-cellulases. Given this, extensive literature have been made about cellulases producing fungi, such as *Trichoderma reesei* RUT-C30 [3], *Trichoderma koningiopsis* FCD3–1 [4], and *Melanoporia* sp. CCT 7736 [5]. Besides, it is also found that the culture and genetically modification of fungi are relatively more difficult to achieve than bacteria, seriously hindering the practical application of fungi and fungi-producing cellulases to hydrolyze celluloses[6, 7]. In general, bacteria were commonly considered a powerful tool for functional modification or genomic operation, for instance, the cloning and expressing of single cellulase or recombinant cellulases. However, the library of bacteria exhibiting good ability to hydrolyze cellulose was not sufficient, thus requiring further enrichment. It has been reported that various of bacteria, such as *Bacillus* sp. BS–5 [8], *Bacillus licheniformis* 2D55 [9], *Bacillus subtilis* BY–4 [10], *Paenibacillus chitinolyticus* CKS1 [11], *Ochrobactrum sp* K38 [12], and *Clostridium thermocellum* [13] were screened, and their ability of cellulose hydrolysis was then further investigated, also suggesting that more bacteria with various species and form different origins should be screened and highlighted.

The Qinling Mountains (32°30′N–34°45′N and 104°30′E–112°45′E) locate in the center of China, 1500 km in length. It is a crucial geographic demarcation line separating semi-arid area and humid regions [14]. It is also generally considered that Qinling Mountains exhibits unique climate, plants, and microorganism resource. Besides, rotten woods originating from Qinling Mountains contains various of biomass degrading microorganisms, providing good materials for screening valuable bacteria to degrade lignocellulose. Thus, in the present study, bacteria exhibiting the capability of degrading cellulose were isolated and identified from Qinling Mountains rotten woods. Subsequently, cellulase activities were assayed and the strains were inoculated into the wheat straw, corn stover and switchgrass to assess the degraded extent of lignocellulosic biomass. Furthermore, by searching target sequence based on NCBI, genes of β-glucosidase and endoglucanase were successfully cloned and expressed on the pET–28a(+) plasmid in *E.coli* BL21 (DE3).

**Results**

**Isolation and identification of cellulolytic bacteria**

A total of 81 strains were isolated from five rotten wood samples, in which 8, 17, 19, 15 and 22 isolates were obtained from weed tree, red birch, poplar, alpine rhododendron and willow, respectively. And then,
55 cellulolytic strains were further screened by Congo red method (Fig. 1), and strains named as *B. subtilis* 1CJ1 and *Bacillus* sp. 1CJ4 had the largest clear zone diameters of 25 mm (Table 1).

The isolated strains were identified according to their 16S rRNA gene, after which phylogenetic tree was established as shown in Fig 2. Results indicated that the strains could be classified to *Bacillus subtilis*, *Bacillus* sp., *Pseudomonas aeruginosa*, *Bacillus licheniformis*, *Bacillus methylotrophicus* and *Bacillus megaterium*.

**Cellulase activities and hydrolysis capability**

The isolated strains were inoculated into sole carbon source medium for 48 h at 37 °C under 120 rpm. Reducing sugar concentration and cellulase activities were determined shown in Supplementary 2. The results were changed into heat map formal (Fig.3) for easy watching by colors.

The crude enzyme extracts were collected to determine reducing sugar content and cellulase activities. Maximum reducing sugar concentration was observed in CMC-Na medium by *B. subtilis* 1AJ3 of 4.83 mg/100mL, followed by *B. subtilis* 1BJ4 of 4.54 mg/100mL and *B. subtilis* 1BJ6 of 4.47 mg/100mL. Compared with CMC-Na medium, a maximum production of reducing sugar (1.61 mg/100 mL) was obtained by *B. subtilis* 3BJ7 in Avicel medium. The results showed that strains selected from CMC-Na medium had a higher enzyme activity than which selected from Avicel, and *B. subtilis* 1BJ4 had both the highest FPase activity (0.0133 U/mL) and CMCase activity (0.0368 U/mL), while *B. licheniformis* 3EJ7 had the highest avicelase activity of 0.010 U/mL. For the majority of strains, it coincided with the general sense. Strains selected from CMC-Na had high CMCase activity while stains from Avicel had higher avicelase activity. It was also found that strains together with CMCase activities and avicelase activities did not appear in proposed study. For example, *B. subtilis* 1BJ4 had the highest CMCase activity, but it didn't exhibit avicelase activity, which was possibly explained by the fact that different strains might produce different cellulases under the same or different carbon source [15]. Interestingly, although *B. subtilis* 3CJ6 had the highest avicelase activity and other two enzyme activities, no reducing sugar was detected.

From the heat map, the difference of intergeneric impacted the cultivation process and which led to the different reducing sugar production and cellulase activities. *B. subtilis* strains had advantages in reducing sugar production, FPase and CMCase activity. *B. methylotrophicus* and *B. licheniformis* performed relatively well in reducing sugar production. In addition, bacteria had higher FPase and CMCase activities isolated from CMC-Na as solo carbon source medium than Avicel medium.
According to the reducing sugar production and cellulase activities, eight strains were further selected out: *B. subtilis* 1AJ2, *B. subtilis* 1AJ3, *B. subtilis* 1BJ4, *B. methylotrophicus* 1EJ7, *B. subtilis* 3BJ4, *B. subtilis* 3CJ6, *B. subtilis* 3CJ8 and *B. methylotrophicus* 3EJ7.

**Reducing sugar production and cellulase activities in different carbon sources**

The selected eight strains were cultured with different carbon sources: wheat straw, corn stover, switchgrass, Avicel and CMC-Na (Fig.4).

Each strain was separately inoculated into the medium with five different carbon sources (wheat straw, corn stover, switchgrass, Avicel, and CMC-Na) for 48 h with 6% seed inoculation. Fig.4 (a) shows reducing sugar concentration in different carbon sources of each strain. *B. subtilis* 1AJ3 and *B. methylotrophicus* 1EJ7 showed strong potential in producing reducing sugar, especially in lignocellulosic biomass without pretreatment (wheat straw, corn stover, and switchgrass), then followed by *B. subtilis* 3BJ4 and *B. subtilis* 1AJ2. The strains showed similar FPase and CMCase activity (Fig.4b and Fig.4c) in different carbon sources. In addition, only *B. subtilis* 1AJ3 and *B. subtilis* 3BJ4 could produce avicelase enzyme in all medium, and *B. subtilis* 1AJ2 could only produce avicelase enzyme in CMC-Na medium. Meanwhile, other strains could produce avicelase enzyme in three or four carbon sources. According to reducing sugar production, cellulase activities and carbon source type, three strains (*B. subtilis* 1AJ3, *B. methylotrophicus* 1EJ7, and *B. subtilis* 3BJ4) were selected for further study.

**Pretreatment of lignocellulosic biomass**

Three strains of *B. subtilis* 1AJ3, *B. methylotrophicus* 1EJ7 and *B. subtilis* 3BJ4 were used to pretreat wheat straw, switchgrass and corn stover separately or mixed-up. After sterilization at 121 °C for 20 min, the beginning reducing sugars concentration was 136.34 mg/100mL, 109.46 mg/100mL, and 39.16 mg/100mL in the medium of corn stover, switchgrass and wheat straw, respectively.

The reducing sugar content in all medium tended to be stable (Fig.5) after culturing with 36 h, and the highest sugar content of 95 mg/100 mL was obtained by *B. methylotrophicus* 1EJ7 in switchgrass. Meanwhile, 73mg/100 mL in wheat straw and 72 mg/mL in corn stover was also obtained by *B. methylotrophicus* 1EJ7, which also indicated that no synergistic effect was observed in the pretreatment of mixture.

SEM could help us to understand the process of the straw degradation by the proposed strains. As one of the major agricultural waste in China, wheat straw has a relatively denser lignocellulosic structure, and
which was selected as the sample to be hydrolyzed by *B. methylotrophicus* 1EJ7. It was found that the epidermis (Fig. 5a) of the wheat straw were dramatically changed (Fig. 5b) after bacteria pretreatment. Specifically, the initial intact structure was destroyed to form some holes and lots of bacteria adhered on the surface. The sunken tiny holes showed that the bacteria could hydrolyze straw and destroy the surface structure of wheat straw, and the similar phenomenon was also observed of corn stover hydrolysis [16].

As the cellulose content affect the crystallinity in most plant biomass, the increase of crystallinity is also an indication of increase of cellulose content and can be used to evaluate the efficiency of the pretreatment [17]. X-ray diffraction was used to analyze crystallinity in wheat straw samples. The Cr I of wheat straw decreased from 41.57 to 40.52 (Fig.5c) before and after pretreatment, which suggested that the degradation of wheat straw could be realized the *B. methylotrophicus* 1EJ7.

**Cellulases clone and expression**

Two cellulases, β-glucosidase of 732 bp and endoglucanase of 1500 bp, were cloned respectively. Universal primer T7 was utilized to amplify the two recombinant plasmids, pET–28a-Bgl and pET–28a-Egl, and then tested the complete sequences.

The pET–28a-Bgl and the pET–28a-Egl recombinant plasmids were constructed and sequenced, after which heterologous expression in *E. coli* BL21 (DE3) was carried out to obtain the enzymes. SDS-PAGE showed that two cellulases were both successfully expressed in *E. coli* BL21 (DE3), and their *Mws* were 28.5 kDa and 56.3 kDa (Fig.6), respectively. The results of crude cellulase activities showed that the activity of crude Bgl and Egl were 1670.15±18.94 U/mL and 0.130±0.002 U/mL, respectively (Supplementary 3).

![Fig. 6](image)

The domains analysis results showed that Bgl belongs to GH16 family (Supplementary 4). Egl contained two domains, catalytic domain (CD) and carbohydrate-binding module (CBM). Catalytic domain belongs to GH5 family and CBM belongs to CBM3 family (Supplementary 5).

Meanwhile, by blast from PDB protein database, the highest identification of Bgl was endo-beta-1,3-1,4 glucanase (PDB id 3O5S_A) from *Bacillus subtilis* 168 with a similarity of 93.55%, and Egl was 94.92% similarity with endo-1,4-beta-glucanase (PDB id 3PZT_A) and 90.41% with CBM3 lacking the calcium-binding site (PDB id 2L8A_A) from *B. subtilis* 168. Compared with Bgl sequence of *Bacillus velezensis* JTYP2, it was found that only four amino acids (70M→V, 96V→A, 156A→K, 204N→T) were different with the Bgl in our study, and the predicted secondary structure didn't obviously affect by these differences. By comparison, the Bgl of *B. subtilis* 168 showed more differences with the proposed Bgl as 22 amino acids were different (Fig.7). The Egl sequence showed that it had a 96.6% similarity with the Egl of *Bacillus velezensis* JTYP2, and there are 51 different bases between the two sequences led to 17 different amino acid changes.
acids (27A→T, 31G→E, 52Q→R, 199P→I, 238S→F, 285K→N, 316S→T, 331S→G, 332N→T, 334S→L, 339A→G, 364S→R, 382T→A, 404F→V, 411I→M, 414S→G, 440K→T), in which most changes appeared on the carbohydrate-binding module (CBM) and the linked peptide [18], and all the changes of the amino acids didn't significantly influence the secondary structure.

← Fig. 7
← Fig. 8

**Bioinformatics analysis and homology modeling**

The recombined Bgl contains 251 amino acids included a His-tag with a molecular weight of 28.47kDa. The computed pI was 6.79, and the negative GRAVY score (−0.491) suggested the protein might be hydrophilic. Bgl showed instability index and aliphatic index of 16.14 and 60.24, respectively. Correspondingly, the recombined Egl contained 507 amino acids including his-tag with a predicted molecular weight of 56.32 kDa. The computed pI was 7.26 and the negative GRAVY score (−0.616) suggested the protein to be hydrophilic. Egl showed instability index and aliphatic index of 29.60 and 73.69, respectively. Instability index less than 40 indicated that both the Bgl and Egl from *B. methylotrophicus* 1EJ7 was stable.

← Fig. 9

Bgl and Egl homology structural models were obtained by the I-TASSER. The information about the active site was obtained through superimposing 3D model structure of the Bgl with the template structure of cellulase from *Paenibacillus macerans* hybrid endo–1,3–1,4-beta-D-glucan 4-glucanohydrolase (PDB id 2AYH) [19], which provided accuracy of homology between two structures and also helped in positioning the conserved active site residues. Active site of Bgl was represented by 6 amino acid residues, Leu103, Phe105, Thr175, Asp179, Tyr188 and Asp236. The 35th to 242th amino acid domain of Bgl included a classical sandwich-like beta-jelly roll fold, and formed by two main, closely packed and curved antiparallel beta sheets, which led to a deep channel harboring the catalytic machinery. Bgl was found to be a catalytic sequence motif similar with GH16 family [18, 20], E-[ILV]-D-[IVAF]-[VILMF] (0, 1)-E, which was formed by amino acid 134th to 138th (EIDIE). The structural analysis of Egl showed that domains of CD and CBM, in which the catalytic domain had the critical TIM-barrel fold structure of GH5 family, consisting of 8 β-strands surrounded by 8 α-helices with the active site located at the cleft, formed by loops from the C-terminal portion of β-strands.

**Discussions**

**The unique cellulolytic bacteria in rotten woods from the Qinling Mountains**
Microbial biodegradation has been employed as an environmental-friendly method in cellulosic materials to generate various valuable compounds. In the past decades, numerous cellulolytic microorganisms have been isolated and characterized. In this study, it was found that the strains isolated from Qinling rotten wood exhibited widely taxonomic coverage (e.g. *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Bacillus licheniformis*, *Bacillus methylotrophicus* and *Bacillus megaterium*), in which *Bacillus subtilis* was found as the most abundant species.

In our previous study, as enrich medium, LB was used to obtain strains from initial material. As expected, *Bacillus* strains was found as the dominant strain in rotten wood. Since *Bacillus subtilis* strains are considered to exhibit a robust enzymes secretory system [21, 22], it may provide a huge potential of cellulase enzymes. Besides, *Bacillus subtilis* has been detected from forest system, such as forest soil[23], freshwater swamp forest [24]. In addition, *Bacillus licheniformis* was another vital species found in wooden trees, exhibiting the ability to degrade cellulose or other natural cellulosic biomass. *Bacillus licheniformis* has been extensively found in hot spring, soil, gut of animals, paddy field and agricultural environment, and high production of cellulase by *Bacillus licheniformis* was reported [25, 26]. It is noteworthy that, *Bacillus megaterium*, *Bacillus methylotrophicus* and *Pseudomonas aeruginosa* in this study were scarcely reported to exhibit the ability to produce cellulase or degrade cellulosic materials. To the best of our knowledge, only *Bacillus megaterium* BM05 has been reported for wheat straw fermentation [27], and *Bacillus methylotrophicus* Y37 [28] were reported to have cellulolytic enzyme, suggesting that the strains screened in this study might have the special potential to develop novel cellulases. Meanwhile, various species of cellulolytic bacteria were also found ( e.g. *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Bacillus licheniformis*, *Bacillus methylotrophicus* and *Bacillus megaterium*) which have been rarely reported. Consequently, the results revealed that samples from special areas (e.g. Qinling mountain) may act as a good source exploit cellulolytic bacterium.

**Hydrolysis capability of the strains and its cellulolytic enzymes**

On the whole, wild bacteria exhibit low enzyme production capacity and generally low enzyme activity. In this study, CMC-Na medium with different stains had the maximum reducing sugar content of 4.83 mg/100mL, while Avicel medium only achieved 1.61mg/100mL reducing content (Fig. 3), revealing that the hydrolysis capability of stains can be affected by the types of cellulose substrates. Also note that the sugar consumption for the strain's growth was also a cause of the low content of reduced sugar in cultivation broth. It was also reported in some literature that no reducing sugars were detected in the final CMC medium when cultured with isolated bacteria [29].

It is noteworthy that different carbon substrates could induce different cellulases and further lead to different capabilities of reducing sugar production. When 8 strains were taken to hydrolyze different carbon source substrates, the strains were found to exhibit a better performance in reducing sugar production in CMC-Na medium than those in Avicel. This phenomenon can be explained from two parts: 1. the different structure of cellulose substrates: Avicel was harder to hydrolyze than CMC-Na by cellulase
for its unique microcrystalline structure [30]; 2. the different action modes of exocellulase and endocellulase: since the type of cellulolytic enzyme is the critical factor for the hydrolysis of different cellulose substrates, the efficiency in hydrolyzing CMC or Avicel was also significantly affected by the enzyme types [31, 32].

Moreover, it was also found that the strains performing good in Avicel and CMC-Na degradation did not show a well performance in hydrolyzing biomass, probably attributed to the complex network of lignin-hemicellulose-cellulose. FPase and CMCase of different strains (Fig.4b and Fig.4c) exhibited similar cellulase activity in different mediums. Furthermore, except for B. subtilis 1AJ3 and the B. subtilis 3BJ4, all the rest strains could produce avicelase in all mediums. Other strains could generate enzyme in limited carbon source medium. For instance, B. subtilis 1AJ2 could produce avicelase enzyme only in CMC-Na medium. Since the avicelase is an inducible enzyme, some carbon sources could induce strain to produce avicelase, whereas some could not. Similar study also revealed that crystalline cellulose or more complex structure were hard to hydrolyze [33].

For the three substrates in this study, the pretreatment of switchgrass, considered major non-food biomass resources developed from US [34], had the highest reducing sugar content. Compared with wheat straw and corn stover, switchgrass acted as the herbaceous plant exhibiting a less tight and crystalline structure. Because switchgrass primarily consists of glucose than xylose or other monomeric sugars, it is relatively easier for strain to utilize, and a similar study has been reported by Sharma R [35]. Compared with acid pretreatment or hot compress water pretreatment [36], bacteria cultivation can be considered a gentle pretreatment method without adding salt ion, acid and alkali, or using high temperature and high pressure. In this study, B. methylotrophicus 1EJ7 was used to pretreat switchgrass, and 0.95 mg/mL reducing sugar was obtained after growth. According to present study, the competitiveness can be improved by optimizing cultivation condition or cooperating with other enzymes. In the meantime, novel technologies such as gene editing and cellulase cloning expression are also feasible.

**Cellulase of B. methylotrophicus**

When talking about B. methylotrophicus, primary studies mainly focused on the following parts: 1. Screening [37]; 2. Utilized as biosurfactant-producer and agricultural agent [38], bioflocculant [39], as well as biofertilizer or biocontrol agent [40]; 3. Cyclic lipopeptides [41] and lipopeptides [42]; 4. Production of enzymes, such as levansucrase [43], α-amylase [44], lactosylfructoside [45], and xylanase [46]. However, there were extremely few reports on cellulases from B. methylotrophicus. Only two types of cellulase have been reported, 1,3--1,4-beta-glucanase [47] and carboxymethyl cellulase [28], both obtained by being purified from strain cultivation broth. In the meantime, cloning and expression of polypeptides or enzymes of B. methylotrophicus have been rarely discussed.

Accordingly, the molecular biology method to clone and express cellulase from bacteria B. methylotrophicus 1EJ7 were employed in this study. Fortunately, two cellulases were successfully cloned
and expressed, and the cellulolytic enzyme activities of cloning peptide were also verified, marking the first time of the cloning and expression of the cellulases (β-glucosidase and endoglucanase) from \textit{B. methylotrophicus} strain. Enzyme activities of recombined crude enzyme Bgl and Egl reached 1670.15±18.94 U/mL and 0.130±0.002 U/mL, respectively. According to the results of SDS-PAGE, the light color band of Egl can be inferred to exhibit a low expression level, which may lead to low enzyme activity. However, it was still higher than the CMCase in strain growth broth in this study or other \textit{Bacillus} [48]. A good expression level of Bgl was also demonstrated under this expression condition and a high crude enzyme activity. Several studies have reported the highest enzyme activity of 560.4 U/mL under optimal expression conditions[49]. Xia et al. used a novel \textit{Trichoderma reesei} expression cassette to get β-glucosidase activity of 25.13 U/mL [50], a data higher than the enzyme activity produced by bacteria, and tens of thousands of times that of β-glucosidase activity of \textit{Penicillium pinophilum} KMJ601(3.2 U/mL) [51] and \textit{Bacillus sp. AS3}( 0.04 U/mL) [48].

As β-glucosidase and endoglucanase had been expressed successfully, more work can be done to obtain stronger hydrolyze ability cellulase. For example, optimize expression conditions or genetic modification in the subsequent study. Higher enzyme activity can also be optimized by factors (e.g. pH, temperature, and metal ions) to achieved. Furthermore, mutations can be made by genetic engineering to increase enzyme activity. Therefore, it laid a foundation of enzymes characters for further study and hydrolyze mechanisms, and also provide a choice of industrial applications via two cellulases.

**Conclusions**

A various of cellulose-degrading bacteria in rotten wood of Qinling Mountains were, identified and then characterized. Among the isolated strains, \textit{Bacillus} were the predominant species, in which \textit{Bacillus methylotrophicus} 1EJ7 exhibited the optimal performance on cellulose degradation. In the meantime, the β-glucosidase and endoglucanase were successfully cloned and expressed from \textit{Bacillus methylotrophicus} for the first time. The strain and the recombinant enzyme have potential application in industrial production.

**Materials And Methods**

**Materials**

Five different rotten wood samples (weed tree, red birch, poplar, alpine rhododendron and willow) were collected from Qinling Mountain in Shaanxi Province, China. Samples were transported to the laboratory and stored at 4 °C. Wheat straw, corn stover, switchgrass were washed by water and dried at 80 °C, and then crushed by high speed pulverizer to 40 mesh. The chemicals used in this study were purchased from Kelong Chemical Reagent Chengdu Co., Aladdin or Sigma.

**Cellulolytic bacteria isolation and identification**
Each sample was broken into pieces, and 1 g was added into LB medium (10 g/L NaCl, 10 g/L tryptone and 5 g/L yeast extract), then incubated at 37 °C for 24 h with a constant shaking speed of 120 rpm. The bacteria suspension was respectively transferred to two selective media. The two selective media, CM and AM, were used CMC-Na and Avicel as single carbon source separately, contained of 2.0 g/L sodium carboxymethyl cellulose (CMC-Na) or Avicel (PH–101), and others of 2.0 g/L (NH₄)₂SO₄, 0.5 g/L MgSO₄•7H₂O, 1.0 g/L K₂HPO₄ at natural pH of 7.20 were the same. The strains were cultured for 48 h at 37 °C with 120 rpm before being spread on the selective media agar plates with 0.4 g/L Congo red. Plates were incubated at 37 °C for 72 h, and then different colonies on the plates were picked.

The strains which probably could produce cellulolytic enzymes had a hydrolyzed circle around the colony. 10 μL broth of each isolated strains was dripped on the Congo red agar plates and the hydrolysis circle diameters of were measured to primarily evaluate the cellulolytic capability. The selected strains were shown in Fig.1.

![Fig. 1](image)

The strains were cultured in broth for 48 h, then the cells were harvested and subjected to genome DNA extraction by a DNA extraction kit (Sangon Biotech, Shanghai, China). The universal primers of 27F and 1492R were utilized to amplify the 16S rRNA gene fragments. Polymerase chain reaction (PCR) was performed in a 25 μL reaction system containing 1 μL DNA template, 1 μL upstream primer (10μM), 1 μL downstream primer (10μM), 12 μL mixture, and 10 μL double-distilled water. The PCR amplification was performed as follows: initial denaturation at 95 °C for 5 min; 35 cycles of 94 °C for 1 min, 58 °C for 30 s, and 72 °C for 1 min; and final extension at 72 °C for 10 min.

Agarose gel electrophoresis was used to confirm target products and the PCR products were sequenced. The sequences were applied to BLAST on the NCBI database (http://blast.ncbi.nlm.nih.gov/). The 16S rRNA gene sequences have been submitted to GenBank (Accession Numbers showed in Supplementary 1). Phylogenetic tree was constructed in iTOL (http://itol.embl.de).

Reducing sugar determination and enzymatic activity assay

Fifty mL of the two selective media were individually transferred into 100 mL flasks and autoclaved under 121 °C for 20 min. The flasks were inoculated with 6 % (v/v) seed bacteria (dilute broth OD₆₀₀ to 1.0) and grow at 37 °C for 48 h under 120 rpm. The cell-free supematant was obtained by centrifugation (11000 rpm, 4°C, and 10min) to examine the activity of crude cellulase. The FPase (filter paper activity), CMCase activity, and avicelase activity were analyzed according to the methods described [52]. All cellulase activities were determined at 50 °C. Reducing sugar of each cultivated liquid was analyzed by DNS method [53]. The activities of recombinant β-glucosidase was determined by p-NPG (p–4-nitrophenyl β-D-glucopyranoside)as substrate at 50°C, 15min with p-NP as standard [54].
Cultivation in different carbon sources

Different carbon source of wheat straw (4.0 g/L), corn stover (4.0 g/L), switchgrass (4.0 g/L), Avicel (2.0 g/L) or CMC-Na (2.0 g/L) was used as sole carbon source. Reducing sugar and cellulase activity were measured.

Single and mixed strain cultivation of cellulosic materials without pretreatment

Three bacteria strains (B. methylotrophicus 1EJ7, B. subtilis 1AJ3 and B. subtilis 3BJ4) were selected to degrade wheat straw, corn stover or switchgrass by single and mixture owing to their higher cellulolytic activity. Mixture of the three strains (1:1:1) had the same inoculum size as the single strain. The inoculum size of the single strain or mixed strains was 6%. The concentrations of wheat straw, corn stover, and switchgrass were 7 % (w/v), after which they were growed at 37 °C, 120 rpm for 72 h. Reducing sugar was determined at intervals of 12 h.

Scanning electron microscopy (SEM) and X-ray diffraction

B. methylotrophicus 1EJ7 was utilized to hydrolyze wheat straw without pretreatment as an example to show morphology changes before and after hydrolysis by SEM method [55].

X-ray diffraction was used to show wheat straw physical structures on a Xian Asn Tech X-ray diffractometer with diffraction angles spanned from 2θ = 5–50°. The radiation was generated at a voltage of 40 kV and a current of 35 mA and a scan step size of 0.033° [17]. Crystallinity Index CRI (%) = [(I_{002} - I_{am}) / I_{002}]×100 (I_{002} is the intensity of crystalline portion of cellulose at 2θ = 22°, and I_{am} is the peak intensity of the amorphous portion at 2θ = 18°).

Cloning and expression of cellulase gene from B. methylotrophicus 1EJ7 in E. coli

B. methylotrophicus 1EJ7 was cultured in LB medium at 37°C for 24 h under 150 rpm. The cells were collected by centrifugation (10,000 rpm, 4°C, and 10 min) and the genomic DNA was extracted using an Ezup column bacteria genomic DNA purification kit (Shanghai Sangon Biotech Co., Ltd.). The extracted DNA was used as a template for PCR amplification. The genes encoding β-glucosidase and endoglucanase were amplified by using primers based on the gene sequences of the β-glucosidase of Bacillus velezensis AS43.3 (CP003838.1) and endoglucanase of Bacillus velezensis strain JTYP2 (CP020375.1). The gene encoding the β-glucosidase was amplified by PCR (94°C for 5min, and then 35 cycles of 94°C for 1min, 65°C for 1min (~0.5°C/c), 72°C 3min, and 72°C for 10min) with a forward primer of 5'- CATGCGATGCCATTTTATGCATTAGAACGAGTG (Ncol site was underlined) and a reverse primer 5'-CCGCAGTAGGTTTTGGGCAACCCA (Xhol site was underlined) using a Takara ExTaqHS
(Takara Bio, Shiga, Japan). The gene encoding the endoglucanase was amplified under the same PCR condition described above with a forward primer of 5′-CATGCCATGGCATTGAAACGTTCAATTTCTATT (NcoI site was underlined) and a reverse primer of 5′-CCGCTCGAGATTGGGTTCTGTTCCCAA (XhoI site was underlined). The amplified genes were double digested with NcoI and XhoI, and inserted into the corresponding site of the pET–28a vector (Novagen) by T4 ligase.

Then, the constructed plasmid was transformed into E.coli BL21 (DE3) by hot hit under 42°C for 90s and correct transformants were identified by PCR amplification and sequencing. The transformant was cultured in 1 L LB medium containing 1 mg/mL kanamycin at 37 °C until the absorbance at 600 nm reached 0.6. Then, expression was induced by adding final density of 0.2 mM IPTG, and the transformant was further cultured at 25 °C for 16 h. The cells were collected by centrifuging (8000 ×g, 4 °C, 10 min), and then suspended in PBS buffer (pH 7.2). Cells were disrupted by ultrasonication under 300W output power, a repeating cycle of 1s ultrasonic treatment and 5s shutdown, for 60 min on a SCIENTZ-IID ultrasonic homogenizer (Ningbo Scientz Biotechnology Polytron Technologies Inc. Zhejiang province, China). The resulting cell lysates were centrifuged (8000 ×g, 4 °C, 30 min). SDS-PAGE was performed to analyze the supernatant and the insoluble fraction of the sonicated whole cell lysate.

**Bioinformatic analysis and homology modeling**

The plasmids of pET–28a-Bgl and pET–28a-Egl were sequenced. The primary sequences of Bgl and Egl protein were obtained by amino acid translation software, and the homology templates were obtained through retrieving in the protein database PDB. Physiochemical characteristics were predicted on ExPASy (http://web.expasy.org/protparam/). Conserved domain was analyzed by CDD of NCBI (https://www.ncbi.nlm.nih.gov/cdd). Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) was used for sequence alignments. Secondary structure and 3D structure were predicted by PSIPRED (http://bioinf.cs.ucl.ac.uk/psipred/) and I-TASSER (https://zhanglab.ccmb.med.umich.edu/I-TASSER/).

**Declarations**

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Authors Affiliations
Lab of Bioresources, College of Food Science and Engineering, Northwest A&F University, Yangling, Shaanxi Province 712100, China

Lingling Ma, Yingying Lu, Hong Yan, Xin Wang, Yanglei Yi, Yuanyuan Shan, Bianfang Liu, Yuan Zhou, and Xin Lü

Authors Contributions
LM, XW, and XL conceived and designed the experiments. LM, YL, YH, and YY performed the experiments. YS, BL, and YZ analyzed the data. LM and XL wrote the manuscript.

Corresponding authors
Correspondence to Xin Lü

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Competing interests
The authors declare that they have no competing interests.

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### Tables

Table 1

Isolated strains growth situation and clear zone size on Congo red plates
| Source organism | No.   | Sole carbon source | Diameter of strain colony (mm) | Diameter of clear zone (mm) | Ratio of clear zone and strain diameter |
|-----------------|-------|--------------------|--------------------------------|-----------------------------|----------------------------------------|
| Weed tree       | 1AJ1  | CMC-Na             | 8.0                            | 20                          | 2.50                                   |
|                 | 1AJ2  | CMC-Na             | 7.5                            | 20                          | 2.67                                   |
|                 | 1AJ3  | CMC-Na             | 7.5                            | 19                          | 2.53                                   |
|                 | 1AJ4  | CMC-Na             | 8.0                            | 21                          | 2.63                                   |
|                 | 3AJ1  | Avicel             | 7.0                            | 22                          | 3.14                                   |
|                 | 3AJ5  | Avicel             | 6.0                            | 19                          | 3.17                                   |
|                 | 3AJ4  | Avicel             | 7.0                            | 15                          | 2.14                                   |
|                 | 3AJ7  | Avicel             | 6.0                            | 19                          | 3.17                                   |
| Red birch       | 1BJ1  | CMC-Na             | 7.0                            | 21                          | 3.00                                   |
|                 | 1BJ2  | CMC-Na             | 8.0                            | 21                          | 2.63                                   |
|                 | 1BJ3  | CMC-Na             | 8.5                            | 21                          | 2.47                                   |
|                 | 1BJ4  | CMC-Na             | 9.0                            | 20                          | 2.22                                   |
|                 | 1BJ5  | CMC-Na             | 9.0                            | 21.5                        | 2.39                                   |
|                 | 1BJ6  | CMC-Na             | 8.5                            | 22                          | 2.59                                   |
|                 | 1BJ7  | CMC-Na             | 9.0                            | 22                          | 2.44                                   |
|                 | 1BJ8  | CMC-Na             | 7.5                            | 20                          | 2.67                                   |
|                 | 1BJ9  | CMC-Na             | 7.0                            | 21                          | 3.00                                   |
|                 | 3BJ2  | Avicel             | 6.0                            | 17                          | 2.83                                   |
|                 | 3BJ3  | Avicel             | 5.5                            | 16.5                        | 3.00                                   |
|                 | 3BJ4  | Avicel             | 8.0                            | 19                          | 2.38                                   |
|                 | 3BJ5  | Avicel             | 6.5                            | 19                          | 2.92                                   |
|                 | 3BJ6  | Avicel             | 7.5                            | 16                          | 2.13                                   |
|                 | 3BJ7  | Avicel             | 6.5                            | 16                          | 2.46                                   |
|                 | 3BJ8  | Avicel             | 6.0                            | 12                          | 2.00                                   |
|                 | 3BJ9  | Avicel             | 6.0                            | 11                          | 1.83                                   |
| Poplar          | 1CJ1  | CMC-Na             | 8.5                            | 25                          | 2.94                                   |
|                 | 1CJ2  | CMC-Na             | 8.0                            | 21                          | 2.63                                   |
|                 | 1CJ3  | CMC-Na             | 9.0                            | 25                          | 2.78                                   |
|                 | 1CJ4  | CMC-Na             | 8.5                            | 20                          | 2.35                                   |
|                 | 1CJ5  | CMC-Na             | 9.0                            | 24                          | 2.67                                   |
|                 | 1CJ6  | CMC-Na             | 8.5                            | 18.5                        | 2.18                                   |
|                 | 1CJ7  | CMC-Na             | 8.0                            | 19                          | 2.38                                   |
|                 | 1CY1  | CMC-Na             | 8.0                            | 11                          | 1.38                                   |
|                 | 1CY2  | CMC-Na             | 9.0                            | 11.5                        | 1.28                                   |
|                 | 1CY3  | CMC-Na             | 9.0                            | 11.5                        | 1.28                                   |
|                 | 3CJ5  | Avicel             | 4.0                            | -                           | -                                      |
|                 | 3CJ6  | Avicel             | 9.0                            | 18                          | 2.00                                   |
|                 | 3CJ7  | Avicel             | 5.5                            | 12                          | 2.18                                   |
|                 | 3CJ8  | Avicel             | 6.5                            | 18                          | 2.77                                   |
|   |   |   |   |   |
|---|---|---|---|---|
| 3CJ9 | Avicel | 7.5 | 16 | 2.13 |
| 3CJ10 | Avicel | 6.0 | 9 | 1.50 |
| 3CY2 | Avicel | 5.0 | 9 | 1.80 |
| 3CY3 | Avicel | 7.0 | 9 | 1.29 |
| 3CY6 | Avicel | 6.5 | 8 | 1.23 |
| Alpine rhododendron |   |   |   |   |
| 1DJ1 | CMC-Na | 8.0 | 11 | 1.38 |
| 1DJ2 | CMC-Na | 9.0 | 11 | 1.22 |
| 1DJ3 | CMC-Na | 8.0 | 11 | 1.38 |
| 1DJ4 | CMC-Na | 8.0 | 11 | 1.38 |
| 1DJ5 | CMC-Na | 8.0 | 10.5 | 1.31 |
| 1DJ6 | CMC-Na | 7.5 | 14 | 1.87 |
| 3DJ1 | Avicel | 6.5 | 13 | 2.00 |
| 3DJ2 | Avicel | 6.0 | 12 | 2.00 |
| 3DJ4 | Avicel | 6.0 | 11 | 1.83 |
| 3DJ5 | Avicel | 6.5 | 11 | 1.69 |
| 3DJ6 | Avicel | 7.0 | 13 | 1.86 |
| 3DJ7 | Avicel | 6.0 | 13 | 2.17 |
| 3DY1 | Avicel | 8.0 | 9 | 1.13 |
| 3DY2 | Avicel | 7.0 | 10 | 1.43 |
| 3DY3 | Avicel | 7.0 | 11 | 1.57 |
| Willow |   |   |   |   |
| 1EJ1 | CMC-Na | 9.0 | 16 | 1.78 |
| 1EJ2 | CMC-Na | 7.5 | 11.5 | 1.53 |
| 1EJ3 | CMC-Na | 7.5 | 14 | 1.87 |
| 1EJ4 | CMC-Na | 7.5 | 13 | 1.73 |
| 1EJ5 | CMC-Na | 8.0 | 15 | 1.88 |
| 1EJ6 | CMC-Na | 9.0 | 12 | 1.33 |
| 1EJ7 | CMC-Na | 7.0 | 19 | 2.71 |
| 1EY1 | CMC-Na | 8.0 | 11 | 1.38 |
| 1EY2 | CMC-Na | 6.5 | 9 | 1.38 |
| 1EY8 | CMC-Na | 8.0 | 9 | 1.13 |
| 3EJ1 | Avicel | 7.5 | 14 | 1.87 |
| 3EJ2 | Avicel | 9.0 | 19 | 2.11 |
| 3EJ3 | Avicel | 8.0 | 18 | 2.25 |
| 3EJ4 | Avicel | 8.5 | 18 | 2.12 |
| 3EJ5 | Avicel | 8.0 | 11 | 1.38 |
| 3EJ6 | Avicel | 8.0 | 7 | 0.88 |
| 3EJ7 | Avicel | 9.0 | 11 | 1.22 |
| 3EJ8 | Avicel | 9.0 | 14 | 1.56 |
| 3EY1 | Avicel | 8.0 | 11 | 1.38 |
| 3EY2 | Avicel | Point colony | 7 | - |
| 3EY3 | Avicel | 8.0 | 11 | 1.38 |
| 3EY4 | Avicel | 8.5 | 14 | 1.65 |
Figures

Figure 1

Hydrolyzed circle of isolates on the Congo red agar plate. (a) Plates with CMC-Na as the sole carbon source. (b) Plates with Avicel as the sole carbon source.
Figure 2

(a) Circular maximum likelihood phylogenetic tree of bacterial of 16S rDNA sequence. The sector band shows genera by different colors. The tree was constructed in iTOL. (b) Strains distribution.

Figure 3

Heat map of reducing sugar production and enzyme activities.
Figure 4

Reducing sugar and cellulase activities of eight strains in different carbon source medium.
Figure 5

Reducing sugar production by different strains in 7% wheat straw, corn stover and switchgrass for 72h. SEM of wheat straw before (5a) and after (5b) 72h fermentation by Bacillus methylotrophicus 1EJ7. X-ray of untreated wheat straw and fermented by Bacillus methylotrophicus 1EJ7 was showed in 5c.
Figure 6

(A) 1% Agarose gel electrophoresis use universal primer of T7 by the recombined plasmid. (B) 12% SDS-PAGE for expression of Bgl and Egl. Lane 1: Marker, lane 2: Bgl protein, lane 3: Egl protein.
Amino acid sequence alignments for Bgl and comparison with same gene in different strains. In which Bgl stand for β-glucosidase in this study; AS43.3 for β-glucosidase gene of Bacillus velezensis AS43.3 (CP003838.1) (complete gene); JTYP2 for Bacillus velezensis JTYP2 (CP020375.1), and 168 for Bacillus subtilis 168 (AL009126.3). Protein secondary structure of Bgl was predicted by PSIPRED. Helix, strand and coil was showed on top of aa sequence.
Amino acid sequence alignments for Egl and comparison with same gene in different strains. In which Egl stand for endoglucanase in this study; JTYP2 for endoglucanase gene of Bacillus velezensis JTYP2 (CP020375.1) (complete gene); AS43.3 for Bacillus velezensis AS43.3 (CP003838.1), and 168 for Bacillus subtilis 168 (AL009126.3). Protein secondary structure of Egl was predicted by PSIPRED. Helix, strand and coil was showed on top of aa sequence.

**Figure 8**
Figure 9

3D structure prediction of Bgl (a) and Egl (b).

Supplementary Files

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