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Isolation and identification of a cellulolytic bacterium from the Tibetan pig's intestine and investigation of its cellulase production

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ABSTRACT

Background: The Tibetan pig is a pig breed with excellent grazing characteristics indigenous to the Qinghai–Tibet plateau in China. Under conditions of barn feeding, 90% of its diet consists of forage grass, which helps meet its nutritional needs. The present study aimed to isolate and identify a cellulolytic bacterium from the Tibetan pig's intestine and investigate cellulase production by this bacterium. The study purpose is to provide a basic theory for the research and development of herbivore characteristics and to identify a source of probiotics from the Tibetan pig.

Results: A cellulolytic bacterium was isolated from a Tibetan pig's intestine and identified based on morphological, physiological, and biochemical characteristics as well as 16S rRNA analysis; it was designated *Bacillus subtilis* BY-2. Examination of its growth characteristics showed that its growth curve entered the logarithmic phase after 8–12 h and the stable growth phase being between 20 and 40 h. The best carbon source for fermentation was 1% corn flour, while 2% peptone and yeast powder compound were the best nitrogen sources. The initial pH during fermentation was 5.5, with 4% inoculum, resulting in a high and stable amount of enzyme in 24–48 h.

Conclusions: The isolated BY-2 strain rapidly grew and produced cellulase. We believe that BY-2 cellulase can help overcome the shortage of endogenous animal cellulase, improve the utilization rate of roughage, and provide strain sources for research on porcine probiotics.

1. Introduction

Plant cellulose, a major component of plant cell walls, is the most abundant and cost efficient renewable energy source, with the maximum annual output [1]. Plant dry weight includes 35–50% cellulose, 20–35% hemicellulose, and 5–30% lignin [2]. Cellulose has a water-insoluble highly crystalline structure, and it is surrounded by a tough lignin layer. Therefore, hydrolysis of cellulose into available glucose sugar is very difficult [3]. At present, plant cellulose is used mainly for fuel, animal feed and manure, and in the paper industry. However, while the utilization of plant cellulose is low, the corresponding environmental pollution is considerable. Although acid, alkali, and steam heating treatment methods produce relatively good results, their applications have been greatly limited [4,5] as they require complex equipment and have disadvantages such as secondary pollution.

Cellulases belong to a large family of glycosyl hydrolases, including endoglucanase (EC 3.2.1.4), exoglucanase or cellobiohydrolase (EC 3.2.1.91), and β-glucosidase (EC 3.2.1.21) [6]. The microorganisms identified thus far to be involved in production of cellulases and related enzymes mainly include bacteria, some fungi, and actinomycetes [7]. Cellulolytic organisms of fungal origin produce cellulase used in food, animal feed, textiles, fuel, the chemical industry, etc. [8,9]. However, because of the slow growth of fungi, cellulase production costs are high for these processes. In contrast, a bacterial culture is simple, grows rapidly, and has a short generation time, and other beneficial characteristics; thus, it has good potential applicability [10].

In the high altitude of the Qinghai–Tibet plateau in China, the Tibetan pig is bred in hypoxic and cold grazing conditions, with excellent characteristics, such as cold and, disease-resistance. Further, this pig shows good grazing characteristics, is an environmentally safe plant-eating pig species, and tastes good. Under conditions of barn feeding, 90% of the diet of this pig consists of forage grass to meet its nutritional needs.

In the present study, a cellulolytic bacterium was isolated from the cecal contents of the Tibetan pig. Examination of the plants consumed

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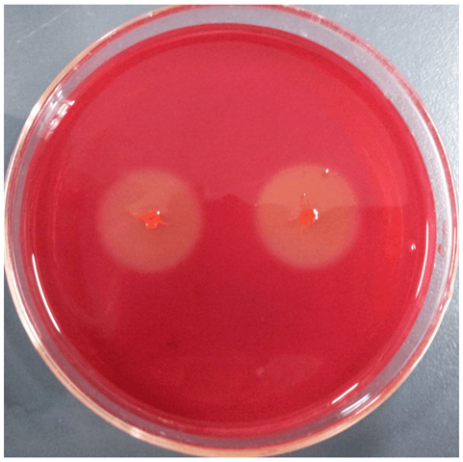

Fig. 1. Clearing zone of cellulolytic bacterium on CMC-Na selective media.

by this pig provided sources of bacterial strains and forms the research basis for the development and utilization of probiotic strains in swine. In addition, the characteristics and production conditions of the enzymes produced were studied to provide a theoretical basis for the reasonable use of cellulase and high-fiber foods in animal production.

2. Materials and methods

2.1. Bacterial strains, plasmids, general reagents, and culture conditions

Competent *Escherichia coli* DH5a cells and the pGEM-T vector were stored in our laboratory. Carboxymethyl cellulose (CMC), microcrystalline cellulose (MCC), isopropyl β-D-thiogalactopyranoside, and X-gal were purchased from Sigma. Tryptone, yeast extract, and agar powder were purchased from OXOID. The bacterial strains were selected from Luria-Bertani (LB) plates and cultured in LB liquid medium. The cellulolytic bacterium was fermented in LB medium supplemented with 1% CMC at 37°C.

2.2. Screening of cellulolytic bacterium

Samples were collected from 8-month-old healthy Tibetan pigs (Shaanxi HuaYi Industrial Co., Ltd.; Tibetan pig breeding base). The pigs were then killed using sodium pentobarbital (50 mg/kg BW) for collection of intestinal samples. The samples were placed in sealed plastic bags surrounded with ice and brought to the laboratory within 2 h. All experiments were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

One gram of cecum contents was diluted with 100 mL distilled water and homogenized in a constant-temperature oscillation water bath at 80°C, for 30 min. After gradient dilution, the samples were coated on the surface of a LB agar plate containing 1% CMC and incubated at 37°C for 24 h [11]. After incubation, the plates were flooded with 1% Congo red for 15–20 min followed by destaining with 1 M NaCl for 15–20 min [12], and the strain with the largest clearing zone was isolated for repeated screening. The strain (inoculum concentration of 1%, 50 mL in a 250 mL flask) was inoculated in liquid LB medium containing 1% CMC at 37°C and grown with shaking at 220 rpm for 24 h. Then, the fermentation extract was centrifuged at 5000 rpm for 15 min, and the clear supernatant was examined for enzyme assays under optimum reaction conditions for screening of highly cellulolytic bacterial strains. Strains showing high cellulase activity were used in subsequent experiments.

Table 1
Identification of physiological–biochemical characteristics of the bacterial isolates.

Characteristics	Results	Characteristics	Results
Catalase	+	Citrate	+
Anaerobic	-	Tyrosine hydrolysis	-
Methyl red test	+	Phenylalanine deaminase	-
V-P measure	+	Nitrate reduction	+
V-P cultures pH 6	+	Formation: benzazole	-
V-P cultures pH 7	+	Growth pH	
Acid production: D-glucose	+	pH 6.8 nutrient bouillon	+
L-arabinose	+	pH 5.7 nutrient bouillon	+
D-xylose	+	Growth NaCL: 7%	+
D-mannitol	+	10%	+
Glucose gas	-	Growth temperature: 5°C	+
Hydrolysis: gelatin	+	50°C	+
Starch	+	65°C	-
Casein	+	Lecithin enzyme	-

+: positive; -: negative.

2.3. Identification and examination of growth characteristics of isolated cellulolytic bacterium

The isolated strain was morphologically identified by Gram staining and malachite green spore staining [13], and physiological–biochemical identification was performed in accordance with Bergey's Manual of Systematic Bacteriology [14]. For further identification, 16S rRNA was amplified by polymerase chain reaction (PCR) from the genomic DNA of the strain using universal primer pair 27F/1492R [15]. The purified PCR products were cloned into the pGEM-T vector and sequenced by BGI Biotechnology. The sequencing results were compared using

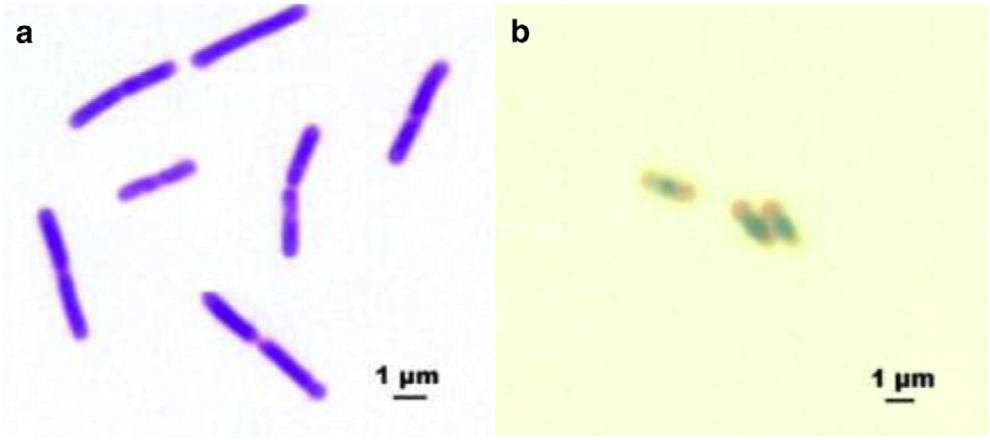

Fig. 2. Morphological identification of the cellulolytic bacterium. (a) Gram staining; (b) malachite green staining of spores.

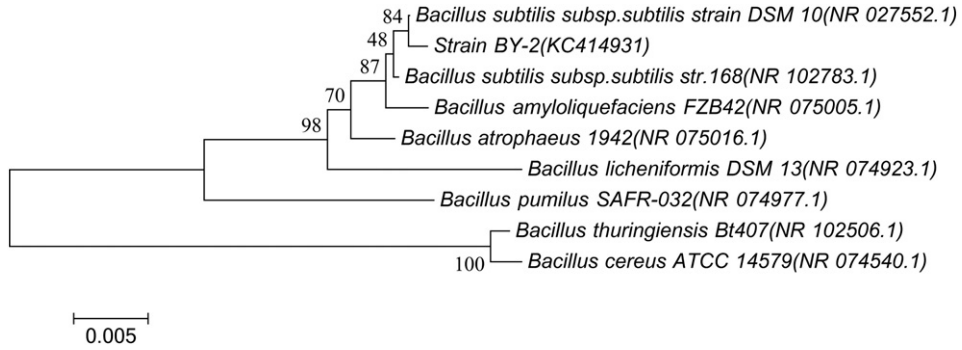

Fig. 3. Phylogenetic dendrogram of the 16S rRNA sequence of the isolated strain.

the Basic Local Alignment Search Tool (BLAST) program on NCBI and 16S rRNA gene sequence homology analysis using GenBank data. A phylogenetic tree was constructed using the neighbor-joining model of the MEGA 5.1 program. Lastly, the microbial optical density (OD) at 600 nm was determined at different growth periods to reflect the growth characteristics of the bacterium.

2.4. Carboxymethyl cellulase activity assay

The activity of carboxymethyl cellulose (CMCase) was determined by measuring the release of reducing sugars from CMC. Standard curves were prepared under assay conditions for glucose, with 1% (w/v) CMC as the substrate, as previously described [16]. For this, 1 mL of culture supernatant was mixed with 1 mL of 1% CMC in 0.1 M sodium acetate buffer (pH 5.5) in a test tube and incubated at 65°C for 5 min. The reaction was terminated by the addition of 2.5 mL dinitrosalicylic acid (DNS), and the mixture was subsequently boiled for 5 min and cooled in ice. The OD at 540 nm was determined. One unit of cellulase activity was defined as the amount of enzyme necessary to release 1 μmol reducing sugar per minute, expressed as units per milliliter.

2.5. Effect of different fermentation conditions on cellulase production

In order to identify the optimal carbon source and nitrogen source for enhanced cellulase production, different carbon sources and nitrogen sources at various concentrations, initial pH values, and inoculum concentrations were evaluated for their effect on enzyme production. Enzyme production at different fermentation times was also simultaneously determined. Cultures (50 mL in 250 mL flasks) were incubated at 37°C on a rotary shaker at 220 rpm for 24 h, the fermentation extract was centrifuged at 5000 rpm for 15 min, and the clear supernatant was used in the enzyme assays.

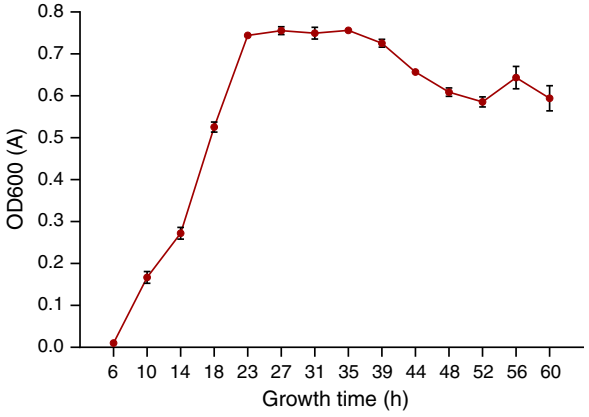

Fig. 4. Growth curve of isolated bacteria. Error bars indicate standard deviation.

3. Results and discussion

3.1. Isolation of cellulolytic bacterium

Using CMC-Na as the sole carbon source in selective media, followed by the Congo red staining method for preliminary isolation of cellulolytic bacterium, is widely considered to be the best method for preliminary screening of cellulolytic bacteria because it is simple and fast [12]. However, plate-screening methods using dyes are not quantitative or adequately sensitive, and because of the poor correlation between enzyme activity and the size of the clearing zone, selective media cannot accurately reflect the cellulolytic activity of bacteria [17], making repeated screening necessary. Many cellulolytic bacteria have been screened from different environments, such as the bovine rumen [12], soil [18], organic waste [19], and ruminant animal waste [17,20]. Most have been identified to be *Bacillus*, *Clostridium*, rumen bacteria, or *Bacteroides* [21,22].

In the present study, the isolated species was cultured at 37°C on CMC-Na solid media for 24 h, and subsequent Congo red staining showed a remarkable clearing zone. In addition, by examining enzyme activity, we selected the strain showing the highest enzyme activity for subsequent research. According to previous research, the enzyme activity of this strain (1.52 U/mL) is higher than that of other strains cultured for 24 h [20,23], indicating that this strain exhibits stable inheritance and has strong CMCase activity (Fig. 1).

3.2. Identification of bacterial isolates

3.2.1. Morphological identification

Morphological examination [13] showed the surface of the colonies to be rough, opaque, and gray or yellowish, indicating that the species is

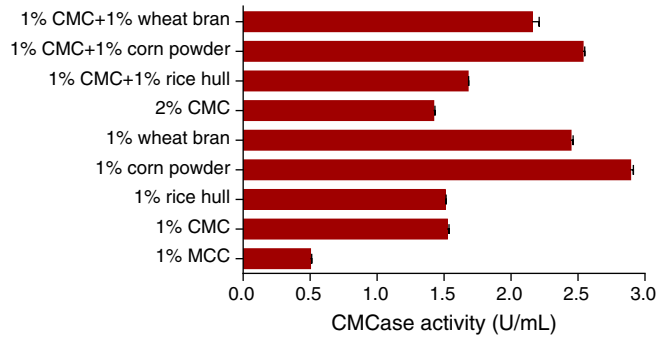

Fig. 5. Effect of different carbon sources on cellulase production. Error bars indicate standard deviation.

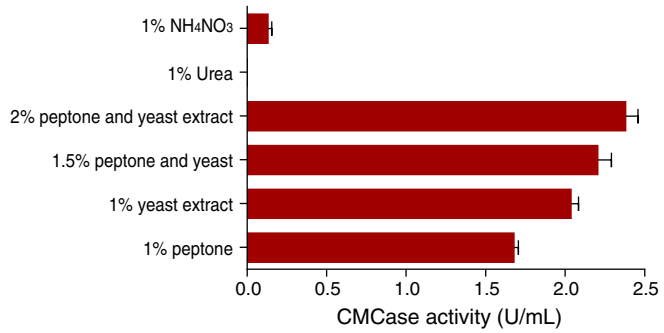

Fig. 6. Effect of different nitrogen sources on cellulase production. Error bars indicate standard deviation.

aerobic. Microscopically, this strain was found to be a Gram-positive, motile bacillus (Fig. 2a). Improved malachite green staining showed a single long, green, oval spore at the center of the bacteria. Trophozoites were stained red (Fig. 2b).

3.2.2. Physiological–biochemical characterization

According to previously reported methods [14], the physiological and biochemical characteristics of the species were identified (Table 1). Preliminary analysis identified the strain as *Bacillus subtilis*.

3.2.3. Analysis of 16S rRNA

The 16S rRNA sequence of the cellulolytic bacterium was 1511 bp long and was submitted to GenBank under accession number KC414931.1. Homology analysis showed that the degree of sequence similarity of this strain to some *Bacillus* species exceeded 99% (Fig. 3). As shown in the phylogenetic tree constructed using MEGA5.1, the strain is related to *B. subtilis* strain DSM 10 (GenBank accession no: NR_027552.1), with a similarity of 99.14%. Thus, all characterization methods showed that the isolated species was a *B. subtilis* strain. The strain was preserved at the Chinese Classic Culture Preservation Center (Wuhan University) under no. CCTCCM 2012535 and was designated *B. subtilis* BY-2.

3.3. Analysis of growth characteristics

Strain BY-2 reached the logarithmic phase at 8–20 h after inoculation, after which its growth reached a plateau and continued for about 20 h (Fig. 4). According to the strain growth characteristics, we concluded that the optimum BY-2 enzyme production time was 20–39 h. Since the bacterial enzyme is most active in the logarithmic phase, we conducted fermentation cultivation for 24 h in a follow-up experiment in order to examine enzyme characteristics.

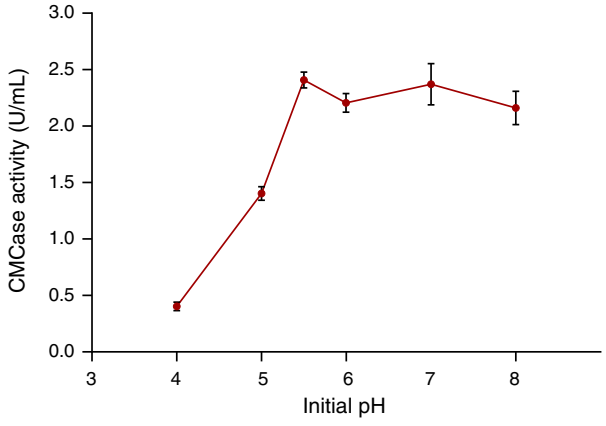

Fig. 7. Effect of initial pH on cellulase production. Error bars indicate standard deviation.

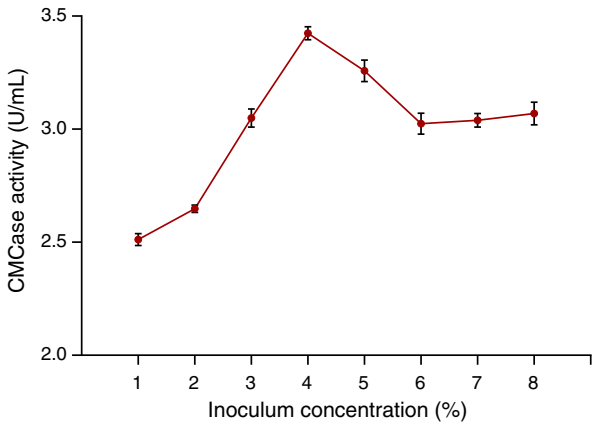

Fig. 8. Effect of inoculum concentration on cellulase production. Error bars indicate standard deviation.

3.4. Analysis of factors affecting BY-2 cellulase production

3.4.1. Effect of carbon source

Because cellulases are inducible enzymes, the medium for cellulase production in fermentation usually contains cellulose-rich substrates as a carbon source [24]. In the present study, different carbon sources at various concentrations were examined to study their effects on BY-2 cellulase production under identical conditions (temperature, 37°C; rotation speed, 220 rpm; seeded culture concentration, 1%; incubation time, 24 h). The results showed that strain BY-2 could utilize various carbon sources, and the maximum CMCase activity (2.91 U/mL) was observed when a mixture of 1% corn powder and 1% CMC was used as the sole carbon source. However, when MCC was used as a sole carbon source, negligible CMCase activity was observed (Fig. 5). Therefore, the inexpensive and easily available corn powder was found to be optimal and used as the carbon source in the following experiments.

3.4.2. Effect of nitrogen source

Various nitrogen sources (1% peptone, 1% yeast extract, a 2:1 mixture of 1.5% peptone and yeast extract, a 1:1 mixture of 2% peptone and yeast extract, 1% urea, and 1% NH_4NO_3) were examined with other conditions identical (as mentioned above). The results showed that strain BY-2 can utilize organic nitrogen sources efficiently, and the maximum CMCase activity (2.38 U/mL) was observed when a 1:1 mixture of 2% peptone and yeast extract was used as the sole nitrogen source. However, the CMCase activity was

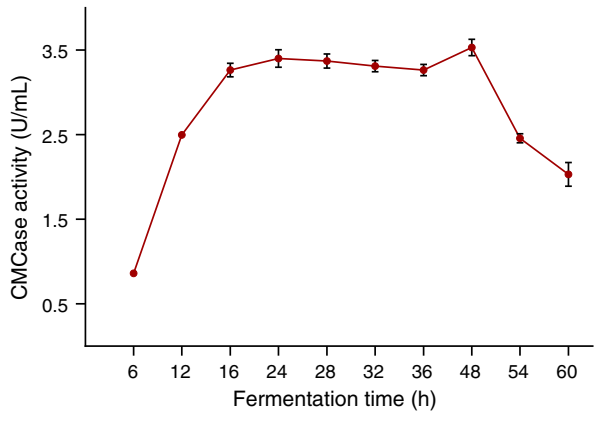

Fig. 9. Effect of fermentation time on cellulase production. Error bars indicate standard deviation.

almost zero when inorganic nitrogen sources (NH_4NO_3 or urea) were used as the sole nitrogen sources (Fig. 6). This could be because the metabolism of inorganic nitrogen contributes to medium acidification, which in turn affects cellulase production. The *Bacillus* species isolated by Rajoka [25] and Ray et al. [26] showed a similar ability to utilize nitrogen sources. However, the cellulolytic *B. subtilis* isolated from elephant dung by Sadhu et al. [27] could utilize both inorganic and organic nitrogen sources. Additionally, the *B. subtilis* strain isolated by Acharya and Chaudhary [28] could not utilize inorganic nitrogen sources when CMC was used as the carbon source, but it could when wheat straw and rice hull were used as the carbon source. The findings collectively demonstrate that both the original environment and medium conditions are closely related to the enzymatic characteristics and productivities of different *Bacillus* strains.

3.4.3. Effect of initial pH

To examine the effect of pH on cellulase production, the initial pH value (5.5) of the medium was adjusted to 4.0, 5.0, 6.0, 7.0, and 8.0. The CMCase activity in each pH-adjusted medium was then measured under identical conditions (temperature, 37°C; rotation speed, 220 rpm; seeded culture concentration, 1%; incubation time, 24 h). The results showed that the minimum CMCase activity (0.5 U/mL) was observed at an initial medium pH of 4.0, while the maximum (2.41 U/mL) was observed at pH 5.5 (Fig. 7). Further, the CMCase activity was stable over a broad range of pH values (5.5–8.0), which are similar to the pH of the cecal contents of the Tibetan pig. These results indicated the potential application of the obtained cellulose in improving the nutritional quality and digestibility of animal feed stocks. The optimal initial pH of strains from different sources varies: for instance, the optimal initial pH for cellulase production by *Bacillus* isolated from artificial manure [29] and *B. subtilis* isolated from a hot spring [28] were 6.0 and 9.0, respectively. Therefore, appropriate pH conditions can promote the growth of strains and enhance the cellulase yield; this may be related to the negative feedback mechanism of enzymes.

3.4.4. Effect of inoculum

Using the optimal pH and carbon and nitrogen sources, the initial inoculum concentration was adjusted to 1%, 2%, 3%, 4%, 5%, 6%, 7%, and 8%. The CMCase activity was then measured under the optimal conditions mentioned above. As shown in Fig. 8, the maximum CMCase activity (3.43 U/mL) was observed when the inoculum concentration was 4%, but it decreased with increasing concentration, possibly because as the inoculum concentration increased, the bacteria grew to the log phase increasingly rapidly. Thus, the inoculum concentration was found to be an important factor for cellulase production. In the present research, 4% inoculum was used in subsequent experiments.

3.4.5. Effect of fermentation time on cellulase production

The CMCase activity was measured at various fermentation times (optimal carbon and nitrogen sources were used; pH, 5.5; temperature, 37°C; rotation speed, 220 rpm; inoculum concentration, 4%). The results showed considerable variation in cellulase production at different times. The strain grew vigorously and the cellulase yield was high when the fermentation time was 6–20 h. Good CMCase activity (3.43 U/mL) was observed after 24 h cultivation, but it decreased continuously during the time from 24 h to 36 h. When the fermentation time was 48 h, the CMCase activity reached the maximum (3.56 U/mL) and then it decreased sharply (Fig. 9). Moreover, the enzyme production ability of BY-2 was found to be consistent with its predicted growth characteristics, indicating a close relationship between these two factors [30]. The fermentation time of BY-2 for the maximum cellulase yield is shorter than that of the *Bacillus* strains isolated by Rastogi et al. [29] and Sadhu et al. [27] and is comparable to that of *Bacillus* species isolated by Acharya and Chaudhary [28]. In the present study, although the cellulase yield of BY-2 was high after 24 h of fermentation, the maximum CMCase activity (3.56 U/mL) was unexpectedly observed at 48 h. This may be related to the high sugar content of the corn powder used as the carbon source. When bacteria grow to the stable phase, spores are formed and most of the nutritional ingredients in the medium are consumed. Although spores are dormant, they can be activated by many normal metabolites such as alanine, adenosine, glucose, and lactic acid, and they start to grow, divide, and multiply under certain nutritional conditions [31]. We found that BY-2 grew to the stable phase after 20 h of cultivation and then began to form spores; the spores were then activated by the high sugar content of the corn powder, which effectively extended the growth cycle of the microbe. When the fermentation time reached 40 h, the new spores began to produce cellulases and the enzyme yield reached the maximum after 48 h of fermentation. Thereafter, however, cellulase production decreased because of the excessive consumption of nutritional ingredients in the medium.

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Authors' contributions

Proposed the theoretical frame: BC; Conceived and designed the experiments: WY; Wrote the paper: JP, PH, FF; Performed the experiments: FM; LM.

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