Electronic Journal of Biotechnology 48 (2020) 29–35

Contents lists available at ScienceDirect

Electronic Journal of Biotechnology

Research article

Screening of cellulose degradation bacteria from Min pigs and optimization of its cellulase production

Feng Li [a,*], Yingjie Xie [a], Xiang Gao [a], Mingxu Shan [a], Changchao Sun [a], Yan Dong Niu [b], Anshan Shan [a,*]

[a] Institute of Animal Nutrition, Northeast Agricultural University, Harbin 150030, PR China
[b] Department of Ecosystem and Public Health, Faculty of Veterinary Medicine, University of Calgary, Calgary, AB T2N 1N4, Canada

ARTICLE INFO

Article history:
Received 22 March 2020
Accepted 2 September 2020
Available online 7 September 2020

Keywords:
Animal production
Cellulase probiotics
Cellulase producing bacteria
Cellulolytic bacteria
Cellulose
Dietary fiber
Feed resource
High-fiber food
Intestinal tract
Isolates
Min pigs
Optimization

ABSTRACT

Background: Cellulose as a potential feed resource hinders its utilization because of its complex structure, and cellulase is the key to its biological effective utilization. Animal endogenous probiotics are more susceptible to colonization in the intestinal tract, and their digestive enzymes are more conducive to the digestion and absorption of feed in young animals. Min pigs are potential sources of cellulase probiotics because of the high proportion of dietary fiber in their feed. In this study, the cellulolytic bacteria in the feces of Min pigs were isolated and screened. The characteristics of enzymes and cellulase production were studied, which provided a theoretical basis for the rational utilization of cellulase and high-fiber food in animal production.

Results: In our study, 10 strains of cellulase producing strains were isolated from Min pig manure, among which the M2 strain had the best enzyme producing ability and was identified as *Bacillus velezensis*. The optimum production conditions of cellulase from strain M2 were: 2% inoculum, the temperature of 35°C, the pH of 5.0, and the liquid loading volume of 50 mL. The optimum temperature, pH and time for the reaction of cellulase produced by strain M2 were 55°C, 4.5 and 5 min, respectively.

Conclusions: Min pigs can be used as a source of cellulase producing strains. The M2 strain isolated from feces was identified as *Bacillus velezensis*. The cellulase from M2 strain had a good activity and the potential to be used as feed additive for piglets.

How to cite: Li F, Xie Y, Gao X, et al. Screening of cellulose degradation bacteria from Min Pigs and optimization of its cellulase production. Electron J Biotechnol 2020;48. https://doi.org/10.1016/j.ejbt.2020.09.001

1. Introduction

Cellulose is the most abundant organic matter on the earth and an inexhaustible renewable resource. However, cellulose has not been effectively utilized because of its complex chemical structure [1]. Most of the fibers of agricultural and sideline products were burned, resulting in the waste of valuable agricultural resources and serious environmental pollution. With the rapid development of economy, the shortage of resources will become the resistance of sustainable economic development. Therefore, the development and utilization of cellulose resources is of great significance to solve the shortage of resources. The degradation of cellulose by cellulase is the key to its biological utilization [2]. Adding cellulase to animal feed can significantly reduce the viscosity of chyme, eliminate some of the anti-nutritional factor and improve the nutrient digestibility [3]. Dairy and beef cattle performance is significantly improved, when diets are supplemented with commercial xylanases and cellulases [4]. Cellulase can promote the production of propionic acid, which can reduce the colonization of pathogenic bacteria and play a positive role on the cecum fermentation process [5]. In monogastric animals, due to the lack of cellulase in the gastrointestinal tract, the reasonable addition of cellulose and cellulase to the diet can reduce the cost of animal diet, increase the feeling of satiety, improve intestinal health, reduce the occurrence of constipation and improve the performance of animals [6].

At present, feed enzyme preparation is mainly produced by fungi, but cellulase producing bacteria have attracted attention because of their strong adaptability and other advantages [7]. Probiotic bacteria are living microorganisms that can reproduce in the intestinal tract and are beneficial to the health of the host [8]. Probiotics were able to promote feed digestion, growth, immune response to infectious intestinal disease in livestock by providing nutrients and enzymes, and inhibit pathogenic microorganisms in the gut [9,10]. *Bacillus* is one of the probiotics that has a great beneficial effect on human and most kinds of farmed animals [11]. The ability of forming spore benefit stability of *Bacillus* makes it more likely to survive in lower gastric pH and other harsh environments than other non-spore

* Corresponding authors.
E-mail addresses: lifeng@neau.edu.cn (F. Li), asshan@neau.edu.cn (A. Shan).
Peer review under responsibility of Pontificia Universidad Católica de Valparaíso.

https://doi.org/10.1016/j.ejbt.2020.09.001

bacteria. The search for *Bacillus*, which can produce cellulase [12], has its practical significance.

More and more attentions have been paid to livestock endogenous microorganisms. In the long-term evolution of natural selection, the beneficial microorganisms formed a good symbiotic relationship with the host. These probiotics are easier to colonize in the digestive tract of animals, and the enzymes from them are more conducive to the digestion, absorption and utilization of animal feed, especially important for young animals with imperfect digestive systems. Min pig, a local pig breed in China, is a kind of long-term extensive breeding pig breed, in which diet crude fiber can account for a very high proportion. Min pig was one of the sources of cellulase producing bacteria due to its strong adaptability to coarse fodder [13]. In this study, the cellulolytic bacteria in the feces of Min pigs were isolated and screened. The characteristics of enzymes and cellulase production conditions were studied, which provided a theoretical basis for the rational utilization of cellulase and high-fiber food in animal production. The aim of this study was to find a superior cellulase-producing bacteria strain that may be used as feed additive for weaned piglets.

2. Materials and methods

2.1. Sample collection

The fresh manure samples were collected into a 50-milliliter aseptic screw pipe separately from six multiparous Min sows (151.2 ± 4.5 kg) at the Lanxi Pig Farm, Suihua City, Heilongjiang Province, China. The samples were immediately placed in the ice box and transported the laboratory within 3 h.

2.2. Preliminary isolation and screening of cellulases producing bacteria

1 g each manure sample from each sow was diluted with 100 mL sterilized water and then oscillated at 80°C for 30 min in a Water Bath Pot (DK-8D, Shanghai Bluepard Instruments Co., Ltd). Serial dilutions from 10^{-3} g/mL to 10^{-6} g/mL were prepared using sterilized water. An aliquot of 50 μL of each dilution was inoculated on primary screening medium agar plates containing (g/L) carboxymethylcellulose sodium (CMC-Na; 10.0), NaCl (10.0), tryptone (10.0), yeast powder (5.0) and agar powder (10.0). The plates were incubated under aerobic conditions at 37°C for 24 h. The Congo-red overlay method [14] was used to have a qualitative screening of cellulase production bacteria. In this method, plates with strains were flooded with 0.1% Congo-red for more than 30 min and then de-stained with 1 mol/L NaCl solution until the clear zones around the colonies were visualized. The ratio of clear zone diameter to colony bacterial diameter was the standard of primary screening. The selected bacterial colonies, of which the ratio was large, were further purified by streaking onto new primary screening medium agar plates for four to five generations. The purified strains were stored at -80°C with glycerol.

2.3. Preparation of stock solution and extraction of crude enzyme solution

The initially cellulase producing bacteria were activated and cultured in Luria-Bertani (LB) solid medium containing (g/L) NaCl (10.0), tryptone (10.0), yeast powder (5.0) and agar powder (10.0). The activated single colony was inoculated in LB liquid medium containing (g/L) NaCl (10.0), tryptone (10.0) and yeast powder (5.0). The medium cultured in Water Bath Pot at 220 rpm and 37°C for 12 h. After that, they were cultured twice in LB liquid medium with 1.0% inoculum. The stock solution was prepared as the optical density (OD) of the bacterial solution was adjusted to 1.0 at 600 nm ($OD_{600} = 1.0$) using LB liquid medium. Stock solution (0.5 mL) was inoculated in 50 mL LB-CMC liquid medium containing (g/L) CMC-Na (10.0), NaCl (10.0), tryptone (10.0) and yeast powder (5.0). The medium was cultured at 220 rpm and 37°C for 24 h. The crude enzyme solution was obtained from the superstratum of the LB-CMC liquid medium centrifuged at 3000 × *g* and 4°C for 15 min.

2.4. Re-screening of cellulase producing bacteria

Dinitrosalicylic acid (DNS) colorimetric method [15] was used for re-screening to determinate the cellulase activity of crude enzyme solution. 1 mL crude enzyme solution was dissolved in 1.5 mL in citric acid buffer (1% CMC-Na) and incubated at 50°C for 30 min. After that, 2.5 mL DNS reagent [15] was quickly added in the citric acid buffer. The mixture was boiled for 5 min, cooled in water bath to room temperature, adjusted to volume of 10 mL with deionized water and shaken well. The OD_{540} of mixture liquid was determined. According to the research of Lowry [16], the standard curves of different concentrations of glucose were drawn. One unit (U) of enzyme activity was defined as the amount of enzyme required for substrate to produce 1 μg of glucose per minute. Strain showing highest cellulase activity was used in subsequent experiments. The carboxymethyl cellulase (CMCase) activity was calculated by the following formula:

$$\text{CMCase activity (U/mL)} = \frac{\text{Glucose production(mg)} \times 1000}{\text{Enzyme dosage(mL)} \times \text{time(min)}}$$

2.5. Identification of the bacterial species

2.5.1. Biochemical characterization

Bacterial isolate was identified using Common bacterial system identification manual [17] Gram staining was done for morphological identification of bacteria, whereas biochemical identification was done by using a series of tests such as heat resistance, catalase, oxidase, indole, methyl red (MR), starch hydrolysis, Voges-Proskauer (VP) glucose fermentation, mannitol fermentation and sugars alcohol (glucose, maltose, fructose, galactose, lactose, sucrose, mannitol, glycerol and ethanol) utilization tests by standard methods [17].

2.5.2. Molecular identification by 16S rRNA gene sequencing

Total DNA was extracted using the Gel Extraction Kit D2500 (Omega, China), following the instructions from the manufacturer. The 16S rRNA gene was amplified by polymerase chain reaction (PCR) from the genomic DNA of the strain using universal primers, 7F(5′-CAGAGTTTG ATCCTGGCT-3′) and 1540R(5′-AGGAGGTGTCCAGCCGCA-3′) [18]. The PCR conditions were as follows: initial denaturation at 94°C for 3 min, followed by 30 cycles with denaturation at 94°C for 1 min each, annealing at 55°C for 1 min, extension at 72°C for 2 min, and a final elongation at 72°C for 10 min. Amplified product was checked for size and purity on 1% (w/v) agarose gel.

PCR products were sequenced through commercial services provided by Sangon Biotech Co., Ltd. (Shanghai, China). The sequence was then compared with Ribosomal database (http://rdp.cme.msu.edu/index.jsp) and used the Basic Local Alignment Search Tool (BLAST) program of NCBI for homology analysis. A phylogenetic tree was constructed using the neighbor-joining model of the MEGA 7.0 program [19].

2.6. Growth curve drawing

Stock solution of screened bacterial strain (0.02 mL) was inoculated in 2 mL LB liquid medium, and the OD_{600} was measured by a microplate reader (M200pro, Tecan, China) after incubation at 37°C for 8 h, and then measured every 2 h until to 48 h. The growth curve of the strain was plotted by measuring the average value obtained from three repeated samples.

2.7. Optimization of cellulase production

The 250-mL Erlenmeyer flask was used in all microbial cultures. Different initial pH values (4.0, 4.5, 5.0, 5.5, 6.0, 6.5 or 7.0) of LB-CMC

liquid media were selected to optimize the pH value for the high yield of cellulase production. The stock solution was inoculated with 1% inoculum in 50 mL LB-CMC liquid medium oscillatory culture on a rotary shaker at 220 rpm and 37°C for 24 h. In order to obtain the optimum temperature for enzyme production, stock solution was inoculated in 50 mL LB-CMC liquid medium (initial pH = 7.0) at 1% inoculum size and incubated on a rotary shaker at 220 rpm and the different temperatures (33, 35, 37, 39 or 41°C) for 24 h. To determine the optimal inoculum size, the following scheme was used: at the optimum temperature, stock solution was inoculated with different inoculations (1%, 2%, 3%, 4% or 5%) in 50 mL LB-CMC liquid medium (initial pH = 7.0) on a rotary shaker at 220 rpm for 24 h. Under the optimum temperature, pH and inoculum size, the LB-CMC liquid medium with different volume (30, 50, 70, 90 or 110 mL) was cultured on a rotary shaker at 220 rpm for 24 h. The CMCase activities of all samples were measured in order to find the optimal conditions. Three repeated samples were used in each experiment and the average values were plotted for each experiment.

2.8. Analysis of the characteristics of crude enzyme

Optimum temperature for the CMCase reaction was determined by the following steps: 1 mL crude enzyme solution was mixed in 1.5 mL 1.0% CMC-Na substrate solution, and then took a water bath for 30 min at 40, 45, 50, 60, 65 or 70°C. Optimum pH for the CMCase reaction was determined by the following steps: CMC-Na was dissolved at 1% (w/v) in different buffers with different pH value (4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0). 1 mL crude enzyme solution was mixed in 1.5 mL 1.0% CMC-Na substrate solution, and then took a water bath at 50°C for 30 min. The optimum time of CMCase reaction was obtained by the following steps: 1 mL crude enzyme solution was mixed in 1.5 mL 1.0% CMC-Na substrate solution, and then took a water bath at 50°C for 5, 10, 15, 20, 25, 30, 35 or 40 min. The CMCase activities were determined by DNS colorimetric method. Three repeated samples were used in each experiment and the average values were plotted for each experiment.

2.9. Statistical analysis

One-way analysis of variance (ANOVA) was done using Statistical Package for the Social Sciences (SPSS 22.0) [20] for the determination of significant differences within different indexes. Tukey test was applied. Three replicates were determined for each index. A significant difference was found when $p < 0.05$.

3. Results

3.1. Isolation and screening of cellulolytic bacteria

Isolation and screening of fiber degrading microbes is of immense importance due to their huge demand in industrial applications. In the present study, ten isolates from Min pigs' manure were initially identified to produce fiber degrading enzymes on CMC agar plates as showed in Fig. 1. Cellulose-solvent zones around colonies are formed in cellulose agar plate after incubating 24 h. The cellulase activities of all screened strains were showed in Table 1. The isolate M2 with high cellulase production was selected for further characterization.

3.2. Physiological and biochemical characterization

The physiological and biochemical characterization of strain M2 has been shown in Table 2.

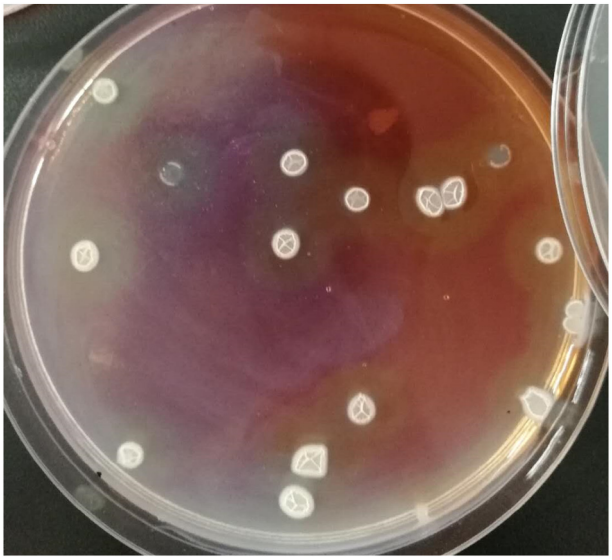

Fig. 1. Cleaning zone of strain M2 on CMC-Na selective medium.

Table 1
The size of transparent circle of the strain and activity of the CMC enzyme.

Isolate's ID	D1 (mm)	D2 (mm)	D1/D2	Cellulase activity (U/mL)
M1	8.26	4.80	1.72	25.96
M2	8.38	3.59	2.33	33.03
M3	8.45	5.80	1.46	25.87
M4	8.74	4.56	1.92	24.70
M5	9.40	4.57	2.06	26.10
M6	8.79	5.83	1.51	25.65
M7	8.54	4.60	1.86	24.06
M8	7.70	5.33	1.44	24.26
M9	8.52	5.34	1.60	24.89
M10	7.81	4.43	1.76	26.16

Note: D1 = the cellulose-solvent zone diameter; D2 = the colony bacterial diameter.

3.3. Phylogenetic analysis

The agarose gel electrophoresis result of the PCR amplification product of strain M2 was shown in Fig. 2. The DNA fragments amplified by PCR were all single bands, and the length of the fragments was about 1500 bp. The PCR products of strain M2 were sequenced to obtain the full-length 1492 bp gene sequence, which was consistent with the result of electrophoresis. The 16S rRNA gene sequence was compared with Ribosomal database (http://rdp.cme.msu.edu/index.jsp), and the homology between M2 strain and *Bacillus velezensis* was 100%. The phylogenetic tree was constructed by

Table 2
Physiological and biochemical characteristics of strain M2.

Characteristic features	Results	Characteristic features	Results
Gram's staining	+	Morphology	B
Glucose utilization	+	Maltose utilization	+
Fructose utilization	+	Galactose utilization	+
Lactose utilization	+	Glycerol utilization	+
Sucrose utilization	+	Ethanol utilization	-
Mannose utilization	+	Mannitol utilization	+
Oxidase	+	Catalase	+
Voges-Proskauer	-	Methyl red	-
Starch hydrolysis	+	Indole	-
Glucose fermentation	+	Mannitol fermentation	+
Growth at 70°C	+	Growth at 80°C	+

Note: B = bacilli; "+" stands for positive and "-" for negative.

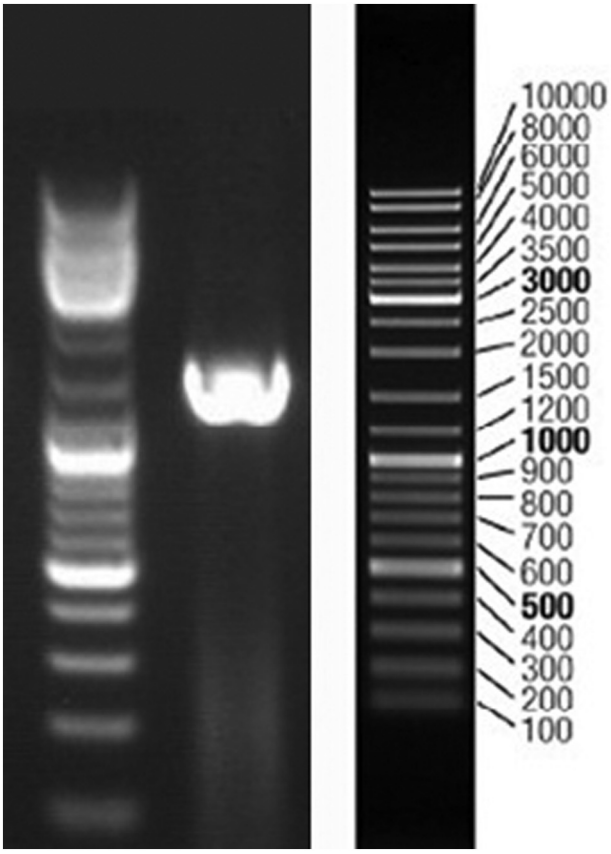

Fig. 2. Electrophoresis of PCR Products of Strain M2.

MEGA7.0 cluster analysis, and the strain was classified as *Bacillus velezensis* as shown in Fig. 3. The strain was preserved at China Center for Type Culture Collection (CCTCC) in Wuhan under no. CCTCC M 2018841.

3.4. Drawing of strain growth curve

The growth curve of strain M2 at 37°C and a pH of 7.0 was shown in Fig. 4. Strain M2 reached the logarithmic phase at 8 to16 h after inoculation, and its growth reached a plateau after 20 h.

3.5. Optimization of cellulase production conditions of strain M2

There were significant efferents ($p < 0.05$) of initial pH, temperature, inoculation concentration and liquid loading amount on cellulase production (Fig. 5). The enzyme production curve of strain M2 with initial pH value was shown in Fig. 5a. The maximum CMCase activity (20.20 ± 0.74 U/mL) was observed at an initial medium pH of 5.0. Further, the CMCase activity was stable (>80% of the maximal level) over a broad range of pH values (from 4.5 to 7.0). The enzyme production curve of strain M2 with temperature was shown in Fig. 5b. The CMCase activity reached the maximum (22.03 ± 0.20 U/mL) at the temperature of 35°C. The CMCase activities at different inoculation concentrations at this optimum temperature were shown in Fig. 5c. The maximum CMCase activity (33.83 ± 0.37 U/mL) was observed when the inoculum concentration was 2%. And then, the activity decreased gradually with the increase of vaccination concentration. Based on the optimum initial pH, temperature and inoculum size, the enzymatic curve of strain M2 varying with liquid (LB-CMC medium) loading was determined and shown in Fig. 5d. The CMCase activity reached the maximum (41.18 ± 1.61 U/mL) at 50 mL.

3.6. Enzymatic properties of CMCase from strain M2

Temperature, pH and time significantly affected ($p < 0.05$) cellulosic enzymatic reaction (Fig. 6). The variation curve of the activity of cellulase from strain M2 at different temperature was shown in Fig. 6a. The optimum temperature of CMCase reaction was 55°C. When the temperature increased to 70°C, the CMCase activity was still more than 80% of the maximum, indicating that the cellulase produced by strain M2 had good thermal stability. The variation curve of cellulase activity at different pH was shown in Fig. 6b. The optimum pH for CMCase reaction was 4.5. And the CMCase activity was still more than 80% of the maximum while pH was from 4.0 to 7.0, indicating that the CMCase had a certain degree of pH stability. The change curve of CMCase activity for different reaction time was shown in Fig. 6c. The CMCase activity was the highest at 5 min, and then decreased gradually with the prolongation of reaction time.

4. Discussion

In this study, cellulose degrading bacteria from Min pigs were isolated and cellulase production of one strain was optimized. At present, protease and cellulase account for a large proportion of industrial enzymes. However, the production of known protease and cellulase series are not enough to meet the needs of industry [21]. Microbial fermentation is still considered to be the most important way to produce enzyme in industry. So far, cellulose degradation bacteria had been isolated by many scholars from animal gastrointestinal tract and feces. Sari et al. [22] isolated a new species of *Enterobacteriaceae* from bovine rumen fluid. Sadhu [23] and Hussain et al. [24] isolated cellulase producing *Bacillus subtilis* from cow dung and soil, respectively. Yang et al. [25] isolated and identified a cellulose decomposing bacterium as *Bacillus subtilis* from Tibetan pig

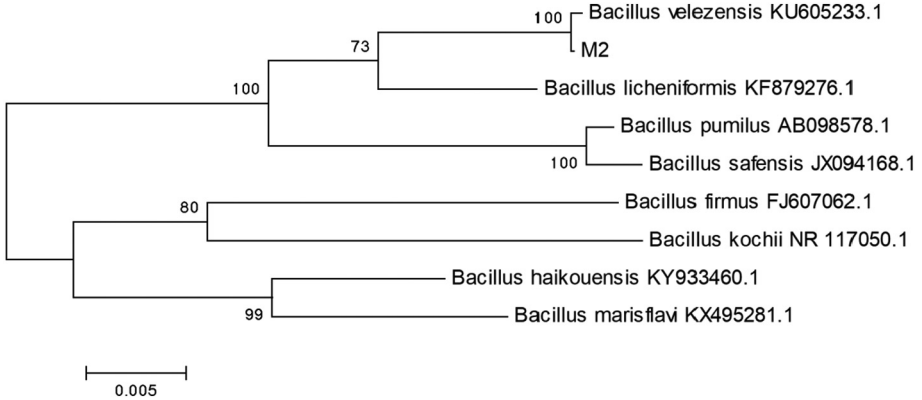

Fig. 3. Phylogenetic tree for the strain M2 and related bacterial strains.

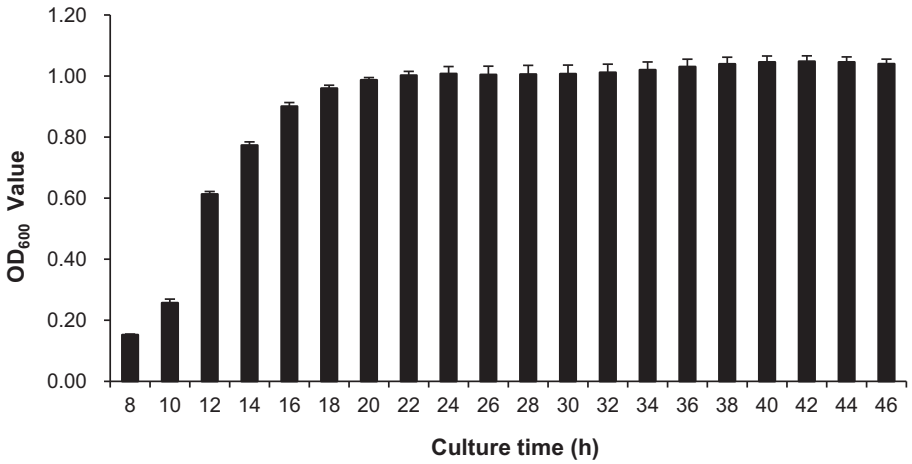

Fig. 4. The growth curve of strain M2.

intestines and its cellulase production was studied. The results of this experiment proved that it was feasible to isolate cellulase producing bacteria from the gastrointestinal tract system of Min pigs. There is a certain relationship between the characteristics of coarse grain feeding and the cellulose-degrading bacteria in the intestinal tract.

In this study, CMC-Na was used as the sole carbon source selective medium, combined with Congo red staining method for preliminary isolation of cellulolytic bacterium. Congo-red and cellulose interact in agar medium. When the bacteria secreted cellulase, the cellulose was degraded into cellulose disaccharide, glucose and organic acid. The pH

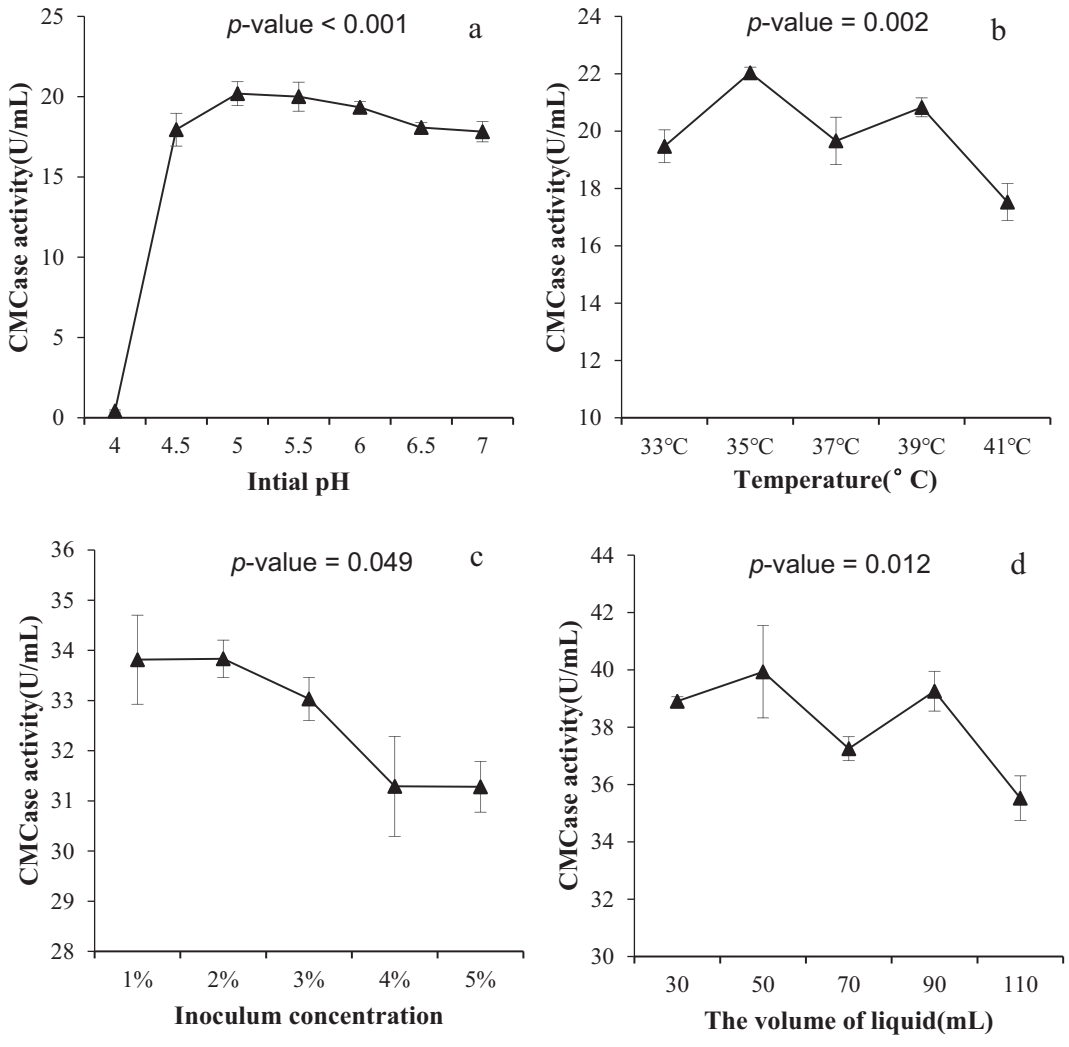

Fig. 5. Effects of different conditions on cellulase production of strain M2.

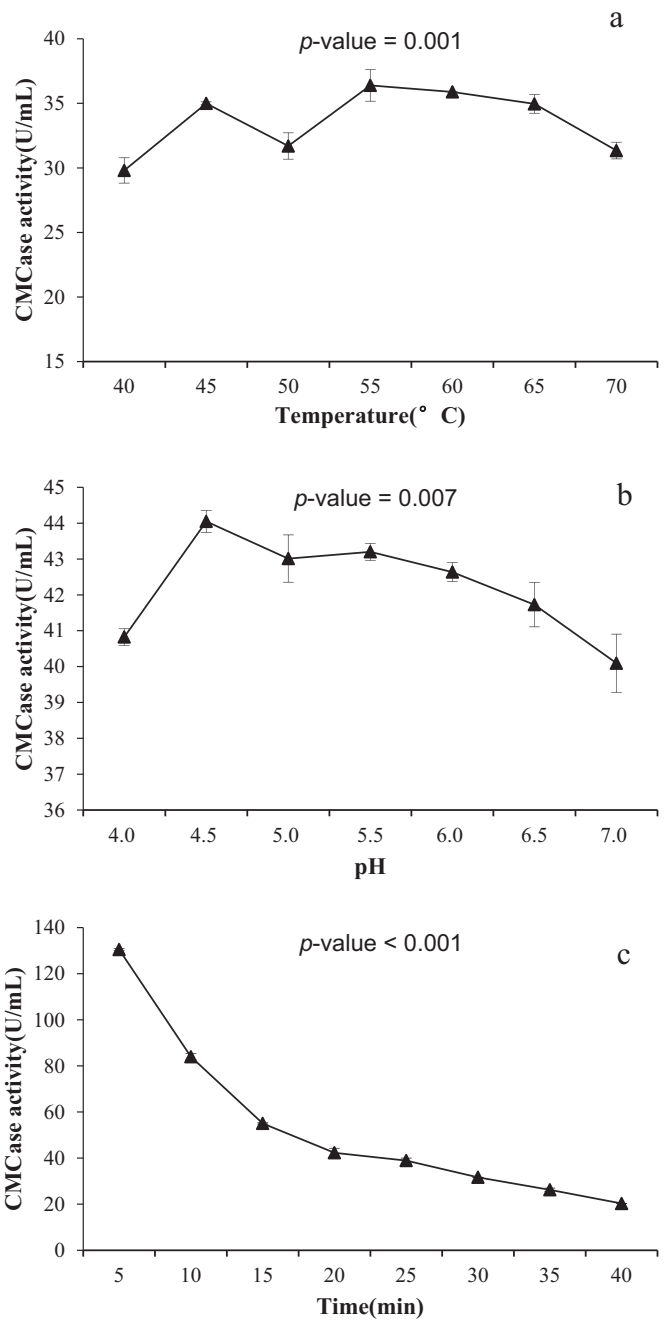

Fig. 6. Effects of different conditions on CMCase activity.

of medium around the colony was reduced, which caused the formation of transparent circle [26]. Considering the excellent tolerance of spore probiotics and their potential probiotic value in livestock, only spore bacteria in Min pig feces were considered in this study. In order to improve the screening efficiency, the spores-free bacteria and some miscellaneous bacteria were killed by a water bath at 80°C in the process of dilution. Through primary screening, 10 isolates were found positive for fiber degrading potential and the M2 strain had the excellence of potential. The primary screen strain was re-screened by the DNS method because the scarlet staining was not quantitative. In this method, free carboxyl groups were released by the oxidation of aldehyde groups of the glucose molecule, which was formed by the action of fiber degrading enzymes. These reduced 3,5-DNS (yellow) to 3-amino-5-nitro salicylic acid (orange) under alkaline conditions [15]. This method is also used in other experiments in this study to determine the activity of the cellulase. The result of enzyme activity in this research also showed that the M2 strain had the greatest enzyme activity among ten isolates.

Results of physiological and biochemical identification and 16S rRNA gene analysis showed that the homology between M2 strain and *Bacillus velezensis* was 100%. The phylogenetic tree was constructed to identify the strain as *Bacillus velezensis*. *Bacillus velezensis* was first isolated from the Estuary of Valle in Malaga, southern Spain [27]. Wang et al. [28] thought that *Bacillus velezensis* was a variety of *Bacillus starch*. *Bacillus velezensis* FZB42 could produce secondary metabolites with antibacterial activity, including lipopeptide (surface protein, fengomycin and Bactericin D), polyketones (macroemulsion, bacillactone and mucin) and peptides (plant azodane, starch cycline and bacillus protease) [29]. This study showed that the *Bacillus velezensis* can produce highly active cellulase, which is consistent with Peixoto et al. [30]. A strain of *Bacillus velezensis* was isolated from the feces of piglets by Ye et al. [31]. Continuous efforts to find new high-efficiency microbial isolates with fiber degradation potential are necessary. In this experiment, the isolate M2 can survive up to 80°C which highlights its industrial importance.

According to the growth, characteristics of the strain (Fig. 4), the M2 is at the growth stage for a long period of time after 20 h. Since the bacterial enzyme is most active in the logarithmic phase, we conducted a cellulase production cultivation for 24 h in a follow-up experiment in order to examine enzyme characteristics. The production of fiber degrading enzymes generally depends on a variety of growth parameters which include inoculum size, pH, temperature, medium additives (carbon and nitrogen sources), aeration, growth and time, and on the presence of various metal ions as activators and inhibitors [26]. Through the determination of CMCase activity of crude enzyme solution in different conditions, it can be judged that M2 strain has the ability to produce enzyme in a wide range of pH values (from 4.5 to 7.0), which suggests the potential application of the strain under different pH conditions. The enzyme production of the microorganism is closely related to the culture temperature. The optimum temperature of M2 enzyme production was 35°C. The results also showed that the optimum inoculum size of enzyme production was 2%. When the inoculation quantity was more than the optimum value, the cellulase production overall showed a downward trend. The excessive inoculation makes the bacterial density too large, resulting in insufficient nutrients and dissolved oxygen in the medium, which ultimately limits the growth of bacteria and reduces the production capacity of enzymes [32]. In this study, 2% inoculum size was used in the subsequent experiments. The suitable amount of liquid loading provided the appropriate amount of oxygen and nutrients [33]. In the space of 250-mL Erlenmeyer flask, the optimum volume of liquid in this study was 50 mL. At this point, the dissolved oxygen and nutrients in the medium reach an optimum value, which makes M2 strain able to produce enzymes more efficiently.

At this study, enzymatic reactions of CMCase were studied by crude enzyme solution. The results showed that the crude cellulase produced by the strain M2 had a good temperature and pH range, enabling it to meet requirement of commercial cellulases. Crude cellulase from *Bacillus subtilis* strain BY-3 has a board of temperature (from 30 to 80°C, above 65% of the maximal level) and pH (from 4.5 to 8.5, above 60% of the maximal level), which has been reported by Meng et al. [33]. The crude CMCase produced by M2 belongs to acidic cellulase, and its optimum pH of enzymatic reaction is 4.5, which can adapt to the acidic environment of gastrointestinal tract. The enzymatic reaction time of CMCase is also an important factor affecting enzyme activity. The enzymatic reaction of cellulase needs the cooperation of enzyme, and the process of its preparation (the diffusion of cellulase, the attachment, the movement of the corresponding active site) takes a certain time [2]. When the enzymatic reaction time of the enzyme was too short, the enzymatic reaction speed was slow because of the uncomplete combination of the cellulase and the cellulose. When the

enzymatic reaction time was too long, the enzyme activity decreased due to the instability of the activity of the cellulase [34]. In this experiment, the CMCase activity was the highest when the reaction lasted 5 min, which indicated that the enzyme bound to substrate quickly.

5. Conclusions

The endogenous cellulose-degrading bacteria of animals are not only easy to colonize in animals, but also can help animals metabolize cellulose to produce small molecules of organic acids to inhibit the invasion of exogenous harmful bacteria. In our study, we screened 10 strains of cellulase producing strains from Min pig manure, among which the M2 strain had the best enzyme producing ability and was identified as *Bacillus velezensis*. The optimum cellulase production conditions of strain M2 were: 2% inoculum, the temperature of 35°C, the pH of 5.0, the liquid loading volume of 50 mL. The optimum temperature, pH and time for the reaction of cellulase produced by strain M2 were 55°C, 4.5 and 5 min, respectively. The role of M2 strain in weaning piglets' diets will be investigated in the future.

Ethical approval

The study protocol was approved by the Ethics Committee of Northeast Agricultural University, with the approval number NEAU-[2011]-9.

Conflict of interest

The authors declare that there is no conflict of interest.

Financial support

This work was supported by the Open Project of Northeastern Science Inspection Station and China Ministry of Agriculture Key Laboratory of Animal Pathogen Biology (DY201708), and the China Agriculture Research System (CARS-35).

Acknowledgments

The authors thank Yi Yang, Xue Yang and Xiaoru Li for their invaluable technical assistance, and also thank all staff of the Lanxi Pig Farm (Suihua City, Heilongjiang Province, China) for their help in sampling process.

References

[1] Zhang JQ, Lin L, Sun Y, et al. Advance of studies on structure and decrystallization of cellulose. Chem Ind Prod. 2008;28(6):109–14. https://doi.org/CNKI:SUN:LCHX.0.2008-06-024.
[2] Wilson DB. Microbial diversity of cellulose hydrolysis. Curr Opin Microbiol. 2011;14(3):259–63. https://doi.org/10.1016/j.mib.2011.04.004 PMid:21531609.
[3] Bhat MK. Cellulases and related enzymes in biotechnology. Biotechnol Adv. 2000;18(5):355–83. https://doi.org/10.1016/S0734-9750(00)00041-0 PMid:21531609.
[4] Rode LM, Mcallister TA, Beauchemin KA, et al. Enzymes as direct-feed additives for ruminants. In: Renaville R, Burny A, editors. Biotechnol Anim Husb. Dordrecht: Springer Netherlands; 2005. p. 301–32. https://doi.org/10.1007/0-306-46887-5_17.
[5] Pazarlioglu NK, Sariişik M, Telefoncu A. Treating denim fabrics with immobilized commercial cellulases. Process Biochem. 2005;40(2):767–71. https://doi.org/10.1016/j.procbio.2004.02.003.
[6] Bimrew A. Effect of common feed enzymes on nutrient utilization of monogastric animals. Int J Biotechnol Mol Biol Res. 2014;5(4):27–34. https://doi.org/10.5897/ijbmbr2014.0191.
[7] Sadhu S, Ghosh PK, De TK, et al. Optimization of cultural condition and synergistic effect of lactose with carboxymethyl cellulose on cellulase production by *Bacillus* sp. isolated from fecal matter of elephant (*Elephas maximus*). Adv Microbiol. 2013;03(03):280–8. https://doi.org/10.4236/aim.2013.33040.
[8] Fuller R. Probiotics in man and animals. J Appl Bacteriol. 1989;66(5):365–78. https://doi.org/10.1111/j.1365-2672.1989.tb05105.x PMid:2666378.
[9] Balcázar JL, de Blas I, Ruiz-Zarzuela I, et al. The role of probiotics in aquaculture. Vet Microbiol. 2006;114(3–4):173–86. https://doi.org/10.1016/j.vetmic.2006.01.009 PMid:16490324.
[10] Rabot S, Rafter J, Rijkers GT, et al. Guidance for substantiating the evidence for beneficial effects of probiotics: Impact of probiotics on digestive system metabolism. J Nutr. 2010;140(3):677S–89S. https://doi.org/10.3945/jn.109.113738 PMid:20107147.
[11] Kewcharoen W, Srisapoome P. Probiotic effects of Bacillus spp. from Pacific white shrimp (*Litopenaeus vannamei*) on water quality and shrimp growth, immune responses, and resistance to *Vibrio parahaemolyticus* (AHPND strains). Fish Shellfish Immunol. 2019;94:175–89. https://doi.org/10.1016/j.fsi.2019.09.013 PMid:31499198.
[12] Rastogi G, Bhalla A, Adhikari A, et al. Characterization of thermostable cellulases produced by Bacillus and Geobacillus strains. Bioresour Technol. 2010;101(22):8798–806. https://doi.org/10.1016/j.biortech.2010.06.001 PMid:20599378.
[13] Liu Y, Yang X, Jing X, et al. Transcriptomics analysis on excellent meat quality traits of skeletal muscles of the Chinese indigenous min pig compared with the large white breed. Int J Mol Sci. 2018;19(1):21. https://doi.org/10.3390/ijms19010021 PMid:29271915.
[14] Ariffin H, Abdullah N, Umi Kalsom MS, et al. Production and characterization of cellulase by *Bacillus pumilus* EB3. Int J Eng Technol. 2006;3(1):47–53. https://doi.org/10.1263/jbb.106.231 PMid:18929997.
[15] Miller GL. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem. 1959;31(3):426–8. https://doi.org/10.1021/ac60147a030.
[16] Lowry OH. How to succeed in research without being a genius. Annu Rev Biochem. 1990;59(1):1–28. https://doi.org/10.1146/annurev.bi.59.070190.000245 PMid:2197974.
[17] Dong XZ, Cai MY. Manual for identification of common bacterial systems. 1st ed.. Bei Jing: Science Press; 2001.
[18] Zhang ZF, Nan ZB. First report of erwinia persicinus causing wilting of *Medicago sativa* sprouts in China. Plant Dis. 2012;96(3):454. https://doi.org/10.1094/pdis-10-11-0909 PMid:30727103.
[19] Kumar S, Stecher G, Tamura K. MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol Biol Evol. 2016;33(7):1870–4. https://doi.org/10.1093/molbev/msw054 PMid:27004904.
[20] Allen P, Bennett K, Heritage B. SPSS statistics version 22: A practical guide. Cengage Learning Australia; 2014.
[21] Percival Zhang YH, Himmel ME, Mielenz JR. Outlook for cellulase improvement: Screening and selection strategies. Biotechnol Adv. 2006;24(5):452–81. https://doi.org/10.1016/j.biotechadv.2006.03.003 PMid:16690241.
[22] Sari WN, Safika, Darmawi, et al. Isolation and identification of a cellulolytic enterobacter from rumen of aceh cattle. Vet World. 2017;10(12):1515–20. https://doi.org/10.14202/vetworld.2017.1515-1520 PMid:29391695.
[23] Sadhu S. Cellulase production by bacteria: A review. Br Microbiol Res J. 2013;3(3):235–58. https://doi.org/10.9734/bmrj/2013/2367.
[24] Hussain AA, Abdel-Salam MS, Abo-Ghalia HH, et al. Optimization and molecular identification of novel cellulose degrading bacteria isolated from Egyptian environment. J Genet Eng Biotechnol. 2017;15(1):77–85. https://doi.org/10.1016/j.jgeb.2017.02.007 PMid:30647644.
[25] Yang W, Meng F, Peng J, et al. Isolation and identification of a cellulolytic bacterium from the tibetan pig's intestine and investigation of its cellulase production. Electron J Biotechnol. 2014;17(6):262–7. https://doi.org/10.1016/j.ejbt.2014.08.002.
[26] Raza A, Bashir S, Tabassum R. Evaluation of cellulases and xylanases production from Bacillus spp. isolated from buffalo digestive system. Kafkas Univ Vet Fak Derg. 2019;25(1):39–46. https://doi.org/10.9775/kvfd.2018.20280.
[27] Ruiz-García C, Béjar V, Martínez-Checa F, et al. *Bacillus velezensis* sp. nov., a surfactant-producing bacterium isolated from the river Vélez in Málaga, southern Spain. Int J Syst Evol Microbiol. 2005;55(1):191–5. https://doi.org/10.1099/ijs.0.63310-0 PMid:15653875.
[28] Wang LT, Lee FL, Tai CJ, et al. *Bacillus velezensis* is a later heterotypic synonym of *Bacillus amyloliquefaciens*. Int J Syst Evol Microbiol. 2008;58(3):671–5. https://doi.org/10.1099/ijs.0.65191-0 PMid: 18319476.
[29] Chen XH, Koumoutsi A, Scholz R, et al. More than anticipated — Production of antibiotics and other secondary metabolites by *Bacillus amyloliquefaciens* FZB42. J Mol Microbiol Biotechnol. 2008;16(1–2):14–24. https://doi.org/10.1159/000142891 PMid:18957859.
[30] Peixoto SB, Cladera-Olivera F, Daroit DJ, et al. Cellulase-producing Bacillus strains isolated from the intestine of Amazon basin fish. Aquacult Res. 2011;42(6):887–91. https://doi.org/10.1111/j.1365-2109.2010.02727.x.
[31] Ye M, Tang X, Yang R, et al. Characteristics and application of a novel species of Bacillus: *Bacillus velezensis*. ACS Chem Biol. 2018;13(3):500–5. https://doi.org/10.1021/acschembio.7b00874 PMid:29309732.
[32] Li ZM, Zhang J, Deng ZY, et al. Selection, identification and optimization of fermented enzyme production conditions for cellulase-producing bacteria. Biotechnol Bull. 2015;31(5):146–52. https://doi.org/10.13560/j.cnki.biotech.bull.1985.2015.05.023.
[33] Meng F, Ma L, Ji S, et al. Isolation and characterization of *Bacillus subtilis* strain BY-3, a thermophilic and efficient cellulase-producing bacterium on untreated plant biomass. Lett Appl Microbiol. 2014;59(3):306–12. https://doi.org/10.1111/lam.12276 PMid:24773580.
[34] Sun Y, Tian Y, Zhao L. Determination of CMC enzyme in cellulase. Food Ind Technol. 2013;34(2):68–72. https://doi.org/10.13386/j.issn1002-0306.2013.02.036.