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IDENTIFICATION AND GROWTH CHARACTERISTICS OF CELLULASE PRODUCING BACTERIA

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Abstract

This study was envisaged to isolate and identify the highly efficient cellulose degrading strains from fresh cow dung and study their biological characteristics. Specifically, the Congo red staining method was used to isolate the bacteria, and screening was done by measuring their endoglucanase activity (CMC enzyme activity), exoglucanase activity (Cex enzyme activity), and filter paper enzyme activity (FPA enzyme activity). Subsequently, the molecular, morphological and growth characteristic were studied. It was observed that 3 strains showed clear transparent circles on the Congo red cellulose medium. LY3 exhibited strong enzyme production ability, with relatively higher enzyme activity than the other two strains. LY3 was found to be a dominant strain that required a temperature of 40 and pH 7.0 for optimal growth. Based on the molecular and morphological characteristics, the three strains were preliminarily identified as *Bacillus*.

Introduction

China has abundant renewable cellulose resources like crop straw and sugarcane (Zhou and Zhuang 2023). Developing and utilizing these cellulose resources can help to solve the current resource shortage problems. Cellulose degrading enzymes are a group of enzymes that degrade cellulose to produce glucose.It is a synergistic multi-component enzyme system that is mainly composed of endoglucanase, exoglucanase, and β-glucosidase, which work together to degrade cellulose into small molecules such as oligosaccharide chains, polysaccharide chains, or glucose. Cellulase can be used to degrade natural cellulose materials, saccharify cellulose like substances, and produce chemical raw materials or food such as fiber ethanol (Gu *et al.* 2016). Currently, the cellulase enzyme has been found an important application in the chemical, food fermentation, industrial washing, and pharmaceutical industries (Tengerdy *et al*. 1991, Pauly *and Keegstra* 2008, Chen *et al.* 2011). Cellulase has a wide range of sources and has been found in various fungi, actinomycetes, and bacteria (Li *et al.* 2015). The production of cellulase in actinomycetes is low, and there has been limited research in this area. Compared to fungi, bacteria grow rapidly and secrete a variety of enzymes, which are more conducive to the synergistic degradation of cellulose by enzymes (Feng *et al.2009*). At the same time, bacteria are more adaptable to the environment and secrete more stable enzymes (Hossain *et al*. 2021, Maki *et al.* 2009). The proportion of the three components of cellulase secreted by different bacterial strains is different, resulting in a significant variation in the activity of their action. Meanwhile, finding the strains with high enzyme production characteristics and isolating cellulases with high enzyme activity has always been an important research focus.

There are many cellulose degrading bacteria in cow dung that can offer a great development value. According to reports of Liu *et al.* (2011), adding microbial agents to cow dung can improve the degradation rate and quality of compost, thereby approaching China's standards for harmless composting and solving environmental pollution problems. This study screened the cellulase

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producing strains from cow dung, determined their cellulase activity, and carried out the molecular identification and morphological studies, in order to provide a theoretical basis for the development and utilization of the cellulose enzyme.

Materials and Methods

About 30 g of fresh cow manure was transferred to a 50 mL sterilized centrifuge tube and stored in a refrigerator at 4°C for further use. In a triangular flask containing 50 ml of enriched culture medium, 1 g of fresh cow dung was thoroughly mixed and cultured at 37°C at 100 r/min for 24 hrs. The gradient of the culture medium was diluted, and a concentration of 10^{-4}~10^{-7} was selected for application into solid LB medium. The medium was inverted at 37°C until the colonies grow.

The selected single cultured colony was placed in 5 ml Luria Bertani (LB) liquid culture medium at 37°C and cultured at 100 r/min for 24 hrs. The culture medium gradient was diluted (10^{-4} - 10^{-7}), and applied on the Congo red cellulose medium plate. After the colonies grew, they were decolorized with 1 mol/l NaCl for 25 min. The size of the hydrolysis zone and the diameter of the colonies were measured to estimate the hydrolysis ability. Finally, the three high-yield enzyme strains based were selected based on the size of the transparent zone, and named LY1, LY2, and LY3, respectively.

Next, the selected single cultured colonies were placed in 50 mL of the enzyme producing fermentation medium and cultured at 37°C on a rotary shaker with 100 r/min for 3 days. The obtained fermentation broth was centrifuged at 8000 r/min at 4°C for 10 min. The supernatant obtained was the crude enzyme broth, which was used for the determination of enzyme activity.

Standard glucose solution (1 mg/ml) was added to 5 ml volumetric flasks to make the final concentration as 0, 0.1, 0.2, 0.3, 0.4, and 0.5 mg/ml, respectively. Subsequently, 3 ml of 3-5-dinitrosalicylic acid(DNS) reagent was added and diluted to 5 mL with distilled water. The absorbance value was measured at 540 nm and a standard curve was obtained with the slope y = 0.7515 x - 0.1384 and linear correlation coefficient (R^2) of 0.99925. The linearity was good and was used for the cellulase activity determination (Fig. 1).

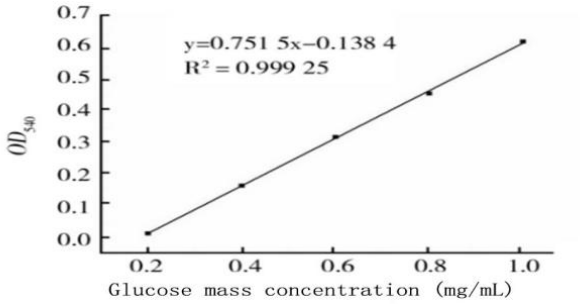

Fig. 1. Glucose standard curve.

The determination of CMC, Cex, and FPA enzyme activity was performed by using the method of Li *et al*. (2016). Determination of endoglucanase (CMC) activity: 0.5 ml of diluted crude enzyme solution was added to a 20 ml test tube (1 control tube, 3 experimental tubes). The control tube was placed in a boiling water bath for 10 min for enzyme inactivation, and 1.5 ml of CMC-Na solution and 1.5 ml of citric acid buffer was added to all the test tubes. The tubes were

subjected to a temperature of 45°C for 30 min and placed in a boiling water bath for 10 min for inactivation. Subsequently, 3 ml DNS reagent was added to it and heated in a boiling water bath for 7 min, immediately cooled to room temperature and the volume was made up to 10 ml. The absorbance of the solution was measured at 540 nm.

Determination of extracellular glucanase (Cex) activity: 0.5 ml of diluted crude enzyme solution was placed in a 20 ml test tube (1 control tube, 3 experimental tubes). The control tube was placed in a boiling water bath for 10 min for enzyme inactivation. Subsequently, 1.5 ml of citric acid buffer was added to the control and experimental tubes, along with 0.1 g of defatted cotton, and the tubes were kept undisturbed at 45°C for 24 h. The color development process and determination steps were consistent with the CMC enzyme activity determination method.

Determination of filter paper enzyme (FPA) activity: 0.5 ml of the diluted crude enzyme solution was placed in a 20 ml test tube (1 control tube, 3 experimental tubes). The control tube was placed in a boiling water bath for 10 minutes for enzyme inactivation. Then, 1.5 ml of citric acid buffer and 0.05 g of starch free filter paper strips were added to all the tubes, and kept at 45°C for 1 h. The color development process and determination steps were consistent with the CMC enzyme activity determination method.

Definition of enzyme activity: The amount of enzyme that can convert 1 micromolar substrate within 1 min under the condition of 45°C was regarded as one enzyme activity unit, expressed in U/ml.

Enzyme activity $E = 1000 \times S \times N/(T \times V)$

where, E is the enzyme activity of the sample (U/ml); S is the glucose content (milligram, mg) corresponding to the average absorbance value of the sample on the standard curve; and N is the dilution ratio of crude enzyme solution; 1000 is the conversion multiple between mg and g; T is the reaction time (min); and V is the volume of crude enzyme solution involved in the reaction (ml).

A single colony was selected and inoculated into the LB liquid culture medium and placed on a rotary shaker at 37°C for 10 h. The bacterial solution was diluted to a concentration of 10^{-4} - 10^{-7}, applied onto the LB solid culture medium and incubated at 37°C for 10 h to observe the colony morphology of the bacteria. Gram staining, flagella staining, and spore staining were conducted in accordance with the "Handbook of Common Bacterial System Identification" compiled by Dong and Cai (2001).

The bacterial DNA was extracted according to the manufacturer's instruction, and the 16S rRNA gene of bacteria was amplified using universal primers F27 and R1542. The F27 sequence was: 5 '- GAGTTTTGATCCTGTC-3', and the R1542 sequence was: 5 '- AGAAAGGGGGGG TGATCCAGC-3'. The sequencing results were compared and analyzed with known 16SrDNA sequences in the NCBI database using the BLAST tool. Cluster analysis was performed using the Neighbor Joining method in MEGA6.0 software, and a phylogenetic tree was constructed (Tamura *et al.* 2013).

The method of Ling *et al.* (2022) was used with slight modifications. The bacterial solution was inoculated overnight with a volume fraction of 0.8% into liquid LB medium on a rotary shaker at 37°C and 100 r/min for cultivation. Samples were taken every 2 hrs, and the absorbance was measured at 600 nm using un-inoculated LB liquid medium as a control. Similar procedure was used to determine the optimum temperature and pH by subjecting the bacterial medium at various temperatures of 15, 20, 24, 28, 32, 36, 40, 44, 50, 55, 60°C, and pH at 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, 10.0, respectively. The un-inoculated LB liquid medium was used as the control. The process was repeated 3 times and the average was considered for analysis.

The experimental data was analyzed using MS Excel 2019 software.

Using the colony morphology studies, distinct colonies were isolated from cow dung on LB agar. They were selected and streaked on the enriched culture medium. Among these, using the Congo red solution, 3 isolates were observed to give a zone of clearance around their colonies and others isolates showed no cellulase activity.

Results and Discussion

It is generally believed that bacteria which can grow in colonies on a cellulose culture media are referred to as cellulose degrading bacteria. All three strains were able to grow on Congo red cellulose sodium screening medium, with clear transparent circles around the colonies (Fig. 2). The transparent circles of LY2 and LY3 bacteria were similar in size, larger than those of LY1 bacteria, indicating that all three strains could decompose cellulose and exhibited the ability to produce cellulase.

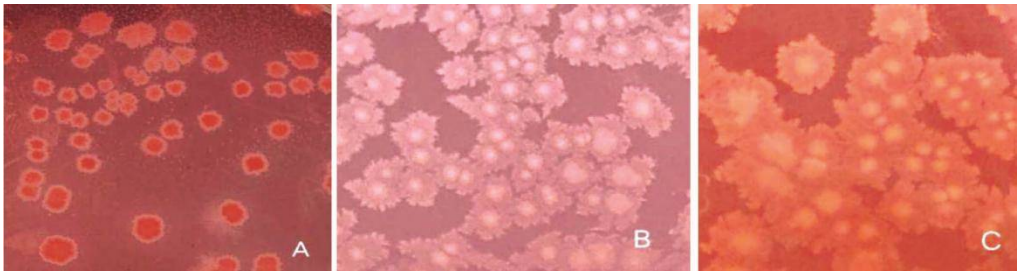

Fig. 2. Growth of bacterial strains on Congo red cellulose medium. A, B, and C represent the strains LY1, LY2, and LY3, respectively.

The CMC, Cex, and FPA enzyme activities of the three strains are showned in Table 1. Table 1 showned that each strain exhibited a certain ability to produce cellulase. Although the strain LY3 did not have the strongest ability to degrade natural filter paper, comprehensive analysis showed that it exhibited the strongest enzyme production ability and a relatively higher enzyme activity than other strains, making it an advantageous strain.

Table 1. Cellulase activity of the three bacterial strains.

Strain	CMC (U/ml)	CEX (U/ml)	FPA (U/ml)
LY1	122.45	4.22	152.73
LY2	208.32	5.92	275.71
LY3	358.28	5.54	278.52

The colony morphology of three strains on LB solid culture medium is shown in Fig 3. The colony formed by strain LY1 on LB agar plate was relatively small, irregular milky white, dry, flat, opaque, and could be picked with an inoculation needle to completely detach from the agar plate. The colony formed by strain LY2 on LB plates was of medium size, circular in shape, moist in texture, with a raised milky white center and a slightly yellow outer ring. The colony formed by

strain LY3 on LB plates was small, circular, moist in texture, and slightly yellow in color. From the Gram staining and microscopic examination, all three bacterial strains were found to be Gram positive, appearing in a straight rod shape, arranged in pairs or chains. Results of flagella staining showed that LY1 and LY3 bacteria exhibited peripheral flagella, while LY2 bacteria presented a single flagella. Spore staining showed that all three strains presented spores that were stained green.

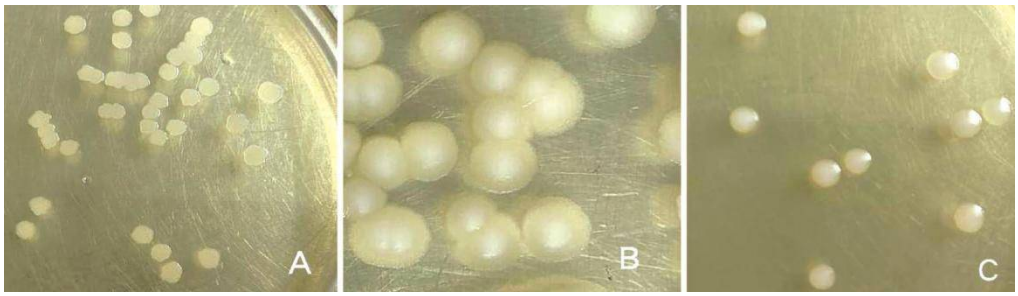

Fig. 3. Morphology of the bacterial colonies. A, B, and C represent LY1, LY2, and LY3 strains, respectively.

After PCR amplification, the 16S rRNA gene obtained approximately 1.5 kb bands. The PCR product was purified using a reagent kit and sent to Shanghai Sangon Biotech Co., Ltd. for sequencing, which resulted in a sequence length of approximately 1.4kb. Comparing the sequencing results with BLAST tool, it was found that all three strains of bacteria exhibited a high homology with the genus *Bacillus* in bacteria, and were named as *Bacillus* sp. LY1, *Bacillus* sp. LY2, and *Bacillus* sp. LY3, respectively. The sequences of three strains were submitted to the NCBI database with login numbers PP094728, PP094735, and PP094740, respectively, and the sequences with high homology were selected from the NCBI database to construct a phylogenetic tree. The clustering of the three strains in the phylogenetic tree is shown in Fig. 4.

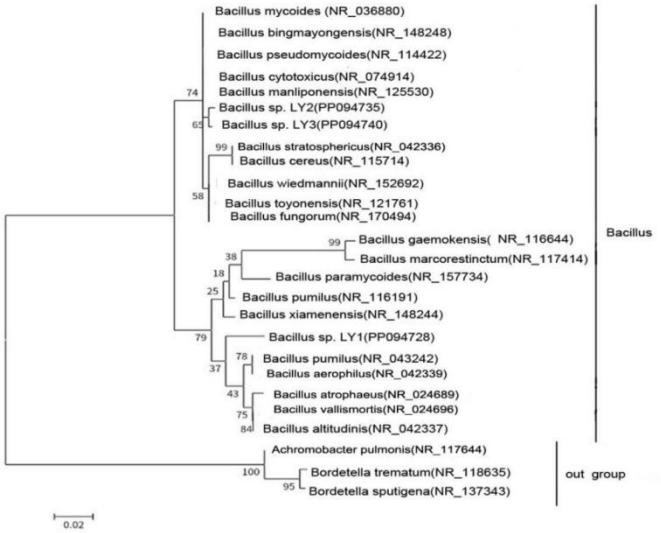

Fig. 4. Phylogenetic tree of the strains.

The experimental data was analyzed and plotted using MS Excel 2019 software, and the results are shown in Fig 5. From Fig 5A, it can be seen that the number of bacteria was stable within 0-10 h with less proliferation, and were in the adjustment period. During this period, sufficient material and energy reserves were available for the bacterial division and proliferation. Within 8-20 , the number of bacteria increased exponentially and was in a logarithmic phase. Within 20-40 , the number of bacteria was relatively flat and in a stable period, but the vitality of the bacterial population changed significantly, producing corresponding metabolites such as cellulose. After 40 h, the bacteria gradually entered in a period of decline. The strain was in a stable period for a long time, indicating a vigorous growth.

The temperature of the environment can affect the biochemical reaction rate of bacteria, thereby affecting their growth rate. At different temperatures, the three strains propagated differently and could grow within 15-60℃ (Fig 5B), with the optimal growth temperature being 36-40℃.

It is known that pH can affect the absorption of nutrients by microorganisms in the environment. Fig 5C shows that the pH of the culture medium had an impact on the growth of the strain, and the bacteria could grow between the pH 4.0 and 8.5. The strains showed maximal growth within the pH range of 5.5-8.0, with an optimal growth pH of around 7.0.

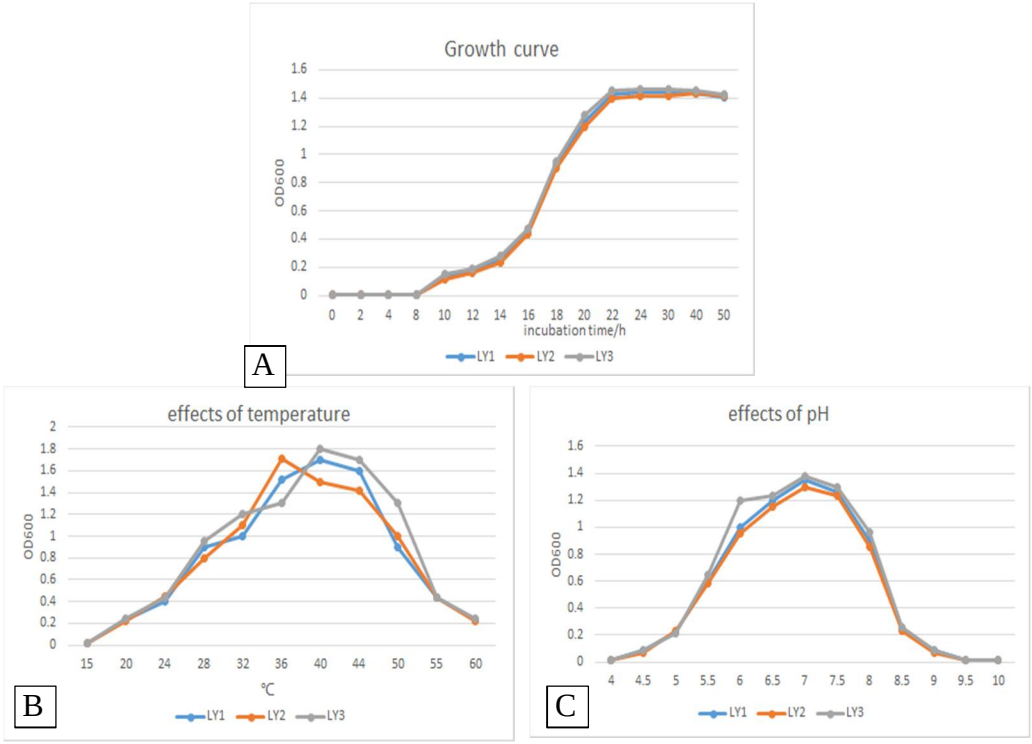

Fig. 5. Growth of strains on culture media(A), at different temperature (B) and pH(C).

This study used three strains isolated from cow dung as the research object. The strains presented clear transparent circles on the Congo red cellulose medium and exhibited the ability to produce cellulase, thus confirming the presence of cellulose degrading bacteria. LY3 exhibited

strong enzyme production ability and a relatively higher enzyme activity than the other two strains, making it a dominant strain with an optimal growth temperature of 40°C and pH of 7.0. It is indeed a cellulase producing strain and may offer a further development potential. Based on the molecular identification results and morphological characteristics, the three strains were preliminarily identified as bacteria belonging to the genus *Bacillus*. At different temperatures, the growth of the three strains varied, and all the three strains could grow within the range of 15-60°C. The optimal growth temperature was 36-40°C. The pH of the culture medium had a significant impact on the growth of the strain, and all strains could grow between the pH range 4.0 to 8.5. The strains showed a good growth within the pH range of 5.5-8.0, with an optimal growth pH of around 7.0. The three strains of *Bacillus* presented certain advantages like alkali resistance, acid resistance, and high temperature resistance, and possess a good potential for application in the industrial production.

Cellulose degrading bacteria are widely present in nature, with the highest production of endoglucanase secreted by bacteria, and the optimal pH of the enzyme is generally neutral to slightly alkaline. In recent years, with the successful application of neutral cellulase and alkaline cellulase in cotton fabric washing and detergent industry, bacterial cellulase preparations have shown good application prospects (Arnold *et al*. 2005, Long *et al*. 2009). *Bacillus* is one of the most common and representative bacterial communities in the environment, with strong adaptability to the environment, and can be used for the degradation of cellulose in environmental protection, textile industry, and food industry.

Our study on the physiological characteristics of strains isolated from cow dung can aid the further investigation of their optimal fermentation conditions and determination of their ability to decompose straw. These strains can be applied to ferment natural cellulose, such as cellulose in crop straw or other wood for conversion into usable resources such as fuel, and applied to composting and fermentation technology of cow dung to promote harmless composting fermentation. This could be of a great significance for achieving the sustainable economic development, thereby reducing the negative impact of fossil fuels on the environment, and maintaining the ecological balance.

In conclusion, this study was aimed to isolate and identify the highly efficient cellulose degrading strains, and study their biological characteristics. Three strains were obtained from cow dung that had clear transparent circles on Congo red cellulose medium and had the ability to produce cellulase, belonging to cellulose degrading bacteria. LY3 has strong enzyme production ability and relatively higher enzyme activity than the other two strains. Based on their molecular identification results and morphological characteristics, the three strains were preliminarily identified as bacteria belonging to the genus *Bacillus*.

Acknowledgements

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