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ISOLATION AND IDENTIFICATION OF CELLULOSE DECOMPOSING BACTERIA FROM THE SOIL OF YUNNAN PROVINCE, CHINA

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Abstract

In this experiment sodium carboxymethyl cellulose (CMC-Na) was used as the only carbon source to isolate cellulose decomposing bacteria from the soil of Qiongzhusi Forest Park in Kunming, Yunnan Province. The ratio of hydrolytic circle diameter and colony diameter formed by the strain on cellulose Congo red medium was determined for primary screening, and then the filter paper disintegration test and CMC enzyme activity assay were used for rescreening. A cellulose decomposing bacterium (HS-2) with high enzyme activity was obtained, and its enzyme activity was up to 16.42 U/ml. (U means the unit of activity of enzymes) Gram stain and Spore stain reaction showed that HS-2 strain was Gram-positive *Bacillus*. The *Bacillus* strain was identified as *Bacillus pumilus* on the basis of physiological and biochemical tests, and 16S rDNA sequence analysis.

Introduction

Yunnan is a large animal husbandry industry province, but the ability of forage is not sufficient. Relying on forage imports has become an important factor affecting the development of Yunnan's animal husbandry industry (Rui and Chi 2023). Crops such as corn, wheat straw and rice straw have high dry matter and protein content, the use of microbial fermentation on the processing of crop residues can transform straw into nutritious, high-quality forage for livestock digestion and absorption (Su and Zhang 2016). However, these crop residues are mainly composed of lignin, cellulose and hemicellulose, which are difficult to be decomposed under natural conditions. It increases the difficulty of forage fermentation (Song *et al.* 2023). Therefore, the most critical problem in straw treatment and forage fermentation is the degradation of cellulose. At present, the most economical and environmentally friendly method is to screen efficient cellulose decomposing bacteria through microbial technology (Zhang *et al.* 2017, Liang *et al.* 2019). Large numbers of workers have conducted research on cellulolytic bacteria. Reese *et al.* (1950) isolated *Trichoderma reesei*, a cellulose-degrading fungus, from the fibrous material of decaying wood. Sheng *et al.* (2012) isolated and screened a strain of *Pseudomonas aeruginosa* with high enzyme activity for cellulose degradation from the intestines of dark gill turtle (*Holotrichia parallela*) larvae. Geng *et al.* (2012) sieved two strains of Bacillus with high efficiency in degrading straw cellulose in soils where wheat and maize were grown in rotation and the straw from both was directly returned to the field. Meng *et al.* (2021) isolated and screened a CB04 strain with an enzyme activity of 114.6 U/ml（U means the unit of activity of enzymes）after

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optimized culture. Zhang *et al.* (2023) screened a strain of actinomycetes with an enzyme activity of 30.5 U/ml, which also have good cellulose degradation capacity. Zhang (2023) isolated an acid-tolerant, cellulolytic bacterial *Bacillus velezensis* strain with high enzyme activity of 6.28 U/ml.

Yunnan Province's grass-fed animal husbandry industry is developing vigorously, but forage supply is seriously insufficient. Therefore, the separation and purification of cellulose decomposing bacteria with high enzyme activity can not only improve the utilization rate of crop straw in Yunnan, but also reduce pollution and protect the ecological environment. In addition, Yunnan has a unique climate, complex and diverse ecological environment. So, selecting strains from local sampling can avoid the environmental inadaptability of foreign strains, and can ensure the stability of enzyme production and high enzyme activity of target strains. Therefore, this study promotes the development of forage fermentation technology to a certain extent, and provides potential strain resources for forage fermentation and research and development of high-quality forage. It lays a foundation for the development of Yunnan animal husbandry industry and has very important research significance. In order to screen out high enzyme activity cellulose decomposing bacteria adapted to the local ecological environment in Yunnan, to ensure the stability of enzyme production and high enzyme activity of the target strain. At the same time, it can promote the development of forage fermentation technology to a certain extent, and provide potential strain resources for the transformation of crop straw resources into high-quality forage.

Materials and Methods

In order to screen out high enzyme activity cellulolytic bacteria adapted to the local ecological environment of Yunnan, and to provide strain resources for the conversion of crop straw resources into high-quality forage, in view of the unique climatic environment of Yunnan, this study collects soil humus from Urizusi Forest Park, Kunming City, Yunnan Province as experimental material to screen, isolate and identify high enzyme activity cellulolytic bacteria. The soil humus was collected about 5 cm below using a shovel. The soil samples were collected in sterilized sealed bag and was taken to the laboratory and stored at 4℃.

5 g of soil samples were weighed and, then put into a sterilised conical flask. 50 ml of sterile water was added, mix up with sufficient oscillation 5 mL of the supernatant after resting was absorbed into the enrichment medium and cultured in a constant temperature at 30℃ and 180 r/min("r/min" is a unit of revolutions, meaning revolutions per minute, 180r/min is 180 revolutions per minute).

The treated medium was diluted to 10^{-5} 10^{-6} and 10^{-7} gradients, and coated in sodium carboxymethyl cellulose medium. The culture plate was inverted for 3 days at 30℃. Bacterial strains with transparent hydrolytic rings were selected, purified and stored in a refrigerator at 4℃. The strains were plated in Congo red medium and cultured incubated for 4 days at 30℃. The cellulase activity of the strain was preliminarily determined based on the ratio of the diameter of the transparent hydrolytic circle (D) to the diameter of the colony (d). The strains with larger diameter ratio (D/d) were selected for further study.

Each cellulolytic bacteria obtained from the preliminary screening was cultured in beef paste peptone liquid medium. After 24 hrs cultivation, 5 ml of different bacterial suspensions were added to the filter paper medium, and incubated in a constant temperature oscillation chamber for 15 days at 30℃ and 120 r/min. For each strain three replications were maintained and filter paper media without bacterial suspensions act as control. The degree of disintegration of filter paper in the medium was observed to compare the cellulose decomposition ability of each strain.

5 ml of cellulose decompositing bacteria were activated and added into the enzyme producing medium and centrifuged at 4 ℃ and 6000 r/min for 15 min. The supernatant was used as the crude

fermentation liquid. Carboxymethyl Cellulose (CMC) enzyme activity was measured with endo-β-1,4 glucanase/cellulase kit.

Strain HS-2 was identified on the basis of morphological, physiological and biochemical reactions with reference to the Manual of Systematic Identification of Common Bacteria (Dong and Cai 2001).

For molecular identification, the DNA of HS-2 strain was extracted-according to the method of bacterial genomic DNA extraction kit. After obtaining the target DNA, PCR amplification was performed. The amplified product was sent to Shanghai Shenggong Biological Company for sequencing analysis. MEGA6.0 software was used to compare and analyze the sequencing results, construct phylogenetic tree, and determine the species.

Results and Discussion

A total of nine cellulolytic bacterial strains with transparent hydrolytic rings were screened using sodium carboxymethyl cellulose. The diameter of transparent circle (D), colony diameter (d) and D/d ratio of the strains on the medium are shown in Table 1. The results showed that the D/d ratio of strain HS-1 was the highest, followed by strain HS-2. In theory, the size of the D/d ratio indicates the size of the ability to decompose cellulose. However, considering the different enzyme characteristics of cellulolytic bacteria, the effect of cellulolytic bacteria cannot be determined solely by the D/d ratio. Therefore, the filter paper disintegration test and CMC enzyme activity determination continue to be used for comprehensive screening.

Table 1. Diameter of transparent circle, colony diameter and their ratio on cellulose Congo red medium owing to different isolates of *Bacillus pumilus*.

Strain Nos	Diameter of transparent circle (D)(cm)	Colony diameter (d) (cm)	Ratio of D/d
HS-1	0.74	0.07	10.59
HS-2	1.40	0.15	9.33
HS-3	1.30	0.15	8.69
HS-4	1.45	0.18	7.97
HS-5	1.40	0.20	7.00
HS-6	0.53	0.08	6.64
HS-7	1.43	0.32	4.47
HS-8	0.91	0.50	1.82
HS-9	0.76	0.45	1.69

The disintegration degree of filter paper can effectively reflect the enzyme activity of cellulose decomposing bacteria. Among the 9 strains obtained from the preliminary screening, 5 cellulose decomposing bacteria with high D/d ratio were selected for disintegration experiment of filter paper. The effect of filter paper disintegration degree is shown in Table 2. It was observed that strains HS-1 and HS-2 have the best degradation effect on filter paper, and the filter paper has been disintegrated into more small balls, the disintegration effect of strains HS-3 and HS-4 was slightly lower than that of the previous two strains, and the disintegration of filter paper in the medium only produces a few small balls. The disintegration effect of HS-5 strain was normal. The enzyme activity of the 5 strains are shown in Table 2. The enzyme activity of strain HS-2 was the highest (16.40 U/ml), followed by HS-1, and the enzyme activity of the others decreased gradually.

Table 2. Degree of disintegration of filter paper and CMC enzyme activity of different strains.

Strain Nos	Degree of disintegration	Enzyme activity (U/ml)
HS-1	++++	14.94
HS-2	++++	16.40
HS-3	+++	6.29
HS-4	++	2.46
HS-5	+	1.68

The disintegration test of filter paper and enzyme activity of each strain showed that HS-2 strain had the best comprehensive enzyme activity. Strain HS-2 was cultured with fermentation enzyme producing medium, and CMC enzyme activity was measured every 24 hrs and the results are shown in Fig. 1. The results showed that the enzyme activity of HS-2 strain increased gradually from 24 hrs of fermentation culture, reached the highest level at about 150 hrs of fermentation culture, and then gradually decreased.

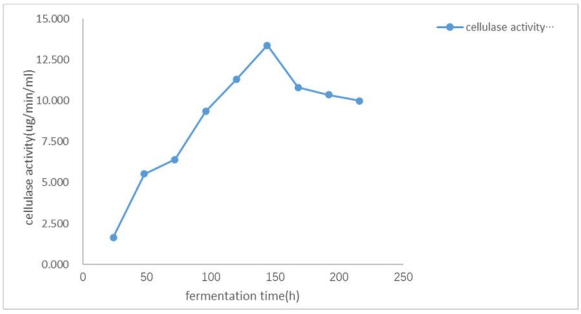

Fig. 1. Cellulase activity in different fermentation time of HS-2 strain.

Colony morphology of HS-2 strain was observed after stripe culture, and the results are shown in Fig. 2. The results of Gram staining showed that strain HS-2 was Gram-positive, and the spores were oval green-but the number of spores was small.

Fig. 2. Colony morphology of HS-2 strain.

The results of physiological and biochemical tests of strain HS-2 are shown in Table 3. HS-2 strain could grow in anaerobic and pH5.7 environment. It can liquefy gelatin and hydrolyze starch. V-P, D-xylose, L-arabinose and D-mannitol were all positive. Citrate, propionate, nitrate reduction and 7% sodium chloride growth were negative. Based on the observation of the morphological results and characteristics of HS-2 strain, it was preliminarily identified as Bacillus strain.

Table 3. Physiological and biochemical results of HS-2 strain.

Name of experiments	Results		Training time
	Positive	Negative	
V-P	+		2-4 days
Nitrate reduction		-	2-4 days
Citrate		-	48 hours
Propionate		-	48 hours
7% NaCl growth		-	2-7 days
Anaerobic growth		-	18-24 hours
Starch hydrolysis	+		48-96 hours
D-xylose	+		2-5 days
L-Arabinose	+		2-5 days
D-mannitol	+		2-5 days
Liquefaction of Gelatine	+		48-96 hours
pH 5.7 growth	+		24-48 hours

"+" represents that the HS-2 strain detection project is positive, while "-" represents that the project is negative.

The DNA of strain HS-2 was extracted by bacterial genome DNA kit. The concentration of DNA was 41.615μg /mlL and the ratio of OD260 to OD280 was 1.79. Then, the fragment of about 1500 bp was obtained by PCR amplification with high purity, as shown in Fig. 3. The target film was sent to Kunming Shenggong Biological Company for sequence sequencing.

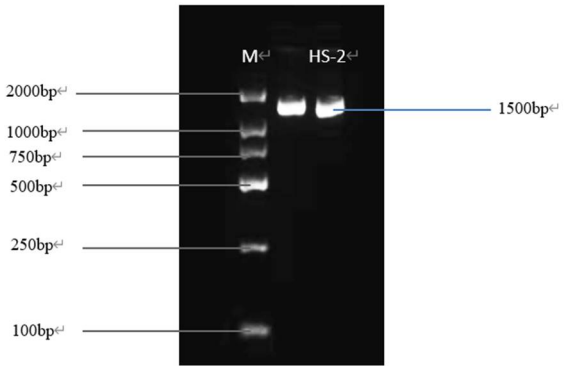

Fig. 3. Gel electrophoresis of DNA PCR amplification product of HS-2 strain.

16S rDNA sequence of HS-2 strain was input into NCBI database for Blast comparison. Then, homology analysis was performed, and the phylogenetic tree of HS-2 strain was constructed by MEGA 6.0 (Fig. 4). The results of homology comparison showed that the homology between HS-2 strain and *Bacillus pumilus* was 99.99%, and HS-2 strain was determined to be *Bacillus pumilus.*

At present, the number and types of cellulolytic bacteria reported in Yunnan are few, and the enzyme activity of one strain of *Bacillus pumilus* screened in this study is 16.40 U/ml. *Bacillus pumilus* is widely used in agricultural production and fermentation. Zhang *et al.* (2011) found that adding *Bacillus pumilus* to marin potato residue for fermentation could optimize the production of single-cell protein feed. Liu *et al.* (2019) used a strain of *Bacillus pumilus* to fermentation biosurfactant with corn straw as raw material and increased the yield by 23%. Yu (2020) found that the application of *Bacillus pumilus* A9 to soybean meal fermentation has a good therapeutic effect on diarrhea in mice.

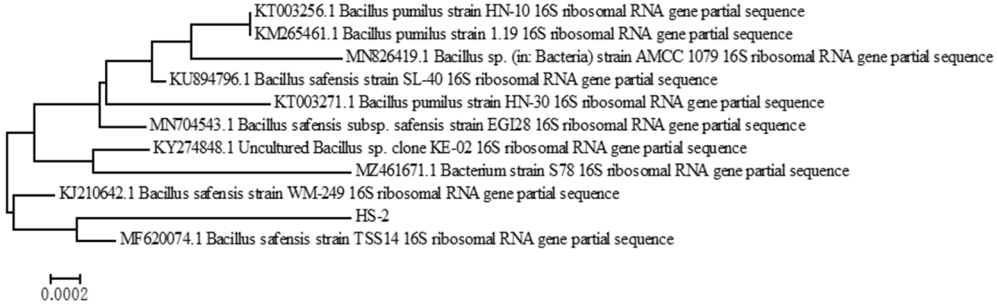

Fig. 4. Phylogenetic tree of 16S rDNA gene sequence of HS-2 strain.

In this study, a strain of *Bacillus pumilus* was screened locally from Yunnan Province which had high enzyme activity. Currently, there are few cellulolytic bacteria isolated ~~locally~~ from Yunnan Province. Tao *et al.* (2010) isolated a strain of *Penicillium oxalate* with cellulose decomposition ability from farmland soil in Yunnan Province, and the enzyme activity was 51.60 U/ml. In order to verify the decomposition ability of the strain on straw cellulose, the enzyme activity of the strain could be improved by mutagenesis culture under ultraviolet irradiation. Through orthogonal experiments, the most suitable culture conditions such as carbon source, nitrogen source, temperature and pH were explored to determine the optimal enzyme production ability of the strain. It is expected that the strain can be applied to Yunnan forage fermentation, improve the conversion rate of forage crude fiber, provide high quality forage for various breeding industries, and promote Yunnan forage fermentation processing technology.

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