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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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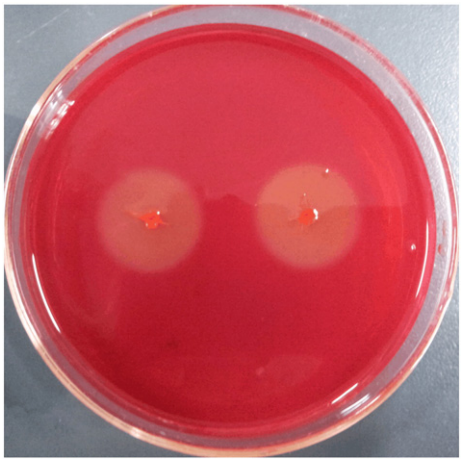

Fig. 1. Clearing zone of cellulolytic bacterium on CMC-Na selective media.

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

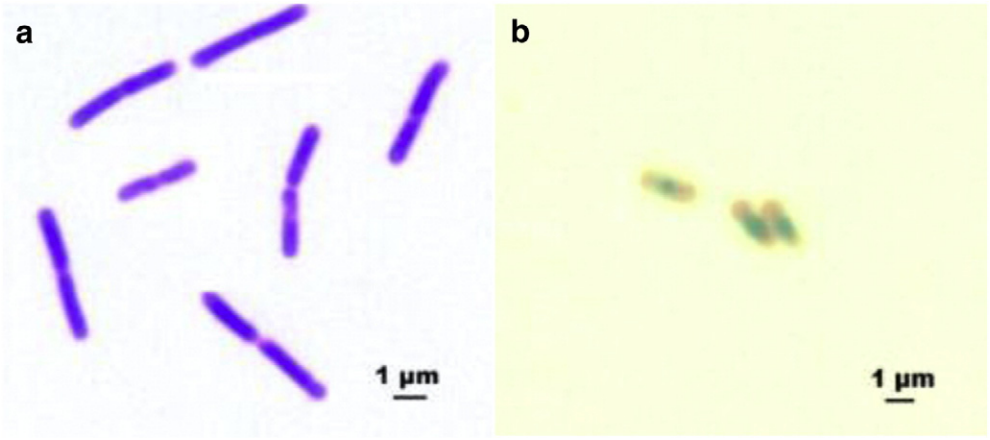

Fig. 2. Morphological identification of the cellulolytic bacterium. (a) Gram staining; (b) malachite green staining of spores.