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A Novel Strain of Moderately Thermophilic *Streptomyces* from the Fenjiu-Flavor Daqu

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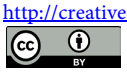

Abstract

Fenjiu-flavour Daqu is an important starter to support growth of microorganisms in the fermented process of Fenjiu-flavor liquor. A novel thermophilic microorganism, designated strain gby1, was isolated from Fenjiu-flavour Daqu from Shanxi Xinghuacun Fenjiu Distillery Company Limited, Fenyang country, Shanxi province. The morphological, physiological, chemical taxonomic and phylogenetic characteristics of the strain were described in this paper. The isolate gby1 exhibited higher heat resistance. The strain is aerobes, non-motile, and spore forming bacteria. The strain can produce amylase, lipase. The physiological tests combined with 16S rDNA-based molecular analysis, gby1 was identified as a moderately thermophilic *Streptomyces*. In the paper, the thermophilic *Streptomyces* sp. gby1 is isolated and identified for the first time in Fen-Daqu. The results offer a reference for the comprehensive understanding of bacterial diversity of Fen-Daqu.

Subject Areas

Microbiology

Keywords

Fen-Daqu, Moderately Thermophilic Actinobacteria, *Streptomyces*

1. Introduction

Baijiu is the national liquor of China and the world's most consumed spirit, which is produced using a unique and traditional solid-state fermentation (SSF) process. Daqu plays a vital role in the formation of Baijiu flavor (Deng *et al.*, 2021 [1]; Ye *et al.*, 2021 [2]). Fen-Daqu is the main source of microorganisms in the brewing process of Fen-flavor liquor, and a rich microbial population will be formed in the traditional Daqu brewing process. Some studies have found that the microorganisms in Daqu include fungi and bacteria (Cao *et al.*, 2015 [3];

Chang *et al*., 2018 [4]; Chen *et al*., 2021 [5]; Luo *et al*., 2013 [6]). As Daqu is in a high temperature environment, heat-resistant groups compete for continuous reproduction and metabolism, temperature has become the main driving force for the formation of Daqu flora. Chinese Fenjiu liquor has been made for 1500 years and is distilled from the product of fermentation using a wild microbial starter, Fenjiu-flavor Daqu. In general, the maximum temperature while making fen-flavor can reach 50˚C. *Bacillus* spp., which are heat-resistant microbe have been considered, however, the roles of other thermophilic microbes in the formation of Fen-Daqu have not been confirmed.

Thermophilic actinobacteria thrive at relatively high temperatures ranging from 40˚C to 80˚C (Tortora *et al*., 2007) [7]. The moderately thermophilic actinobacteria require 45˚C - 55˚C for optimum growth (Jiang and Xu, 1993) [8]. Many thermophilic strains have been reported in thermophilic actinobacteria. Thermophilic actinobacteria are known to possess unique metabolic rates and physical properties that prove to be beneficial in a variety of ecological roles such as composting, antimicrobial activity, plant growth promotion, nitrogen fixation, hypersensitivity pneumonitis (Shivlata and Satyanarayana, 2015 [9]; Wu *et al*., 2016 [10]; You *et al*., 2013 [11]). Thermophilic actinobacteria have been proven as a potential source of bioactive compounds and richest source of secondary metabolites. They are the most economically and biotechnologically valuable microorganisms (Singh *et al*., 2012) [12].

The aim of this work was to investigate the thermophilic actinobacteria from Daqu ecosystems by the culture-dependent methods. Therefore, we explored the thermophilic actinobacteria in Fen-Daqu. The characterization of thermophilic actinobacteria was achieved by analysis of morphology, optimum temperature, enzyme production activity, antibacteia activity, and 16S rRNA gene sequence.

2. Materials and Methods

2.1. Materials

2.1.1. Daqu Samples

Daqu samples were provided from Shanxi Xinghuacun Fenjiu Distillery Company Limited, Fenyang country, Shanxi province. The Company produces typical light-liquor brewing by Daqu.

2.1.2. Medium

The actinobacteria medium was prepared as described previously (Wei *et al*., 2019 [13]; Zhang *et al*., 2019 [14]). IPS2 medium (composed of Yeast extract 4 g, Malt extract 5 g, Dextrose 4 g, Agar 18 g, pH 7.3, Nutrient solution (Daqu 10 g, Corn flour 10 g), ddH_2O 1 L, pH 7.3. Gauss No. 1 medium (composed of composed of starch soluble 20 g, KNO_3 1 g, K_2HPO_4 0.5 g, $MgSO_4{\cdot}7H_2O$ 0.5 g, NaCl 0.5 g, $FeSO_4{\cdot}7H_2O$, 0.01 g, Agar 15 - 20 g, ddH_2O 1 L, adjusting pH to 7.4 - 7.6). Gauss No.2 medium (composed of glucose 5 g, peptone 2.5 g, NaCl 2.5 g, Agar 10 g, ddH_2O 500 mL, Nutrient solution 5 ml, pH 7.2). IPS4 medium (composed of starch soluble 5 g, K_2HPO_4 0.5 g, $MgSO_4{\cdot}7H_2O$ 0.5 g, NaCl 0.5 g, $(NH_4)_2SO_4$ 1

g, $CaCO_3$ 1 g, $FeSO_4 \cdot 7H_2O$ 0.0005 g, $MnSO_4 \cdot 7H_2O$ 0.0005 g, Agar 10 g, Nutrient solution 5 ml, ddH_2O 500 mL).

2.2. Methods

2.2.1. Isolation of Thermophilic Actinobacteria from Daqu

Serial dilution and spread-plate techniques (Williams *et al*., 1965) [15] were used to isolate actinomycetes from Daqu samples. Four agar media were tested: IPS2, Gauss No. 1, Gauss No. 2, IPS4 medium. 10 g Daqu sample with 90 ml sterile water, set the original solution number as 10^{-1}, shake and dilute the original sample of bacteria to be tested fully, then transfer 1 ml original sample with sterile pipette to 9 ml sterile water, 10^{-2}, and dilute to 10^{-6}, 0.2 ml of diluted bacterial solution were added to the corresponding numbered solid medium. All media were supplemented with 25 $mg \cdot mL^{-1}$ Nalidixic acid and 10 $mg \cdot mL^{-1}$ Amphoteric to inhibit the growth of bacteria and fungi. All plates were incubated at 45˚C for 3 days. Actinomycete colonies were identified by visual examination of the cultural and morphological characteristics; microscopic examination was performed if needed. The isolated and purified high-temperature resistant strains were streaked on the prepared solid plate and cultured at 45˚C for 4 days to observe the growth of each strain. According to the size of the colony, the color of mycelia and the number of aerial mycelia, the growth state of the strain in different media was judged, and the appropriate basic media was selected.

2.2.2. Temperature Range of Actinomycetes

The strains were inoculated on ISP2 medium for activation for 3 days, and cultured at seven temperature gradients of 25˚C, 30˚C, 40˚C, 45˚C, 50˚C, 55˚C and 60˚C, respectively. Three groups were set at each temperature. The activated strains were cultured for 3 days, and the colony size was observed. After that, the cross measuring method was used to measure the size of the colony diameter to determine the optimum growth temperature of the strain.

2.2.3. Morphological Characterization and Biochemical Tests

The selected isolate was grown in ISP2 solid medium in preparation for colony morphology observation. Cultures grown on ISP2 agar for 3 days at 45˚C was observed by light microscopy. Scanning electron microscopy was used to observe mature spores on aerial mucelium of the isolates grown on ISP2 for 3 days at 45˚C. Gram staining was performed as described by Harrigan *et al.* (1968) [16]. The assays for enzyme activity (Li *et al*., 2016) [17] is used for inferring the function in Daqu. The antimicrobial activity of gby1 isolated on ISP2 was analyzed using an agar block method (Stern *et al*., 2006) [18] against 3 bacterial species, which were provided by the Microbiology Laboratory in Shanxi Normal University. The bacterial species included *Escherichia coli*, *Staphylococcus aureus*, and *Bacillus subtilis*.

2.2.4. Phylogenetic Analysis

The isolate was identified by 16S DNA-based sequence analysis. Actinomycete

DNA was extracted from pure isolates using the method described by Saito and Miura (Saito and Miura, 1963) [19]. Partial 16S rRNA gene fragments were amplified by polymerase chain reaction (PCR) using the forward primer used was p27f (5'-AGAGTTTGATCCTGGCTCAG-3'), whereas the reverse primer was p1492r (5'-TACGGCTACCTTGTTACGACTT-3'). PCR reaction (50 μL) contained the following: a hot start performed at 95˚C for 5 min and 30 cycles at 95˚C for 30 s, 56˚C for 1 min, and 72˚C for 2 min, followed by a final extension performed at 72˚C for 10 min. PCR reactions were purified and sequenced by Beijing Tsingke Biotech Co., Ltd. MEGA5.0 was used to analyze the phylogeny and molecular evolution of the strain. The sequences were then compared with BLAST search sequences from the National Center for Biotechnology Information (NCBI) to find similar nucleotide sequences. The obtained sequence was compared with available reference sequences in the EMBL/GenBank/DDBJ databases and deposited in GeneBank under the accession No. MZ156984.

3. Results

The suspension was spread onto the surface of ISP2 agar plates and incubated at 45˚C for 3 days. Strain gby1 was isolated from this medium.

3.1. The Cultural Characteristics of the Isolate gby1 on Different Media

The cultural characteristics of the isolate gby1 are summarized in Table 1. There were great differences in the growth state of the strain in different media. The isolate grew well on ISP2 medium, followed by Gauss No.2. It grows poorly in ISP4 and Gauss No.1. The isolate did not produce diffusible pigments on any medium (Table 1).

3.2. The Optimum Temperature of *Streptomyces* sp. gby1

Growth temperature was measured on ISP2 medium at 25˚C - 60˚C. The strain *Streptomyces* sp. gby1 could grow at 25˚C - 55˚C and the optimum temperature is 45˚C (Figure 1). The results showed gby1 belongs to the moderately thermophilic actinomycetes.

Table 1. The colonies characteristics of gby1 on four different media at 45˚C.

Medium Type / Characteristics	ISP2	ISP4	Gauss No.1	Gauss No.2
Aerial mycelium	Dense and exuberant	Less	Less	More
Substrate hyphae	Yellowish brown	White	White	Light yellow
Spores	White	White	White	White
Colony size	Larger	Smaller	Smaller	Larger

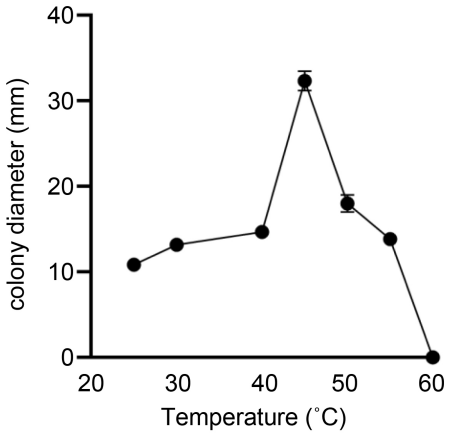

Figure 1. The growth of *Streptomyces* sp. gby1 on ISP2 medium at different temperatures.

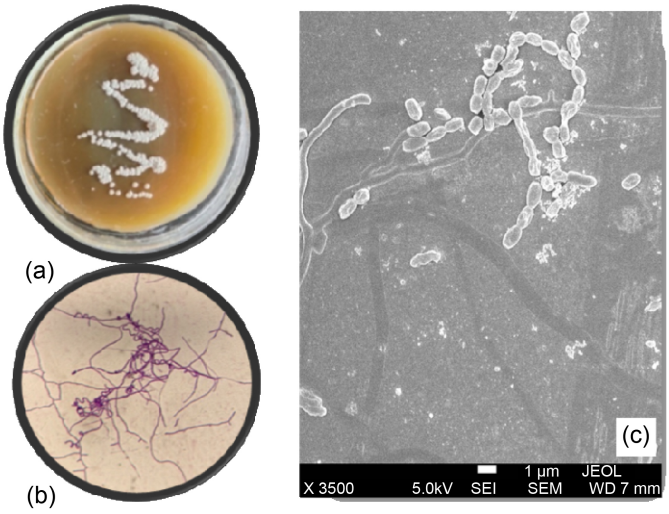

Figure 2. The morphological characterization of the isolate gby1 on ISP2 medium. (a) Colony morphology; (b) Aerial mycelium formation and spore filament (10 × 100); (c) Scanning electron microphotograph of the isolate gby1.

3.3. Morphological Characterization and Biochemical Tests

Morphological characteristics were observed under light microscopy (**Figure 2(b)**) and scanning electron microscopy (**Figure 2(c)**) after incubation for 3 days on ISP2 medium at 45 ˚C, it could be observed that the colony morphology was small, with fine and dense mycelia. The colony surface was dry and powdery, difficult to stir up (**Figure 2(a)**). The experimental results showed that the strain did not produce protease and cellulose. It was discovered the isolate gby1 markedly suppressed *Escherichia coli* and *Staphylococcus aureus* (**Table 2**).

3.4. 16S rDNA Sequencing and Phylogenetic Analysis of gby1

We used a 16S rDNA gene sequence-based strategy to identify the isolate gby1. The 16S rDNA was sequenced and is available at GenBank under accession

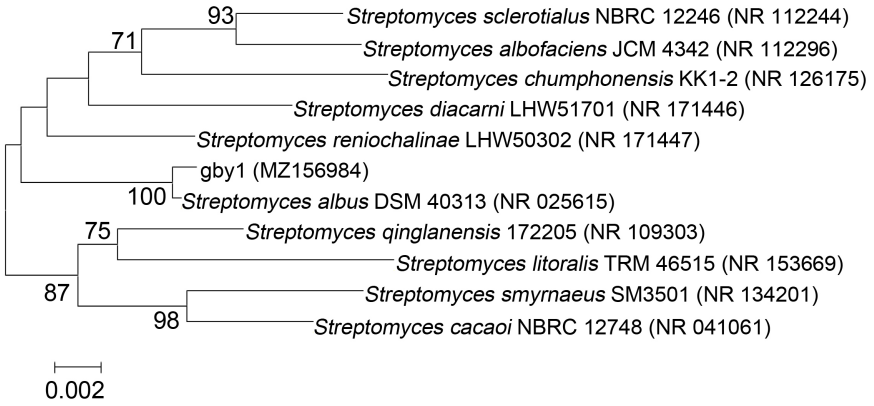

Figure 3. Phylogenetic tree constructed after multiple alignments using CLUSTAL-X of the 16S rDNA gene sequences available from the GenBank/EMBL/DDBJ database (accession numbers are given in parentheses) (Thompson *et al.* 1997) [20]. the tree was constructed with MEGA 5 using the neighbor-joining with bootstrap values calculated from 1000 trees based on 1377 base pairs of the 16S rDNA gene of sequences of the isolate gby1 (GenBank accession number MZ156984). The scale bar indicates the 0.002 evolutionary distance unit.

Table 2. The morphologica, biochemical characteristics and antagonistic activity.

Characteristics	Result
Gram staining	Positive
Shape and growth	Filamentous aerial growth
Range of temperature for growth	25˚C - 55˚C
Optimum temperature	45˚C
Range of pH for growth	6 - 8
Amylase	+
Protease	−
Lipase	+
Cellulase	−
Tested microbes	
Escherichia coli	+
Staphylococcus aureus	+
Bacillus subtilis	−

MZ156984. A phylogenetic tree was constructed based on an alignment of the sequences (**Figure 3**). Based on 16S rRNA gene sequence analysis, sequence similarity calculations indicated that the isolate gby1 showed the greatest degree of similarity to *Streptomyces albus* (NR025615) 99.93%.

4. Discussion

Traditional Chinese liquor (Baijiu) solid state fermentation technology has lasted for several thousand years. The microbial communities that enrich in liquor starter are important for fermentation. However, the microbial communities

are still under-characterized (Huang *et al*., 2017) [21]. Some thermophilic microbes were investigated using culture-dependent and culture-independent technology. Xiao *et al*. pointed that bio-heat functioned as a primary endogenous driver promoting the formation of functional MT-Daqu microbiota. The thermotolerant strains, survived or kept on growing from day 4 to day 12, might contribute to the formation of flavor metabolites (Xiao *et al*., 2017) [22]. In 2014, the thermotolerant and thermophilic microbes were showed that using DGGE technology in the Chinese "Baiyunbian" liquor Daqu, the most dominant bacterial species were *Bacillus* and *Virgibacillus*, followed by *Lactobacillus* and *Trichococcus* (Xiong *et al*., 2014) [23]. In 2021, *Thermoactinomyces daqus* H-18^{T} was isolated at 55˚C from a high-temperature Daqu sample collected from the manufacturing process of a sesame-flavoured liquor in Shandong province, China (Yao *et al*., 2014) [24]. Some thermophilic microorganisms were showed using metatranscriptomics method from Chinese *Luzhou-flavor baijiu*. The authors inferred that thermophilic microorganisms might bring significant effects in aged pit mud (Zhou *et al*., 2021) [25].

In our previous studies, we found that most of the medium temperature actinomycetes from Fen-jiu Daqu belong to *Streptomyces* (Zhang *et al*., 2019) [14]. Interestingly, the thermophilic actinomycetes gby1 also belong to *Streptomyces.* However, the comparison of 16S rDNA sequences showed that there were significant differences between the thermophilic *Streptomyces* sp. gby1 and medium temperature *Streptomyces* spp. In 2019, Wei found 3 Thermoactinomycetaceae strains including *Shimazuella kribbensis*, *Kroppenstedtia sanguinis* and *Kroppenstedtia eburne* in Niulanshan-flavor baijiu (Wei JW., 2019). In the paper, the thermophilic *Streptomyces* sp. gby1 is isolated and identified for the first time in Fen-Daqu. Further studies are required to clarify the role and mechanism of action of the thermophilic actinomycetes, which needs to be further studied.

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Conflicts of Interest

The authors declare no conflicts of interest.

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