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EVALUATIONOF THE POTENTIAL OF DAQU-DERIVED ACTINOBACTERIA FOR LIGHT-FLAVOUR CHINESE LIQUOR

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ABSTRACT:*The 15 Actinobacteria strainsisolated from the Daqu for light-flavour Chinese liquor, were identified on the basis of 16S rDNA gene sequence analysis and investigated from morphological, physiological and biochemical characteristics to analyze the role in Fenjiu liquor brewing. The research results demonstrated the 10 strains have the ability to produce exo-enzymes, amylase, protease, cellulose simultaneously. The 15 strains showed a wide spectrum of antifungal and antibacteria propertieson tests of antagonisma against phytopathogenic fungi and bacteria. The 16S rDNA sequencing analysis revealed that Streptomyceswasis the predominant genus in culture-dependent Actinobacteriastrains inDaqu ecosystem. We speculated the Actinobacteria play a very important role on two aspects: one is the ability to produce amylase, protease, cellulase to participate in the process of saccharification; the other is the inhibition of harmful microorganisms in the production of Daqu, and these isolates may have strong biotechnological potential in agriculture.*

KEYWORDS: Daqu, Actinobacteria, Antibacterial, Antifungal, *Streptomyces*

INTRODUCTION

Daqu, a traditional fermentation starter that is used for Chinese liquor production, is manufactured through a traditional spontaneous solid-state fermentation process with no selected microorganisms are inoculated **(**Li et al, 2017**).**The microbial community structures of different Daqu samples exhibited some differences. These may be ascribed to the raw material constituents, different peak production temperatures, and microhabitats around the liquor enterprises **(**Zheng et al. 2014; Tang et al. 2017). For a long time, the research on microbes in Daqu has focused on bacteria, yeasts and molds **(**Zheng et al. 2012; Chen et al. 2014; Zheng et al.2014; Zhang et al. 2016; Li et al. 2018**).** However, In recent years, many research results from culture-independent methods indicated that actinobacteria was the predominant bacteria in different kind of Daqu (Li et al. 2014;Zhang et al.2014; Wang et al. 2017; Zou et al. 2018).

Fen-Daqu, used for famous Chinese light-flavour liquor brewing, includes three kinds of Daqu (Qingcha, Hongxin and Houhuo). In 2014, We First revealed the bacterial composition in Fen-Daqu by high-throughput sequencing. The result was shown the actinobacteria is 23.1% in QC, 17.9% in HX, 1.1% in GT, respectively (Zhang et al. 2014). Up to date, however, not much is known about the specific contributions of the Actinobacteria to the baijiu production except for reports of *streptomyces*

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producing geosmin (Du et al. 2013; Yan et al. 2016).

The aim of this work was to investigate Actinobacteria from Daqu ecosystems by the culture-dependent methods. Therefore, we performed an extensive screening exercise with the objective to explore the function of Actinobacteria in Fen-Daqu. Characterization was achieved by analysis of morphology, enzyme activity, antibacteia activity, and 16S rRNA gene sequence.

MATERIALS AND METHODS

Sample Collection and Medium

Sample collection

Daqu samples were provided from Shanxi Xinghuacun Fenjiu Distillery Company Limited, Fenyang country, Shanxi province. The company produce typical light-liquor brewing by Daqu (Fig 1). HX, QC and GT were used to isolated Actinobacteria strains.

Medium

Five different types of mediums were selected to isolate strains.

GS medium (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).

GW1 medium (composed of caseins 5 g,mannitol1 g, $NaHCO_3$ 2 g, $CaCO_3$ 0.2 g, $(NH_4)_2SO_4$ 2 g, KNO_3 2 g, K_2HPO_4 1 g, $MgSO_4 \cdot 7H_2O$ 2 g, $FeSO_4 \cdot 7H_2O$ 0.02 g, KCl 0.01 g, ddH_2O 1 L； PH7.2-7.4).

R2A medium (composed of yeast extract 0.5 g, peptone 0.5 g； caseins 0.5 g, glucose 0.5 g, starch soluble 0.5 g, Sodium pyruvate 0.3 g, K_2HPO_4 0.3 g, $MgSO_4$ 0.024 g, agar 15 g, ddH_2O 1 L, PH 7.2).

CMKA medium (composed of caseins 0.5 g, Mannitol 1.5 g, KNO_3 1.0 g, K_2HPO_4 0.5 g, $(NH_4)_2SO_4$ 2 g, $CaCO_3$ 0.5 g, agar 15 g, ddH_2O 1 L； composed salt (15%NaCl, 5% KCl, 1% $MgCl_2$), PH7.2-7.4.

HV medium (composed of humic acid 5 g, $CaCO_3$ 0.02 g, Na_2HPO_4 0.5 g, KCl 1.7 g, $FeSO_4 \cdot 7H_2O$ 0.01 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, agar 5 g, ddH_2O 1 L, pH 7.2-7.4. The mediums were added with sterilized $K_2Cr_2O_7$ (80 mg/L). Sterilize by autoclaving (121°C, 30 min).

Isolation of Actinobacteria strains

Daqu was ground into a powder in laboratory, 5 g power was transferred to bottle with 95 ml sterile ddH_2O. Appropriate serial dilutions were prepared and the samples were spread on five medium respectively. The plates were incubated at 28°C for one week. The actinobacteria were selected and purified according to their morphological characteristics. Single colonies were selected and stored on GS agar plate.

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Assays for enzyme activity

Prepare a basal medium (H1 medium composed of glucose 10.0 g, peptone 5.0 g, yeast extract 5.0 g, $MgSO_4 \cdot 7H_2O$ 0.2 g, K_2HPO_4 1.0 g, agar 15 g， ddH_2O 1 L, pH7.2-7.4) containing 10 g/L starch soluble for the ability of an organism to produce amylase (Li et al. 2016). After growth occurs for 2-3 days, flood the plate with the iodine solution. Starch stains blue with iodine, so look for colorless areas around the microbial growth; Prepare H1 basal medium containing 20 g/L skim milk for the ability of an organism to produce Protease. Appearance of zone indicates protease production (Li et al. 2016); Prepare H1 basal medium containing 10 g/L cellulose for the ability of an organism to produce cellulase by congo red-polysaccharide method (Teathe et al. 1982; Li et al. 2016).

Assays for antimicrobial activity

The test organisms included two Gram−positive bacteria (*Staphyloccocus aureus* and *Bacillus subtilis*), two Gram−negative bacteria (*Escherichia coli, Kiebsiella* sp.), and seven phytopathogenic fungi strains (*Penicillium* sp., *Fusarium graminearu, Ceratobasidium cornigerum, Thanatephorus cucumeris, Botrytis cinerea* Pers, *Alternaria solani, Sclerotinia sclerotiorum*).The fungin strains were grown and maintained on potato dextrose agar (PDA), and the bacteria strains were on Luria-Bertani solid medium (LB).

Cross steak method for antimicrobial activity **(**Williston et al. 1947). The absence of growth or a less dense growth of test bacteria near the growth of isolate was considered positive for the production and secretion of antibacterial metabolite. And the results according to Li et al (2016): no antibacterial activity (no zone of inhibition); weak antibacterial activity (diameter of zone, 6-15 mm), strong antibacterial activity (diameter of zone, >15 mm).

Amplification and sequencing of 16S rDNA and construction of the phylogenetic trees

The 16S rDNA amplification was applied for the molecular identification. The forward primer used was p27f (5'−AGAGTTTGATCCTGGCTCAG−3'), whereas the reverse primer was p1492r (5'−TACGGCTAC CTTGTTACGACTT−3'). PCR reaction (50 µL) contained the following: a hot start performed at 95℃for 5min and 30 cycles at 95℃ for 30 s, 55℃ for 30 s, and 72℃ for 90 s, followed by a final extension performed at 72℃ for 10 min. The calculations of level of sequence similarity for the 16S rRNA gene sequences were carried out using the EzTaxon server 2.1 (Yoon et al. 2017). The phylogenetic trees were constructed from evolutionary distance matrices by the neighbour-joining method using the software package MEGA 5.The 16S rDNA gene sequences have been deposited in GenBank under the accession Nos. MH549241-MH549258.

RESULTS

Isolation of Actinobacteria strains

In order to obtain more actinobacteria strains, five different selective media were used. Finally, the 15 strains were isolated, which maximum number of isolates were obtained in GS medium from HX and QC followed by HV medium and GW1, R2A, CMKA medium respectively (Table 1).

The previous investigation showed the number of active strains was found basis on many factors influenced like method of screening, the medium and so an (Hayakawa et al. 1987; Zucchi et al. 2011;

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Sharma et al. 2012; Arumugam et al. 2017). The present result are consistent with the previous conclusion by high throughput sequencing that Actinobacteria content is relatively low in GT(Zhang et al. 2014).

Table 1 the isolated results from the on three types of Daqu

Daqu type / Medium	QC	HX
GS	ZYP3, ZYP10	ZYP6,ZYP7, ZYP12, ZYP13, ZYP15, ZYP16, ZYP17, ZYP18
GW1		ZYP11
R2A		ZYP9
CMKA		ZYP8
HV	ZYP14	ZYP1

Potential of isolates in extracellular enzymes

The enzyme-producing ability of isolated strains was explored, which helps us understand the potential of isolated strains in Daqu. Microbiology of Daqu affects its characteristics and functions by their metabolic products, such as amylase, protease, cellulose, and so on. The results showed strains ZYP1, ZYP6, ZYP9, ZYP11, ZYP 12, ZYP14, ZYP15, ZYP16, ZYP17, ZYP18 were found to secret amylase, protease, cellulase simultaneously, which implied that the actinobacteria participate in the process of saccharification which is the basic functional process in Daqu.

Antagonistic potential of the isolates

The Antimicrobial potential of active isolates was screened against fungi strain and against four bacterial strains. It was observed the 15 strains showed different affection on tests of antagonism against phytopathogenic fungi and bacteria. Strains ZYP1, ZYP7, ZYP13 exerted broad-spectrum anti-bacteria activity (Table 2); Strains ZYP6, ZYP11, ZYP12, ZYP16 exerted broad-spectrum anti-fungal activity (Table 3).The results demonstrated the isolates in Daqu can inhibit the pathogenic organisms that are harmful to human beings in the environment.

Phylogenetic analysis of isolates

The phylogenetic tree was shown in Figure 1, based on publicly available 16S rRNA gene sequences. 16S rRNA gene sequence analysis indicated the 15 strains belong to *Streptomyces*, which means *Streptomyces* is the dominant genus in isolated strains from Daqu. The actinobacteria were found to be the most predominant genera from the high throughput sequencing, which is in accordance with our results.

Table 2 Inhibition affection of 15 strains to four bacteria

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Test strains / Strains	*Escherichia coli*	*Staphylococcus aureus*	*Bacillus subtilis*	*Kiebsiella* sp.
ZYP1	—	+	++	+++
ZYP3	—	—	—	+++
ZYP6	—	+	+	—
ZYP7	+	—	+	++
ZYP8	—	—	++	++
ZYP9	—	—	—	—
ZYP10	—	—	—	—
ZYP11	—	—	—	—
ZYP12	—	—	—	—
ZYP13	++	—	++	++
ZYP14	—	—	++	—
ZYP15	—	—	++	—
ZYP16	—	—	+	—
ZYP17	+	+	—	—
ZYP18	—	+	—	—

notes：“+” indicated the diameter of inhibition is less than 15 mm，“++” means the diameter of inhibition is between 15-20 mm，“+++” indicated the diameter of inhibition is more than 20 mm, “—” means not determined.

Table 3 Inhibition affection of the 15 strains to seven fungal phytopathogens

	1	3	6	7	8	9	10	11	12	13	14	15	16	17	18
T. cucumeris	++	—	+	—	—	—	+	—	—	—	—	+	+	—	—
S. sclerotiorum	+	+	+++	+	+	+	+++	++	+	—	—	++	+	+	—
Penicillium sp.	+	+	+	+	+	+	—	+	+	+	+	—	+	+	+
B. cinerea Pers	—	—	++	—	+	—	—	+	+	—	+	+	—	+	+
A. solani	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—
C. cornigerum	—	+	+	—	—	+	—	+	+	—	+	—	+	—	+
F. graminearu	—	++	—	—	—	—	—	++	++	—	—	—	+	—	—

notes：“+” indicated the diameter of inhibition is less than 15 mm，“++” means the diameter of inhibition is between 15-20 mm，“+++” indicated the diameter of inhibition is more than 20 mm

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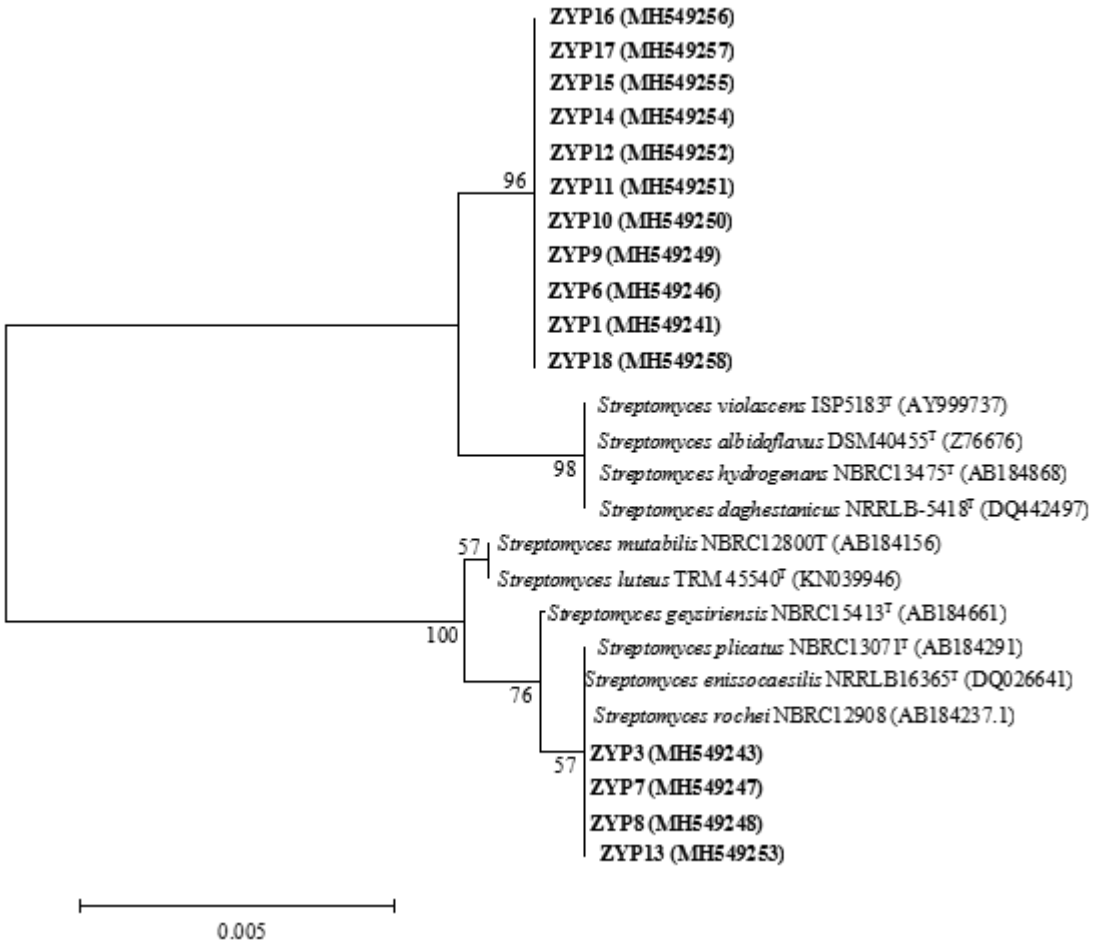

Figure 1. Phylogenetic tree based on 16S rRNA partial gene sequences. Comparision obtained using the neighbor-joining method, showing the position of the isolates and representative species in Daqu. bootstrap values (expressed as percentage of 1000 replications) are shown at the branch points. Bar, 5 nt substitutions per 1000 nt.

DISCUSSION

Actinomycetes have attracted wide attention for producing a large number of secondary metabolites, and *Streptomycetes* are among Nature's most competent chemists and produce a stunning multitude and diversity of bioactive secondary metabolites (Barka et al. 2016), Now, many researchers began to isolate actinobacteria from different environments (Golinska et al. 2015; Zhang et al.2016; Baoune et al.2018; Kumar et al., 2018).

In recent years, the species of microorganisms in Daqu have become clear gradually by high-throughput sequencing. Many research results indicated that actinobacteria is the dominant bacteria groups from the different type Daqu. However, there is no report of the biocontrol function of the actinobacteria from the Fen-Daqu or other different type Daqu. Zhao et al used the Fen-Daqu to produce the Bio-organic fertilizers to manipulation of the rhizosphere microbial community. They found that the soil actinobacteia and proteobacteria increased (Zhao et al. 2018). The studies revealed

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that the isolated filamentous bacteria are belonged to *Actinobacteria*, our present research work indicated *Streptomyces* is the dominant genus in actinomycetes species from Daqu, and showed remarkable potential of antimicrobial properties. Which implied *Streptomyces* spp. from Daqu be used in medicine, industry and agriculture, and so an.

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