Original article

Cultivable bacterial diversity and amylase production in three typical Daqus of Chinese spirits

Zuming Li,^{1,2} Zhihui Bai,^{2*} Deliang Wang,³ Wujiu Zhang,³ Michael Zhang,⁴ Francis Lin,⁴ Liping Gao,¹ Bodi Hui¹ & Hongxun Zhang²

1 College of Arts and Sciences of Beijing Union University, Beijing 100191, China

2 Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, China

3 China National Research Institute of Food and Fermentation Industries, Beijing 100027, China

4 Department of Physics and Astronomy, University of Manitoba, Winnipeg, MB R3T 2N2, Canada

(Received 22 June 2013; Accepted in revised form 28 August 2013)

Summary Both culture-dependent method and molecular technique were firstly used to simultaneously investigate the cultivable bacterial diversity and amylase production in three typical Daqus of Chinese spirits. The results showed that both cultivable bacterial diversity and amylase production were obviously different. The species of nine bacteria from Deshan, nine from Baisha and six from Wuling Daqus were identified. The total bacterial strains of 17, 15 and 14, and 9, 16 and 10 could produce α -amylase and glucoamylase, respectively, from the Daqus, and the enzyme yields were different. *Bacillus licheniformis*, *Bacillus subtilis* and *Bacillus amyloliquefaciens* not only were dominant bacteria in the Daqus, but also possessed high activities of α -amylase and glucoamylase. By comparison, *Bacillus cereus* and *Bacillus oleronius* were found to be another predominant bacterial species and good producers of α -amylase and glucoamylase in Deshan and Wuling Daqus, respectively.

Keywords 16S rRNA gene, bacterial diversity, Daqu, glucoamylase, α -amylase.

Introduction

Chinese spirits are commonly known as ‘Chinese liquor’, which is one of the six most well-known distilled spirits in the world (Wang *et al.*, 2011). The total annual production of Chinese spirits was estimated at approximately 5 million metric tons per year, and the reason of the popularity of Chinese spirits is the diversity of flavours (Zheng *et al.*, 2012). According to the flavours, Chinese spirits can be roughly classified into strong flavour, light flavour, sauce flavour and strong sauce flavour (Shi *et al.*, 2009; Liu *et al.*, 2012). The spirits are made from cereals, mainly sorghum, by fermentation, distillation and maturation. Mixed culture starters are locally called ‘Daqu’. Daqu, which is made from raw wheat, barley and/or pea, is used as inoculum for the fermentation of Chinese spirits (Zheng *et al.*, 2012).

Daqu is an important saccharifying and fermenting agent for the production of Chinese spirits, associated with the degradation of starch to produce alcohol, and also is a determinative factor for the flavouring of

spirits (Zheng *et al.*, 2011; Liu *et al.*, 2012; Zheng *et al.*, 2012). During Daqu-making process, the raw materials are typically milled, mixed with water, pressed into a brick-shaped block and then incubated under nonsterile conditions (Zhang *et al.*, 2011). Daqu contains microbes and enzymes, and both glucoamylase and α -amylase are two important amylases for starch hydrolysis (Yoshizaki *et al.*, 2010; Qian *et al.*, 2011). In brewing industry, the hydrolysates are used as nutrients in microbial fermentation for ethanol production after enzymatic hydrolysis (Dhale & Vijay-Raj, 2009). However, amylase production of bacteria from Daqus is poorly understood. During fermentation, degradation of biopolymers such as starch and formation of aromatic compounds are usually done by microbes (Wang *et al.*, 2008). Therefore, it is important to check the exact composition of the natural product microflora, characterise and distinguish them at a species level (Chen *et al.*, 2011). However, very little is known about the specific microbes associated with Daqus, despite the fact that the subject has been studied by some microbiologists (Li *et al.*, 2011).

Traditional microbiological methods, such as isolation and enumeration in selective media, are effective

*Correspondent: Fax: +86 10 62923563;
e-mail: zhbai@rcees.ac.cn

in qualitative and quantitative analysis (Wang *et al.*, 2008; Shi *et al.*, 2009). The microorganisms were isolated from Daqus and then physiological and morphological tests are typically employed for identification. However, phenotypic tests required prolonged periods, and microbial phenotypic characteristics are often negatively affected by culture conditions. Those inaccurate descriptions of characteristics may lead to the misidentification of microbial species (Liu *et al.*, 2012). Molecular methods provide a precise insight into microbial diversity and allow a rapid, high-resolution description of microbial communities by targeting ribosomal genes, which have been used to distinguish or identify the bacteria and fungi associated with Chinese spirits and other foods (Shi *et al.*, 2009; Li *et al.*, 2011; Liu *et al.*, 2012; Zheng *et al.*, 2012). 16S rRNA gene sequence analysis is commonly used for identifying bacteria and constructing bacterial phylogenetic relationships (Huang *et al.*, 2012). PCR-based molecular marker methods have widely been applied in many fields such as in food, agriculture and medicine. In food processing and food preservation, the PCR is a very good tool for quality assurance and food microbe detection (Minh & Rakshit, 2009). For example, the microbial diversity of Irish edible brown seaweeds had been investigated by combined culture-dependent method and 16S rRNA molecular technique (Gupta *et al.*, 2010). The microbial population densities and speciation of bacterial communities in dried dairy dietary supplements had been investigated using serial dilution plating methods and 16S rRNA molecular techniques, respectively (Hayes *et al.*, 2012). In this study, we firstly report an investigation of the cultivable bacterial diversity and amylase production from three typical Daqus of Chinese spirits.

Materials and methods

Daqu samples

The three Daqus were obtained from three main factories of Chinese spirits in Hunan province, and they are famous Chinese spirits in China. The sampling data are shown in Table 1. Three parallel samples were collected from the top, middle and bottom of the store room. After milling and mixing, samples were then transferred to sterile bags, sealed and stored at 4 °C for further use.

Strain isolation and screening

Serial dilution plating method was used for the isolation. Ten grams of Daqu powder was homogenised in 90 mL of sterile 0.9% NaCl solution in a rocking incubator for 30 min at 180 rev min⁻¹. The homogenised samples (1.0 mL) were serially diluted 10-fold in sterile 0.9% NaCl solution, from which aliquots

Table 1 Samples of three typical Daqus of Chinese spirits

Sample no.	Name of Daqu	Flavour type	Highest temperature inside the Daqu pile (°C)	Factory and Region (province, city)
W	Wuling	Sauce flavour	65	Hunan Wuling Spirits Co., Ltd., Hunan, Changde
B	Baisha	Strong sauce flavour	60	Changsha Baisha Spirits Co., Ltd., Hunan, Changsha
D	Deshan	Strong flavour	55	Hunan Deshan Wine Industry Co., Ltd., Hunan, Changde

(0.1 mL) were spread on beef extract peptone agar plates containing (w/v) 0.5% beef extract, 1.0% peptone and 0.5% NaCl. Inoculated plates were incubated at 37 °C for 24–48 h for the isolation and screening on the basis of different colony morphologies (diameter, shape, colour, surface and spore).

DNA extraction and amplification

A previously published extraction method was used to extract the genomic DNA of the isolates (Li *et al.*, 2005). The genes coding for bacterial 16S rRNA (16S rDNA) were amplified from the genomic DNA of the isolates with the primer pair 27f (20 bp) (5'-AGAG TTGATCMTGGCTCAG, M = C:A) and 1492r (22 bp) (5'-TACGGYTACCTTGTTACGACTT, Y = C:T). These are standard primers appropriate for most eubacteria, and the product represents >97% of the 16S rDNA sequence (*Escherichia coli* standard). The amplified product of the appropriate size was about 1.5 kbp. PCR cycling was performed with an Applied Biosystems Gene Amp PCR system 2700. PCR amplification reaction conditions were as previously described (Li *et al.*, 2005, 2012).

DNA sequencing and nucleotide accession number

The amplified product of the appropriate size (1.5 kbp) was sequenced by BGI LifeTech Co., Ltd., Beijing, China. 16S rRNA gene sequence data of the isolates reported in this article have been submitted to GenBank nucleotide sequence databases with the accession numbers.

Amylase production

The isolates from the Daqus were diluted in sterile 0.9% NaCl solution and then plated onto starch agar

plates containing 2.0% soluble starch, 1.0% peptone and 0.5% NaCl. Inoculated plates were incubated for 1–3 days at 37 °C to obtain colonial growth. The incubated plates were stained with 0.3% iodine solution, and the colonies with clear zones formed by the hydrolysis of starch were evaluated as amylase producers.

The medium used for liquid inoculum culture contained (w/v) beef extract 0.3%, peptone 1.0%, NaCl 0.5%, pH 7.2. The medium was sterilised by autoclaving at 121 °C for 20 min. Medium (20 mL) in the 150-mL Erlenmeyer flasks was inoculated and incubated at 37 °C by shaking at 180 rev min⁻¹ for 24 h.

The medium in a 500-mL Erlenmeyer flask contained (w/v) soluble starch 2.0%, beef extract 0.5%, peptone 1.0% and NaCl 0.5%, pH 7.2. After sterilisation, the medium (100 mL) was inoculated with 6 mL of a 24-h-old inoculum culture, incubated at 37 °C by shaking at 180 rev min⁻¹ for 48 h, and then was centrifuged at 8000 rev min⁻¹ at 4 °C for 15 min to remove the cells and debris. The supernatant was designated as the crude enzyme for future enzyme activity assays.

Enzyme assays

For α -amylase assays, the starch-iodine method was used (Xie *et al.*, 2007; Shankar *et al.*, 2009). The crude enzyme (0.1 mL) was added to 4.0 mL of 0.2 M Na₂HPO₄/C₆H₈O₇·H₂O buffer solution (pH 6.0) containing 1.0% soluble starch at 60 °C. After 30 min of enzyme reaction, 10 mL of 0.5 M HAc was added to the solution. Then 1 mL of the enzyme reaction mixture was added to 10 mL of iodine solution. Absorbance was measured at 620 nm using spectrophotometer (WFZ UV-2000; Unico [Shanghai] Instrument Co., Ltd, Shanghai, China). One unit of α -amylase activity was defined as the amount of enzyme that hydrolysed 1 mg of starch per hour under the assay conditions. The experiments were run in triplicate, and all standard deviations were under 5%.

Glucoamylase was determined by measuring released reducing sugars using DNS (dinitrosalicylic acid) method (Smith & Zahnley, 2005; Kumar & Satyanarayana, 2009). Activity of glucoamylase was evaluated using 1.0% of starch solution in 0.2 M Na₂HPO₄/C₆H₈O₇·H₂O buffer (pH 6.0) as substrate. A known volume of 0.5 mL of crude enzyme was added to 1.5 mL of substrate solution that had been pre-incubated at 40 °C for 5 min. The enzyme–starch mixture was incubated for 30 min at 40 °C and then 1.5 mL of DNS reagent was added. Samples were boiled for 15 min, cooled to room temperature and diluted with 21.5 mL H₂O. The absorbance of the samples at 540 nm was determined using spectrophotometer (WFZ UV-2000, Unico [Shanghai] Instrument Co., Ltd). One unit of glucoamylase activity was defined as the amount of enzyme that liberated 1 mg of reducing

sugar as glucose per hour under the assay conditions. The experiments were run in triplicate, and all standard deviations were under 5%.

Results and discussion

Bacterial diversity

A total of thirty-four, twenty-four and fifty-six bacterial isolates were randomly screened and identified by sequencing the 16S rDNA from Deshan, Baisha and Wuling Daqu samples, respectively. The sequences had been submitted to the GenBank database with accession numbers (Tables 2–4). For the three bacteria, the sequences fell into nine, nine and six species. *Bacillus* genus was predominant in the Daqus. Indeed, 85.3% of the sequences identified from Deshan Daqu belonged to the species of *B. licheniformis*, *B. subtilis*, *B. cereus* and *B. amyloliquefaciens*, while 70.8% of the sequences identified from Baisha Daqu belonged to the species of *B. licheniformis*, *B. subtilis* and *B. amyloliquefaciens*. Other bacteria such as *B. megaterium*, *Geobacillus stearothermophilus*, *B. altitudinis*, *Brevibacillus parabrevis* and *B. anthracis* were encountered in Deshan Daqu. Meanwhile, *B. cereus*, *B. fordii*, *B. flexus*, *B. methylotrophicus*, *Staphylococcus kloosii* and *B. sonorensis* were found in Baisha Daqu. In contrast, 94.6% of the sequences were identified from Wuling Daqu, which belonged to the species *B. licheniformis*, *B. subtilis*, *B. amyloliquefaciens* and *B. oleronius*. Other bacteria such as *B. circulans* and *B. sonorensis* were observed in Wuling Daqu.

To understand the phylogenetic position of these strains from the typical Daqus, we constructed phylogenetic trees based on the comparison of 16S rDNA sequences of the strains from the Daqus and those of reference bacteria (Figs 1a,b, 2 and 3a,b).

Daqu is a mixture of grains, microorganisms, enzymes and aroma precursors. Using Daqu as a starter culture is the unique characteristic of the Chinese spirits. The specific metabolic characteristics of the microorganisms in Daqus can greatly affect the yield and quality of the Chinese spirits; for example, α -amylase and glucoamylase produced from microorganisms in Daqus play a key role in starch degradation for supplying fermentable sugars for the fermentation process of the Chinese spirits. Owing to differences in the climate of the brewing geographical origins, production techniques, fermentation process, etc., bacterial diversity, amylase production and Daqu intensity of flavours between the three Daqus were obviously different (Qin *et al.*, 2012).

Bacillus species were the most frequently isolated bacteria from Deshan, Baisha and Wuling Daqus (Figs 1 including a and b, 2 and 3 including a and b). This observation is consistent with those of previous studies (Wang *et al.*, 2008; Shi *et al.*, 2009; Zheng

Table 2 Bacterial diversity and amylase production in Deshan Daqu

Strain no.	Related GenBank sequence	Closest relative (NCBI accession no.)	Identity /%	HC value	Activities of α -amylase U ⁻¹ mL ⁻¹	Activities of glucoamylase U ⁻¹ mL ⁻¹
D1	KC441750	<i>B. licheniformis</i> HT18 (JN013203)	100	–	–	–
D2	KC441751	<i>B. amyloliquefaciens</i> WAS2-1 (JF496509)	99.9	1.3 $\pm$ 0.1	16.1 $\pm$ 0.6	30.1 $\pm$ 1.3
D3	KC441752	<i>B. subtilis</i> LXA7 (GQ861467)	99.9	1.8 $\pm$ 0.2	5.6 $\pm$ 0.3	28.4 $\pm$ 1.2
D4	KC441753	<i>B. licheniformis</i> LZBL-9 (JX847115)	100	–	–	–
D5	KC441754	<i>B. megaterium</i> SZ-3 (HM061611)	99.9	–	–	–
D6	KC441783	<i>Geobacillus stearothermophilus</i> DDKRC4 (JN641292)	99.9	–	–	–
D7	KC441755	<i>B. licheniformis</i> LZBL-13 (JX847119)	99.9	1.4 $\pm$ 0.1	6.4 $\pm$ 0.3	15.9 $\pm$ 0.7
D8	KC441756	<i>B. amyloliquefaciens</i> SDF004 (JQ062537)	99.9	–	–	–
D13	KC441757	<i>B. subtilis</i> LXA7 (GQ861467)	99.9	1.6 $\pm$ 0.2	15.4 $\pm$ 0.5	31.2 $\pm$ 1.3
D15	KC441758	<i>B. amyloliquefaciens</i> Nt1-7 (HQ831418)	99.9	2.6 $\pm$ 0.3	36.2 $\pm$ 1.6	43.8 $\pm$ 1.9
D18	KC441759	<i>B. amyloliquefaciens</i> Ht1-1 (JF899275)	99.9	1.9 $\pm$ 0.2	20.4 $\pm$ 0.8	31.8 $\pm$ 1.4
D19	KC441760	<i>B. cereus</i> Cr-50 (JF895490)	98.9	–	–	–
D20	KC441761	<i>B. amyloliquefaciens</i> T004 (HQ840415)	100	–	–	–
D21	KC441762	<i>B. cereu</i> CP1 (HQ833025)	99.5	1.7 $\pm$ 0.2	14.2 $\pm$ 0.5	–
D23	KC441763	<i>B. subtilis</i> LXB3 (GQ861468)	99.9	–	–	–
D24	KC441764	<i>B. amyloliquefaciens</i> BAB-807 (KC250199)	100	2.1 $\pm$ 0.3	11.9 $\pm$ 0.4	12.3 $\pm$ 0.4
D26	KC441765	<i>B. cereus</i> JY7 (HQ833025)	99.7	–	–	–
D28	KC441766	<i>B. subtilis</i> CBZ-2 (KC142124)	100	1.7 $\pm$ 0.2	7.7 $\pm$ 0.3	19.1 $\pm$ 0.8
D29	KC441767	<i>B. subtilis</i> HT19 (JN013204)	100	2.1 $\pm$ 0.3	21.3 $\pm$ 0.8	38.9 $\pm$ 1.7
D30	KC441768	<i>B. cereus</i> KVP109 (JX290089)	100	1.5 $\pm$ 0.1	15.2 $\pm$ 0.5	10.1 $\pm$ 0.4
D31	KC441769	<i>B. subtilis</i> SY-A4 (JN656163)	100	–	–	–
D34	KC441770	<i>B. altitudinis</i> JF31 (KC172006)	100	–	–	–
D35	KC441771	<i>B. subtilis</i> YT2 (HQ143571)	99.9	–	–	–
D36	KC441772	<i>Brevibacillus parabrevis</i> NBRC 12335 (AB680275)	99.9	1.7 $\pm$ 0.2	1.9 $\pm$ 0.1	–
D38	KC441773	<i>B. licheniformis</i> BaDB24 (JX237858)	100	1.4 $\pm$ 0.1	5.8 $\pm$ 0.3	64.9 $\pm$ 2.8
D39	KC441774	<i>B. licheniformis</i> SCC112026 (JN998723)	99.9	–	–	–
D40	KC441775	<i>B. anthracis</i> SH123(KC172044)	99.9	1.7 $\pm$ 0.2	10.3 $\pm$ 0.4	20.1 $\pm$ 0.8
D41	KC441776	<i>B. amyloliquefaciens</i> FJWHG14 (HQ853016)	100	2.2 $\pm$ 0.3	26.5 $\pm$ 1.1	57.5 $\pm$ 2.5
D42	KC441777	<i>B. cereus</i> FM10 (DQ289077)	99.4	–	–	–
D43	KC441778	<i>B. licheniformis</i> WAS3-5 (JF496512)	99.9	1.5 $\pm$ 0.1	13.3 $\pm$ 0.4	31.3 $\pm$ 1.3
D46	KC441779	<i>B. licheniformis</i> KJN-1 (JQ619622)	99.9	–	–	–
D48	KC441780	<i>B. licheniformis</i> ISA7 (HQ189751)	100	–	–	–
D49	KC441781	<i>B. cereus</i> CP1 (JX544748)	98.9	–	–	–
D50	KC441782	<i>B. licheniformis</i> NJ-5 (FJ435674)	100	1.2 $\pm$ 0.1	10.7 $\pm$ 0.4	14.6 $\pm$ 0.5

–, means the value of parameter was not detected.

et al., 2012). The *Bacillus* dominant in the Daqus was an endospore-producing bacterium, which was able to endure an unfavourable environment, and had a better ability than other bacteria to survive under low moisture content (13–14%) and high temperature (60–65 °C) (Liu *et al.*, 2012). *Bacillus* species are important sources of proteases and amylases, and the hydrolytic capabilities of these microorganisms can result in a precursor-rich environment, which is useful for subsequent reactions leading to flavour production (Wang *et al.*, 2008). In addition, *Bacillus* spp. produce nitrogenous flavour compounds such as diverse pyrazines to contribute to Daqu flavour (Zheng *et al.*, 2012). We noted that *B. licheniformis* and *B. subtilis* were the dominant bacteria in the Daqus (Figs 1, 2 and 3). This is in accordance with earlier reports (Wang *et al.*, 2008; Shi *et al.*, 2009; Zheng *et al.*, 2012).

Although Deshan, Baisha and Wuling Daqus were all produced in Hunan province, they are strong flavour Daqu, strong sauce flavour Daqu and sauce flavour Daqu, respectively. The cultivable bacterial diversity was obviously different between them. It was observed that the dominant bacteria existing in the Daqus were *B. licheniformis*, *B. subtilis* and *B. amyloliquefacien* (Figs 1, 2 and 3). *Bacillus amyloliquefaciens* was also found in Daqu of Maotai spirit (Liu *et al.*, 2012). We also found that *B. cereus* and *B. oleronius* were another dominant bacterial species in Deshan and Wuling Daqus, respectively (Figs 1 and 3). But *B. oleronius* was not the dominant bacterial species in Maotai Daqu, which is a sauce flavour Daqu from Guizhou Province (Liu *et al.*, 2012). Other species, such as *B. anthracis*, *B. altitudinis*, *B. megaterium*, *Geobacillus stearothermophilus* and *Brevibacillus parabrevis*, were all in Deshan

Table 3 Bacterial diversity and amylase production in Baisha Daqu

Strain no.	Related GenBank sequence	Closest relative (NCBI accession no.)	Identity /%	HC value	Activities of α -amylase U ⁻¹ mL ⁻¹	Activities of glucoamylase U ⁻¹ mL ⁻¹
B2	KC441726	<i>B. licheniformis</i> BaDB24 (JX237858)	100	1.5 ± 0.1	8.3 ± 0.3	9.3 ± 0.3
B5	KC441727	<i>B. licheniformis</i> LZBL-9 (JX847115)	99.9	1.2 ± 0.1	4.5 ± 0.2	36.2 ± 1.6
B8	KC441728	<i>B. licheniformis</i> 5.22 (HE801988)	100	1.6 ± 0.2	6.8 ± 0.3	–
B10	KC441729	<i>B. fordii</i> 2-6AIA (FN397482)	99.9	–	–	–
B11	KC441730	<i>B. licheniformis</i> DQgbc4 (GQ470399)	100	1.1 ± 0.1	10.9 ± 0.4	–
B12	KC441731	<i>B. licheniformis</i> 30N1-9 (JN366720)	99.9	–	–	–
B18	KC441732	<i>B. flexus</i> WY2 (JQ936679)	99.9	1.6 ± 0.2	4.3 ± 0.2	10.3 ± 0.4
B30	KC441733	<i>B. licheniformis</i> HT18 (JN013203)	100	–	–	–
B32	KC441734	<i>B. methylotrophicus</i> JF38 (KC172013)	100	–	–	–
B39	KC441735	<i>B. subtilis</i> Aj080718IA-25 (HQ272971)	99.7	2.3 ± 0.3	26.5 ± 1.1	4.5 ± 0.2
B40	KC441736	<i>B. licheniformis</i> TSM2 (JX847115)	99.9	–	–	–
B47	KC441737	<i>B. amyloliquefaciens</i> KtVC2-2 (KC122196)	100	1.7 ± 0.2	11.4 ± 0.4	12.5 ± 0.4
B49	KC441747	<i>B. licheniformis</i> (JN166722)	99.7	1.8 ± 0.2	12.6 ± 0.4	11.9 ± 0.4
B51	KC441749	<i>B. licheniformis</i> SD2 (HM055611)	99.6	–	–	–
B53	KC441738	<i>B. subtilis</i> FS106 (KC179631)	100	1.9 ± 0.2	13.4 ± 0.5	50.1 ± 2.2
B54	KC441739	<i>B. subtilis</i> VITM6 (JX981919)	100	1.8 ± 0.2	14.1 ± 0.5	8.3 ± 0.3
B57	KC441740	<i>B. amyloliquefaciens</i> BAB-807 (KC250199)	99.9	2.1 ± 0.3	18.5 ± 0.7	6.3 ± 0.3
B59	KC441741	<i>B. subtilis</i> NS04 (JX126865)	99.9	1.2 ± 0.1	4.6 ± 0.2	–
B64	KC441742	<i>B. cereus</i> C (JX544747)	100	1.6 ± 0.2	10.8 ± 0.4	–
B68	KC441743	<i>B. licheniformis</i> 2J-1 (FJ493045)	99.9	1.2 ± 0.1	3.5 ± 0.1	–
B69	KC441744	<i>Staphylococcus kloosii</i> S7-447 (JQ660231)	99.9	–	–	–
B71	KC441745	<i>B. licheniformis</i> HT17 (JN013202)	100	–	–	–
B81	KC441746	<i>B. flexus</i> MDLD1 (FJ861081)	100	–	–	–
B83	KC441748	<i>B. sonorensis</i> HT1 (JN013186)	100	–	–	–

–, means the value of parameter was not detected.

Daqu, while *B. fordii*, *B. flexus*, *B. methylotrophicus* and *Staphylococcus kloosii* were only observed in Baisha Daqu. Additionally, *B. circulans* were found only in Wuling Daqu. Although both Maotai Daqu and Wuling Daqu belong to sauce flavour Daqu, the cultivable bacterial diversity between them is obviously different. Nested PCR–DGGE analysis of bacterial DNA from the Maotai Daqu highlighted the presence of various species, for example, *B. licheniformis*, *B. subtilis*, *B. sonorensis*, *Bacillus* sp., *Virgibacillus* sp. and *Lactobacillus buchneri* (Liu et al., 2012). Bacteria in Maotai Daqu, which was revealed by culture-dependent method, consisted of *Bacillus*, *Acetobacter*, *Lactobacillus* and *Clostridium*, among which *Bacillus* strains were found to be predominant (Wang et al., 2008). A clear advantage of culturing is that a collection of pure cultures is obtained that can be used for further experimentation (Zheng et al., 2012). It is similar that *Bacillus* is a dominant bacterial genus between them, while *B. subtilis* and *B. licheniformis* are their predominant bacterial species too (Wang et al., 2008; Liu et al., 2012).

Amylase production

Following staining with iodine solution, the isolates from the three Daqus, which exhibited visible clear

zones around the colonies on agar plates with starch as the sole carbon source, were selected as good producers of amylase (Tables 2–4).

A total of seventeen and fifteen bacterial isolates from Deshan Daqu could produce extracellular α -amylase and glucoamylase with the maximum yield of 36.2 U mL⁻¹ by *B. amyloliquefaciens* D15 and 64.9 U mL⁻¹ by *B. licheniformis* D38 (Table 2). The activity of α -amylase and glucoamylase produced by *B. licheniformis*, *B. subtilis*, *B. amyloliquefaciens* and *B. cereus* accounted for 15.2%, 28.2%, 20.9% and 26.1%, and 46.5%, 39.0%, 12.3% and 2.2%, respectively. Furthermore, *B. anthracis* could produce both α -amylase and glucoamylase, while *Brevibacillus parabrevis* could produce merely α -amylase.

Fourteen and nine bacteria could be isolated from Baisha Daqu, which could produce α -amylase and glucoamylase (Table 3). *Bacillus subtilis* B39 produced the maximum α -amylase yield of 26.5 U mL⁻¹, and the maximum glucoamylase yield of 50.1 U mL⁻¹ was produced by *B. subtilis* B53. The activity of α -amylase and glucoamylase produced by *B. licheniformis*, *B. subtilis* and *B. amyloliquefaciens* accounted for 31.0%, 38.4% and 39.0%, and 42.1%, 19.9% and 12.6%, respectively. In addition, *B. flexus* could produce both α -amylase and glucoamylase, whereas *B. cereus* could produce merely α -amylase.

Table 4 Bacterial diversity and amylase production in Wuling Daqu

Strain no.	Related GenBank sequence	Closest relative (NCBI accession no.)	Identity /%	HC value	Activities of α -amylase U ⁻¹ mL ⁻¹	Activities of glucoamylase U ⁻¹ mL ⁻¹
W1	KC441816	<i>B. subtilis</i> C8-4 (EU257436)	99.7	2.1 ± 0.3	20.8 ± 0.8	12.6 ± 0.4
W4	KC441817	<i>B. licheniformis</i> HT9 (JN013194)	100	1.7 ± 0.2	12.9 ± 0.4	2.8 ± 0.1
W5	KC441818	<i>B. oleronius</i> 30N3-3 (JN366766)	98.5	1.4 ± 0.1	8.4 ± 0.3	20.7 ± 0.8
W6	KC441819	<i>B. licheniformis</i> DQgbc4 (GQ470399)	99.9	—	—	—
W7	KC441820	<i>B. licheniformis</i> HT9 (JN013194)	100	—	—	—
W8	KC441821	<i>B. licheniformis</i> LZBL-13 (JX847119)	100	—	—	—
W10	KC441822	<i>B. amyloliquefaciens</i> BAB-807 (KC250199)	99.9	—	—	—
W11	KC441823	<i>B. licheniformis</i> LZBL-13 (JX847119)	100	1.2 ± 0.1	1.4 ± 0.1	4.5 ± 0.2
W12	KC441824	<i>B. licheniformis</i> LZBL-13 (JX847119)	99.9	—	—	—
W13	KC441825	<i>B. licheniformis</i> LZBL-13 (JX847119)	99.9	—	—	—
W14	KC441826	<i>B. licheniformis</i> 2J-1 (FJ493045)	99.9	—	—	—
W15	KC441827	<i>B. licheniformis</i> ML1 (HQ850703)	99.6	—	—	—
W16	KC441828	<i>B. amyloliquefaciens</i> SWM1 (JN851189)	99.9	—	—	—
W17	KC441829	<i>B. licheniformis</i> HU14 (EF101734)	99.9	—	—	—
W18	KC441830	<i>B. licheniformis</i> LZBL-9 (JX847115)	100	1.5 ± 0.1	3.2 ± 0.1	0.9 ± 0.1
W19	KC441831	<i>B. oleronius</i> ATCC 700005 (NR_043325)	99.5	—	—	—
W20	KC441832	<i>B. licheniformis</i> LZBL-13 (JX847119)	99.7	—	—	—
W21	KC441833	<i>B. oleronius</i> Gx8 (JF833092)	99.4	1.6 ± 0.2	9.6 ± 0.4	—
W22	KC441834	<i>B. oleronius</i> ATCC 700005 (NR_043325)	99.7	1.7 ± 0.2	10.7 ± 0.4	—
W23	KC441835	<i>B. subtilis</i> 9A (KC196261)	99.9	1.8 ± 0.2	6.5 ± 0.3	6.2 ± 0.3
W24	KC441836	<i>B. amyloliquefaciens</i> BAB-807 (KC250199)	100	1.9 ± 0.2	8.8 ± 0.3	3.1 ± 0.1
W25	KC441837	<i>B. amyloliquefaciens</i> BAB-807 (KC250199)	99.5	2.0 ± 0.3	14.3 ± 0.5	30.3 ± 1.3
W27	KC441838	<i>B. licheniformis</i> HT13 (JN013198)	99.8	—	—	—
W28	KC441839	<i>B. licheniformis</i> LZBL-13 (JX847119)	99.9	—	—	—
W30	KC441840	<i>B. licheniformis</i> LZBL-13 (JX847119)	99.9	—	—	—
W31	KC441841	<i>B. licheniformis</i> KJN-1 (JQ619622)	100	—	—	—
W32	KC441842	<i>B. licheniformis</i> LZBL-13 (JX847119)	100	—	—	—
W33	KC441843	<i>B. licheniformis</i> HT13 (JN013198)	99.9	—	—	—
W34	KC441844	<i>B. licheniformis</i> HT13 (JN013198)	99.9	—	—	—
W35	KC441845	<i>B. licheniformis</i> HT9 (JN013194)	99.9	1.3 ± 0.1	3.6 ± 0.1	—
W36	KC441846	<i>B. licheniformis</i> 2J-1 (FJ493045)	99.9	—	—	—
W37	KC441847	<i>B. licheniformis</i> LZBL-9 (JX847115)	99.9	1.4 ± 0.1	2.5 ± 0.1	—
W41	KC441848	<i>B. oleronius</i> 30N3-6 (JN366730)	99.7	—	—	—
W42	KC441849	<i>B. oleronius</i> 30N3-6 (JN366730)	99.7	—	—	—
W44	KC441850	<i>B. licheniformis</i> LZBL-13 (JX847119)	99.9	—	—	—
W45	KC441851	<i>B. licheniformis</i> LZBL-13 (JX847119)	99.9	—	—	—
W46	KC441852	<i>B. oleronius</i> 30N3-6 (JN366730)	99.9	—	—	—
W47	KC441853	<i>B. circulans</i> C2-37c-4 (JX517212)	99.9	—	—	—
W48	KC441854	<i>B. subtilis</i> FS05 (JQ403532)	99.9	1.4 ± 0.1	13.8 ± 0.5	0.19 ± 0.04
W49	KC441855	<i>B. amyloliquefaciens</i> BAB-807 (KC250199)	99.9	1.2 ± 0.1	6.9 ± 0.3	—
W50	KC441856	<i>B. subtilis</i> 9A (KC196261)	99.9	—	—	—
W51	KC441857	<i>B. licheniformis</i> BaDB24 (JX237858)	100	—	—	—
W52	KC441858	<i>B. licheniformis</i> F3 (EU256500)	99.9	—	—	—
W53	KC441859	<i>B. sonorensis</i> HT1 (JN013186)	99.9	—	—	—
W55	KC441860	<i>B. licheniformis</i> LZBL-13 (JX847119)	99.7	—	—	—
W56	KC441861	<i>B. licheniformis</i> HT9 (JN013194)	100	1.6 ± 0.2	4.5 ± 0.2	2.6 ± 0.1
W57	KC441862	<i>B. licheniformis</i> LZBL-13 (JX847119)	99.9	—	—	—
W58	KC441863	<i>B. licheniformis</i> HT9 (JN013194)	99.9	—	—	—
W59	KC441864	<i>B. licheniformis</i> 55N2-8 (JN366726)	100	—	—	—
W60	KC441865	<i>B. licheniformis</i> LZBL-9 (JX847115)	99.9	—	—	—
W61	KC441866	<i>B. licheniformis</i> BPRIST039 (JF700489)	99.9	2.2 ± 0.3	22.1 ± 0.9	—
W62	KC441867	<i>B. licheniformis</i> LZBL-13 (JX847119)	99.9	—	—	—
W63	KC441868	<i>B. licheniformis</i> (JN166722)	99.7	—	—	—
W64	KC441869	<i>B. licheniformis</i> LZBL-13 (JX847119)	100	—	—	—
W65	KC441870	<i>B. licheniformis</i> BaDB24 (JX237858)	100	—	—	—
W66	KC441871	<i>B. sonorensis</i> HT1 (JN013186)	99.9	—	—	—

—, means the value of parameter was not detected.

Figure 1 Phylogenetic relationships between bacterial 16S rDNA sequences in Deshan Daqu and previously reported sequences (a: main tree, b: subtree of *Bacillus licheniformis*). The number on each branch indicates the percentage of 1000 replicates that included the branch. Sequences determined in this study are shown in bold, and GenBank accession numbers are shown in parentheses for all of the related sequences. The scale bar of 0.02 (a) and 0.005 (b) represents a 2% and 5‰ nucleotide substitution rates according to the Jukes–Cantor evolutionary distance, respectively.

Figure 2 Phylogenetic relationships between bacterial 16S rDNA sequences in Baisha Daqu and previously reported sequences. The number on each branch indicates the percentage of 1000 replicates that included the branch. Sequences determined in this study are shown in bold, and GenBank accession numbers are shown in parentheses for all of the related sequences. The scale bar of 0.02 represents a 2% nucleotide substitution rate according to the Jukes–Cantor evolutionary distance.

To determine diversity in phenotypic functions, extracellular amylase screening assay was conducted among all fifty-six bacterial isolates from Wuling Daqu. A total of sixteen and ten isolates demonstrated α -amylase and glucoamylase production, respectively (Table 4). The maximum α -amylase yield of 22.1 U mL⁻¹ was obtained from *B. licheniformis* W61, whereas *B. amyloliquefaciens* W25 produced the maximum glucoamylase yield of 30.3 U mL⁻¹. The activity of α -amylase and glucoamylase produced by

B. licheniformis, *B. subtilis*, *B. amyloliquefaciens* and *B. oleronius* accounted for 33.5%, 12.9%, 27.4% and 22.6%, and 20.0%, 39.8%, 19.1% and 24.7%, respectively. However, *B. circulans* and *B. sonorensis* could produce neither α -amylase nor glucoamylase.

The variety of bacteria found in the Daqus produced complicated enzyme systems that converted the Daqus into unique glycation and fermented substances, which are useful for subsequent reactions leading to flavour production (Shi *et al.*, 2009). The variety of bacteria

Figure 3 Phylogenetic relationships between bacterial 16S rDNA sequences in Wuling Daqu and previously reported sequences (a: main tree, b: subtree of *Bacillus licheniformis*). The number on each branch indicates the percentage of 1000 replicates that included the branch. Sequences determined in this study are shown in bold, and GenBank accession numbers are shown in parentheses for all of the related sequences. The scale bar of 0.02 (a) and 0.002 (b) represents a 2% nucleotide substitution rate according to the Jukes–Cantor evolutionary distance, respectively.

observed in the three Daqus could produce amylase, but the compositions of bacteria for producing amylase were obviously different, although they were produced in Hunan province (Tables 2–4). As the dominant bacterial species between the three Daqus, *B. licheniformis* and *B. subtilis* also possessed high activities of α -amylase and glucoamylase that enabled them to utilise raw starch as a carbon source, which is a very important nutrient during fermentation (Tables 2–4). *Bacillus licheniformis* and *B. subtilis* may facilitate the conversion of starch into fermentable carbohydrates due to amylolytic activity, which may generate suitable substrates for the second stage of spirit production, that is, the alcoholic fermentation (Zheng *et al.*, 2012). *Bacillus amyloliquefaciens* was one of the dominant bacterial species producing amylase between Deshan, Baisha and Wuling Daqus. *Bacillus amyloliquefaciens*, which is one of the most widely used species for the bulk production of amylase, is a potent producer of amylase and other industrial exoenzymes (Tanyildizi *et al.*, 2007; Liu *et al.*, 2012). Moreover, *B. cereus* was also

one of the main bacterial species producing amylase in Deshan Daqu. It was reported that amylase was produced by 83% and 46% of *B. cereus* strains isolated from cheese in Turkey and legume-based Indian fermented foods (Roy *et al.*, 2007; Molva *et al.*, 2009). *Bacillus oleronius* was also another predominant bacterial species producing amylase in Wuling Daqu, which was also detected in Maotai Daqu (Liu *et al.*, 2012). However, other bacterial species, for example, *B. anthracis*, could produce both α -amylase and glucoamylase merely in Deshan Daqu. By comparison, *B. flexus* could secrete both α -amylase and glucoamylase only in Baisha Daqu. Additionally, no other bacterial species could produce either α -amylase or glucoamylase in Wuling Daqu. It was observed that different bacterial species could produce different yields of α -amylase and glucoamylase between the three Daqus. The maximum α -amylase yields produced by *B. amyloliquefaciens* D15 and *B. subtilis* B39 are 63.8% and 19.9% higher than that by *B. licheniformis* W61. Meanwhile, the maximum glucoamylase yield secreted by *B. licheniformis* D38 and *B. subtilis* B53 is 2.1-fold and 1.7-fold higher than that by *B. amyloliquefaciens* W25.

The bacterial diversity in the three Daqus was closely related to the types of spirits. Moreover, the bacterial diversity in the Daqu was affected by the environmental condition, production techniques, raw materials and storage time. Many intrinsic and extrinsic factors of Daqu production influence the richness and structure of the bacterial community including raw material variety, moisture content, temperature control, geographical location, climate and water (Wang *et al.*, 2011). Zhang *et al.* (2013) studied the potential of *Bacillus licheniformis* as a starter culture for aroma concentration improvement in the fermentation of Chinese Moutai-flavour liquor. Yan *et al.* (2013) reported that *B. licheniformis* and *B. subtilis* can influence the organic acids in Daqu significantly and differently. The three typical Daqus related to flavour production should be studied in the future to establish the connection between the cultivable bacterial diversity, amylase production and the flavour intensity or the volatile compounds.

Conclusion

This study is the first application using culture-dependent method and 16S rRNA molecular technique to simultaneously investigate the cultivable bacterial diversity and amylase production in the three typical Daqus of Deshan, Baisha and Wuling spirits. The results showed that the indigenous bacterial communities in the three Daqus were complex and consisted of a wide variety of bacteria. Both cultivable bacterial diversity and amylase production were obviously different. Each Daqu contained some unique bacteria

Figure 3 (Continued)

species, which could produce different activities of amylase. Different bacterial species could produce different yields of α -amylase and glucoamylase in the three Daqus. *Bacillus licheniformis*, *Bacillus subtilis* and *Bacillus amyloliquefaciens* not only were the dominant bacteria in the three Daqus, but also possessed high activities of α -amylase and glucoamylase that

enables them to utilise raw starch as a carbon source. The diversity of bacterial species and metabolisms might be linked to the flavour intensity. Further investigation needs to be carried out for obtaining more detailed information on the specific function of each individual microorganism and its contribution to the final formation of the unique aroma in Chinese spirits.

Acknowledgments

The authors are thankful to those who provided the Daqu samples for this research. This research was supported by the National Key Technology R&D Program of China (No. 2012BAK17B11) and Beijing Municipal Commission of Education and Subsidy for Outstanding People of Beijing (No. KM201311417007; PXM2013_014209_07_000082; 2011A005022000004).

References

- Chen, T.T., Wang, M.J., Jiang, S.Y., Xiong, S.Q., Zhu, D.C. & Wei, H. (2011). Investigation of the microbial changes during koji-making process of Douchi by culture-dependent techniques and PCR-DGGE. *International Journal of Food Science and Technology*, **46**, 1878–1883.
- Dhale, M.A. & Vijay-Raj, A.S. (2009). Pigment and amylase production in *Penicillium* sp. NIOM-02 and its radical scavenging activity. *International Journal of Food Science and Technology*, **44**, 2424–2430.
- Gupta, S., Rajauria, G. & Abu-Ghannam, N. (2010). Study of the microbial diversity and antimicrobial properties of Irish edible brown seaweeds. *International Journal of Food Science and Technology*, **45**, 482–489.
- Hayes, M.M., Hughes, T.A. & Greene, A.K. (2012). Bacterial diversity in dried colostrum and whey sold as nutraceutical products. *Journal of Food Science*, **77**, M359–M363.
- Huang, J.J., Wang, S.Y., Zhang, L.Y. et al. (2012). Isolation and identification of high pressure-resistant bacteria naturally contaminating strawberry pulp. *International Journal of Food Science and Technology*, **47**, 2620–2626.
- Kumar, P. & Satyanarayana, T. (2009). Overproduction of glucoamylase by a deregulated mutant of a thermophilic Mould thermomucor indiciae-seudaticae. *Applied Biochemistry and Biotechnology*, **158**, 113–125.
- Li, Z.M., Bai, Z.H., Zhang, B.G. et al. (2005). Newly isolated *Bacillus gibsonii* S-2 capable of using sugar beet pulp for alkaline pectinase production. *World Journal of Microbiology and Biotechnology*, **21**, 1483–1486.
- Li, X.R., Ma, E.B., Yan, L.Z. et al. (2011). Bacterial and fungal diversity in the traditional Chinese liquor fermentation process. *International Journal of Food Microbiology*, **146**, 31–37.
- Li, Z.M., Bai, Z.H., Zhang, B.G. et al. (2012). Purification and characterization of alkaline pectin lyase from a newly isolated *Bacillus clausii* and its application in elicitation of plant disease resistance. *Applied Biochemistry and Biotechnology*, **167**, 2241–2256.
- Liu, X., Guo, K.L. & Zhang, H.X. (2012). Determination of microbial diversity in Daqu, a fermentation starter culture of Maotai liquor, using nested PCR-denaturing gradient gel electrophoresis. *World Journal of Microbiology and Biotechnology*, **28**, 2375–2381.
- Minh, H.K.Q. & Rakshit, S.K. (2009). Use of specific PCR-based molecular markers for discrimination, rapid analysis of purity and identification of six fragrant rice. *International Journal of Food Science and Technology*, **44**, 1959–1965.
- Molva, C., Sudagidan, M. & Okuklu, B. (2009). Extracellular enzyme production and enterotoxigenic gene profiles of *Bacillus cereus* and *Bacillus thuringiensis* strains isolated from cheese in Turkey. *Food Control*, **20**, 829–834.
- Qian, J.Q., Chen, X.Y., Ying, X.X. & Lv, B.F. (2011). Optimisation of porous starch preparation by ultrasonic pretreatment followed by enzymatic hydrolysis. *International Journal of Food Science and Technology*, **46**, 179–185.
- Qin, H., Huo, D.Q., Zhang, L. et al. (2012). Colorimetric artificial nose for identification of Chinese liquor with different geographic origins. *Food Research International*, **45**, 45–51.
- Roy, A., Moktan, B. & Sarkar, P.K. (2007). Characteristics of *Bacillus cereus* isolates from legume-based Indian fermented foods. *Food Control*, **18**, 1555–1564.
- Shankar, M., Priyadharshini, R. & Gunasekaran, P. (2009). Quantitative digital image analysis of chromogenic assays for high throughput screening of α -amylase mutant libraries. *Biotechnology Letters*, **31**, 1197–1201.
- Shi, J.H., Xiao, Y.P., Li, X.R., Ma, E.B., Du, X.W. & Quan, Z.X. (2009). Analyses of microbial consortia in the starter of Fen Liquor. *Letters in Applied Microbiology*, **48**, 478–485.
- Smith, M.R. & Zahneley, J.C. (2005). Characteristics of the amylase of *Arthrobacter psychrolactophilus*. *Journal of Industrial Microbiology & Biotechnology*, **32**, 439–448.
- Tanyildizi, M.S., O'zer, D. & Elibol, M. (2007). Production of bacterial α -amylase by *B. amyloliquefaciens* under solid substrate fermentation. *Biochemical Engineering Journal*, **37**, 294–297.
- Wang, C.L., Shi, D.J. & Gong, G.L. (2008). Microorganisms in Daqu: a starter culture of Chinese Maotai-flavor liquor. *World Journal of Microbiology and Biotechnology*, **24**, 2183–2190.
- Wang, H.Y., Gao, Y.B., Fan, Q.W. & Xu, Y. (2011). Characterization and comparison of microbial community of different typical Chinese liquor Daqus by PCR–DGGE. *Letters in Applied Microbiology*, **53**, 134–140.
- Xie, Z., Zhang, Z.L., Hanzlik, S., Cook, E. & Shen, Q.J. (2007). Salicylic acid inhibits gibberellins-induced alpha-amylase expression and seed germination via a pathway involving an abscisic-acid-inducible WRKY gene. *Plant Molecular Biology*, **64**, 293–303.
- Yan, Z., Zheng, X.W., Chen, J.Y., Han, J.S. & Han, B.Z. (2013). Effect of different Bacillus strains on the profile of organic acids in a liquid culture of Daqu. *Journal of the Institute of Brewing*, **119**, 78–83.
- Yoshizaki, Y., Susuki, T., Takamine, K., Tamaki, H., Ito, K. & Sameshima, Y. (2010). Characterization of glucoamylase and α -amylase from *Monascus anka*: enhanced production of α -amylase in red koji. *Journal of Bioscience and Bioengineering*, **6**, 670–674.
- Zhang, C.L., Ao, Z.H., Chui, W.Q., Shen, C.H., Tao, W.Y. & Zhang, S.Y. (2011). Characterization of volatile compounds from Daqu—a traditional Chinese liquor fermentation starter. *International Journal of Food Science and Technology*, **46**, 1591–1599.
- Zhang, R., Wu, Q. & Xu, Y. (2013). Aroma characteristics of Moutai-flavour liquor produced with *Bacillus licheniformis* by solid-state fermentation. *Letters in Applied Microbiology*, **57**, 11–18.
- Zheng, X.W., Tabrizi, M.R., Nout, M.J.R. & Han, B.Z. (2011). Daqu—a traditional Chinese liquor fermentation starter. *Journal of the Institute of Brewing*, **1**, 82–90.
- Zheng, X.W., Yan, Z., Han, B.Z. et al. (2012). Complex microbiota of a Chinese “Fen” liquor fermentation starter (Fen-Daqu), revealed by culture-dependent and culture-independent methods. *Food Microbiology*, **31**, 293–300.