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Determination of the Microbial Origin of Geosmin in Chinese Liquor

Hai Du and Yan Xu*

State Key Laboratory of Food Science and Technology, Key Laboratory of Industrial Biotechnology of Ministry of Education, School of Biotechnology, Jiangnan University, 1800 Lihu Avenue, Wuxi, Jiangsu, China 214122

ABSTRACT: Geosmin is the major cause of the common earthy off-flavor in light-aroma type Chinese liquor and, thus, highly detrimental to the aromatic quality. To find out its origin, the evolving process of geosmin in light-aroma type liquor making was monitored, and microbial analysis of *Daqu* containing geosmin was carried out. The results showed that geosmin appeared in all the fermented sorghums at different fermentation periods. About 57% geosmin in the fermented sorghums was distilled into liquor. During the distillation process, the peak of geosmin concentration appeared when alcohol content was 50−60% vol. More importantly, high geosmin content was observed during the *Daqu*-making process. Furthermore, five Streptomyces strains were isolated from different types of *Daqu* used for the fermentation of light-aroma type liquor. All of them produced only geosmin as the main volatile metabolite but no 2-methylisoborneol (2-MIB). It appears that microorganisms developing in *Daqu* are responsible for the presence of geosmin in liquor. Because of the relatively low detection threshold estimated at 110 ng/L in 46 vol % hydroalcoholic solution, the presence of geosmin in *Daqu* may pose a risk for Chinese liquor producers.

KEYWORDS: *streptomyces, geosmin, microbial origin, earthy odor, Chinese liquor, Daqu*

INTRODUCTION

Geosmin is a well known microbial metabolite produced by several species of cyanobacteria, streptomycetes, and some fungi, which is responsible for the characteristic smell of moist soil or freshly plowed earth, and is mainly found in soil but also in odor-polluted water, wheat grain, beets, apple juice, cheese, nuts, cabbage, fish, or wine.[1−5] It smells undesirably musty or earthy even at a very low concentration owing to an exceptionally low threshold for human detection (less than 10 ng/L in water).[1] Besides its characteristic earthy aroma, geosmin is also associated with an undesirable musty odor or off-flavor, making its detection and elimination important in the management of water and food quality.

On the basis of that, the origin of the off-flavor is interpreted in order to control it effectively. For instance, Lu et al.[6] found that geosmin presents in beets because red beets are capable of endogenous synthesis of geosmin. La Guerche et al.[7] reported that two groups of strains of *Botrytis cinerea* ([bot +] and [bot −]) induced significantly higher production of geosmin from *Penicillium expansum*. The authors suggest that an ethanol-precipitable fraction, probably a polysaccharide, which was synthesized by *B. cinerea* [bot −] instead of [bot +], inhibited geosmin production. In an effort to eliminate this compound in water, many studies have been aimed at preventing the growth of such organisms as cyanobacteria and *Streptomyces* based upon the isolation and identification of odor-producing organisms and the conditions promoting their growth in natural lakes and ponds.[8−11]

In former studies, we have identified geosmin in Chinese liquor presenting a strong earthy odor and quantified it at concentrations of up to several micrograms per liter, especially higher in light-aroma type liquor.[12] Chinese liquor is fermented from cooked grains. First, *Daqu*, as the main source of microbes for fermentation, is prepared by natural inoculation of molds, yeasts, and bacteria on the grains.[13] Raw wheats, barley, and/or peas are ground and mixed with water. The wet mixture is compressed by a molding press to form a *Daqu* adobe, and then the adobe is incubated to generate a succession of microorganisms. Generally, maximum temperatures reached during the light-aroma type *Daqu* incubation period range between 40 and 50 °C as a result of natural metabolism. Freshly made *Daqu* is stored for maturation. Usually, the maturation process takes about 6 months. Then the mature *Daqu* is ground and mixed with cooked grains to start the solid-state fermentation. Traditionally, the fermentation of the light-aroma type liquor lasts for one month in a jar. Finally, the fermented grains are taken out, and distillation is performed to obtain distilled liquor. Apart from ethanol, different microingredients distilled from fermented grains have an enormous effect on the quality and flavor of the final distilled product.[14−17]

The Chinese liquor-making process is a relatively open system.[14] The production of quality liquor requires great attention to possible chemical and microbiological contamination during the *Daqu*-making, fermentation, and aging processes. Usually, the contaminant in Chinese liquor is associated with the development of microorganisms. The microbes may be introduced from anywhere concerned, such as grains, water, tools, *Daqu*, and so on. The identification of geosmin in aged liquor before blending led us to suppose that the presence of this compound in Chinese liquor could be attributable to the development of contaminants during *Daqu*-making or the fermentation process.[12]

The aim of the present research was to determine whether the origin and transfer process of geosmin occurred in the course of making the light-aroma type liquor. For this purpose, the processes of *Daqu*-making, fermentation, and distillation were tested through a large-scale geosmin screening by a

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headspace−solid phase microextraction−gas chromatography−mass spectrometry (HS-SPME-GC-MS) method. Geosmin-producing microorganisms were also isolated and identified.

MATERIALS AND METHODS

Chemicals. Geosmin (99%, 2 mg/mL in methanol) was purchased from Sigma-Aldrich (Shanghai, China). L-(−)-menthol (98.0%, internal standard, IS) was purchased from Acros Organics (New Jersey, USA). Absolute alcohol was purchased from Tedia (Ohio, USA). Both analytes and IS spiking solutions were prepared in ethanol. All standard solutions were kept in the freezer at −20 °C in the dark. Sodium chloride was purchased from China National Pharmaceutical Group Corporation (Shanghai, China).

Microbiological media Luria−Bertani (LB), Rose Bengal agar (RBA), and potato dextrose agar (PDA) were provided by Oxoid (Hampshire, England). All media were supplied with antibiotic supplements. PCR was performed with C1000 Thermal Cycler provided by Bio-Rad Laboratories (Hercules, CA, USA). All reagents for PCR amplification and DNA purification, including the Taq DNA polymerase, were from Takara Biotechnology (Dalian, China).

Samples. Different stage samples of cultivating *Daqu* and storing *Daqu*, fermented sorghums, and distillates were obtained from *Laobaigan* Distillery Co. Ltd. in (Hengshui, China). The mature *Daqu* made by different technologies, *Qingcha* (QC), *Hongxin* (HX), and *Houhuo* (HH) were obtained from *Fenjiu* Distillery Co. Ltd. (Fenyang, China). The mature *Qingcha Daqu* made by different light-aroma type liquor distilleries, *Fenjiu* (FJ), *Baofeng* (BF), and *Laobaigan* (LBG) were obtained from *Fenjiu* Distillery Co. Ltd. (Fenyang, China), *Baofeng* Distillery Co. Ltd. (Pingdingshan, China), and *Laobaigan* Distillery Co. Ltd. (Hengshui, China), respectively.

Sample Preparation for SPME Extraction. Each liquor sample was diluted with fresh redistilled−deionized water to a final ethanol content of 5% vol for the extraction of SPME. As for the solid samples such as *Daqu* and fermented sorghums, 1 g of each sample was applied to ultrasonic treatment at 40 °C for 30 min, followed by being soaked in distilled water three times (30 mL, 20 mL, 10 mL, respectively). The supernatants were combined after centrifugations, and then their volatiles were extracted by SPME.

Quantification of Geosmin by Headspace−Solid Phase Microextraction−Gas Chromatography (HS-SPME-GC-MS). For the SPME, an automatic headspace sampling system (Multi Purpose Sample MPS 2 with a SPME adapter, from GERSTEL Inc., Baltimore, MD, USA) with a 50/30 μm DVB/CAR/PDMS fiber (2 cm, Supelco Inc., Bellefonte, PA, USA) was used for analyte extraction. Before analysis, the fiber was conditioned by inserting it into the GC injector at the 250 °C for 2 h to prevent contamination. A total of 8 mL of the diluted sample was transferred to a screw capped headspace vial with a 15 mL volume and spiked with 10 μL of L-(−)-menthol (internal standard, IS), 4 mg/L, in ethanol. The diluted solution was saturated with NaCl. The vial was tightly capped with a Teflon-faced silicone septum. The samples were equilibrated at 60 °C for 5 min and extracted for 45 min at the same temperature under stirring (250 rpm). After extraction, the fiber was inserted into the injection port of the GC (250 °C) for 5 min to desorb the analytes.

For the GC-MS analysis, an Agilent 6890N GC coupled with an Agilent 5975 mass selective detector (MSD) was used. The capillary column used was a CP-Wax column (60 m in length, 0.25 mm in i.d., 0.25 μm in film thickness, Varian Inc., Palo Alto, CA, USA). The injector temperature was 250 °C, and the splitless mode was used. The conditions were as follows: the starting temperature was 80 °C (holding for 2 min), then raised to 230 °C at the rate of 8 °C/min, and held at 230 °C for 10 min. The column carrier gas was helium with a purity of 99.9995% at a constant flow rate of 2 mL/min. The electron impact energy was 70 eV, and the ion source temperature was set at 230 °C. Full-scan acquisition was used in the ranges of masses (30—350 amu) to characterize the compounds, which were clearly identified by comparison with reference spectra (NIST05a.L, Agilent Technologies, Inc.) and with pure standards.

The selected mass fragments for identification and quantification were 112 for geosmin. A calibration curve was derived by plotting the peak area ratios against the concentration ratios of geosmin (m/z 112) to IS (m/z 81) as described in our previous paper.[12] The concentration of geosmin in the sample was quantified by comparing the ratio of the peak areas with the calibration curve. Every determination was made in triplicate.

Isolation and Identification of Geosmin-Producing Microorganisms. Microorganisms were isolated by using a conventional agar dilution method. *Daqu* samples (2 g) were mixed with 50 mL of sterile physiological saline (0.85% w/v sodium chloride) under agitation for 30 min. Then, 0.1 mL of the dilution was spread on the surface of the isolation plate. LB plates were supplemented with nystatin (final concentration = 60 μg mL^{-1}) and used for bacterial isolation. Chloramphenicol-supplemented (100 μg mL^{-1}) RBA and PDA were used for the isolation of fungi and yeasts. After incubation at 30 °C, the separate colonies appeared on the plate. Colonies of different shapes and colors were selected and repeatedly stroked on fresh medium agar plates until pure cultures were obtained. The pure strains were picked and then vaccinated into a tube with 1/3 volume of bran mixed with 70% water. After culturing at 30 °C for 7 days, these products were tested by smelling by a 30-person jury to find the earthy odor. All of the people were trained with geosmin standard solution. The total training time is over 30 h. When the tubes with earthy odor were picked out, one part of the culture was analyzed to confirm the presence of geosmin by GC-MS. If it was confirmed that geosmin was present in the cultured products, the strains in the other part were isolated and preserved.

PCR amplification was performed with primers 27F and 1492R[18] for bacterial strains and with ITS1 and ITS4[19,20]for fungi. Colonies were picked directly from agar plates, and nucleic acids were extracted using a modified method of Hoffman et al.[21] Purified DNA was quantified at 260 nm using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Amplification reactions were contained in a total volume of 25 μL: 1 × PCR buffer (Mg^{2+} Plus), 0.8 mM dNTP mixture, 0.3 μM of each primer, and 2.5 U of Taq DNA polymerase. Extracted DNA (10−100 ng) was added as template for the PCR reactions. The sizes of the PCR products were checked in TAE agarose gels (1%) and GoldView staining. Finally, DNA sequencing was conducted by Sangon Biotech (Shanghai, China). Similarity searches were performed by using BLASTn software (http://www.ncbi.nlm.nih.gov/BLAST/), and the results were used for the identification of isolates.[23]

RESULTS AND DISCUSSION

Detection of Geosmin in Different Stages of Fermentation and Distillation Processes. In former studies, we concluded that geosmin is the source of the earthy odor in Chinese liquor. Its concentrations are high in *light-aroma* type liquors, especially in *Laobaigan* liquor.[12] To determine the source of this earthy compound, the process of *Laobaigan* liquor making was studied. First, geosmin concentrations in fermented sorghums at different fermentation periods were measured. Geosmin was detected in all fermented sorghums. At the beginning of the fermentation process, geosmin concentration increased to 1.96 μg/kg dry fermented sorghums until day 12 but decreased to 0.72 μg/kg dry fermented sorghums by day 24 (Figure 1). We infer that geosmin-producing microbes may exist in the fermented sorghums and that they may be aerobic microorganisms. During the fermentation process, as the normal growth and metabolism of aerobic microorganisms took place, dissolved oxygen in fermented sorghums was exhausted, forming the anaerobic microenvironment for the growth of anaerobic microorganisms. Meanwhile, there may be some anaerobic geosmin-degrading microbes activated at the end of fermentation.

Distillation is the key step of isolating the volatile compounds from fermented sorghums. At the earlier stage of distillation,

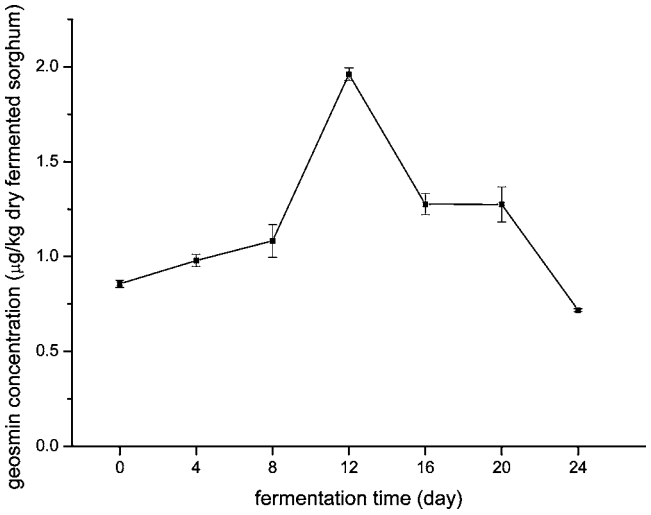

Figure 1. Concentrations of geosmin in fermented sorghums during the fermentation process.

the temperature is 85−95 °C. At the end of distillation, it is 105 °C. The geosmin concentrations of the fermented sorghums before and after distillation were measured, which were sampled from the 3 batches. Before distillation, the average of geosmin concentration in the fermented sorghums was 1.24 μg/kg dry fermented sorghums, while after distillation, it was 0.53 μg/kg dry fermented sorghums. About 57% geosmin was distilled out from fermented sorghums after distillation and introduced into Chinese liquor as other flavor compounds were distilled out. Moreover, different fermentation batches of the distillation process were tested. On the basis of the liquor-making technology of *Laobaigan* liquor, 5 batches of fermented grains are distilled. Among them, the first 3 batches of fermented grains are the same, which are with high starch content during the fermentation process. The fourth batch contains less starch content, while the fifth batch contains the least starch content. So we have tested first, fourth and fifth batches. In practice, the distillates above 40% vol are collected and aged. During 10 min or so, most of the alcohol and flavor compounds were distilled out. Therefore, alcohol contents decreased with distillation time. In all batches, the peak concentration of geosmin appeared when alcohol content was 50−60% vol (Figure 2). It suggests that geosmin exists in fermented sorghums and that it can be distilled out and transferred into liquor. Moreover, for distillates collected according to alcohol content, the kinetic connection between geosmin concentration and alcohol content facilitates the control of geosmin concentration in fresh distillates. On the whole, the highest amount of geosmin and alcohol content appeared in the distillation of the first batch with the highest starch content among the three batches. We can conclude that higher starch content will result in more geosmin and alcohol. It suggests that geosmin as alcohol is the result of microbial metabolism.

Sorghums, the main raw material of fermentation, can also be contaminated and affect liquor quality.[22] Nevertheless, sorghums are presteamed for about 30−40 min to get rid of off-odors and harmful microbes.[14] In most of the cases, sufficiently steamed sorghums probably do not represent a major source of geosmin or geosmin-producing microbes. We also measured geosmin in sorghums. There was no detected geosmin. It confirms that the sorghum is not the origin of geosmin in fermented sorghums.

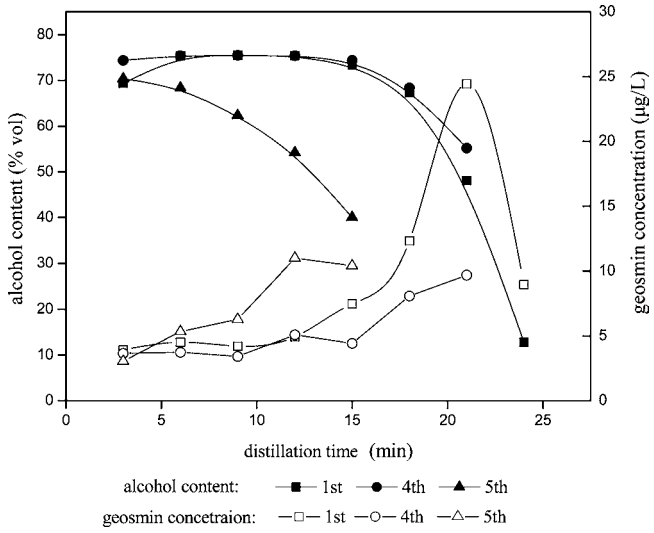

Figure 2. Changes of geosmin concentration and alcohol content during the distillation time of 3 batches of fermented sorghums (first, fourth, and fifth).

Kinetics of Geosmin Formation in the *Daqu*-Making Process. Before fermentation, *Daqu* powder, the most widely used fermentation starter and substrate complex to initiate the fermentation of Chinese liquor, was mixed into cooked sorghums at a ratio of 0.2.[23] To prove whether geosmin is introduced by *Daqu*, the concentrations of geosmin in *Daqu* were measured during the *Daqu*-making period. As Figure 3 shows,

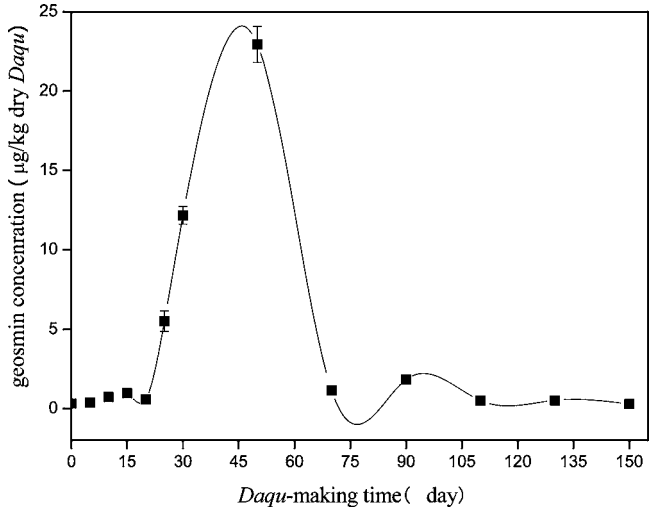

Figure 3. Kinetics of geosmin formation during the *Qingcha Daqu*-making process.

geosmin was not detected in *Daqu* adobe made from crushed wheat and water, and at the early stage of culture in house, there was a low level (approximate to 0) of geosmin found in *Daqu*. After *Daqu* was cultivated for about 25 days, obvious concentrations of geosmin in *Daqu* were observed. At that time, in-house *Daqu* cultivation was close to the end, and soon, the cultivated *Daqu* was stored indoors with cool ventilation for about 3−4 months before being used as the fermentation starter. An obvious rise of geosmin content was also observed at the earlier stage of storage. After storing for 20 days, geosmin in

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Daqu reached its peak concentration followed by an ascending trend.

The incubation of *Daqu* was set in warm and moist conditions, which are extremely favorable for microbial growth. The number of viable microbes increased tremendously during the incubation period and remained high during the storage period.[24] Geosmin, whose concentration varied greatly among different stages during the *Daqu*-making process, seemed evidently to come from the metabolites of microbes growing in *Daqu*. Because of the diverse environmental conditions, such as humidity, temperature, and air composition, microbes proliferate differently in different stages of the *Daqu*-making process, leading to different microflora compositions (microecology) and metabolites.

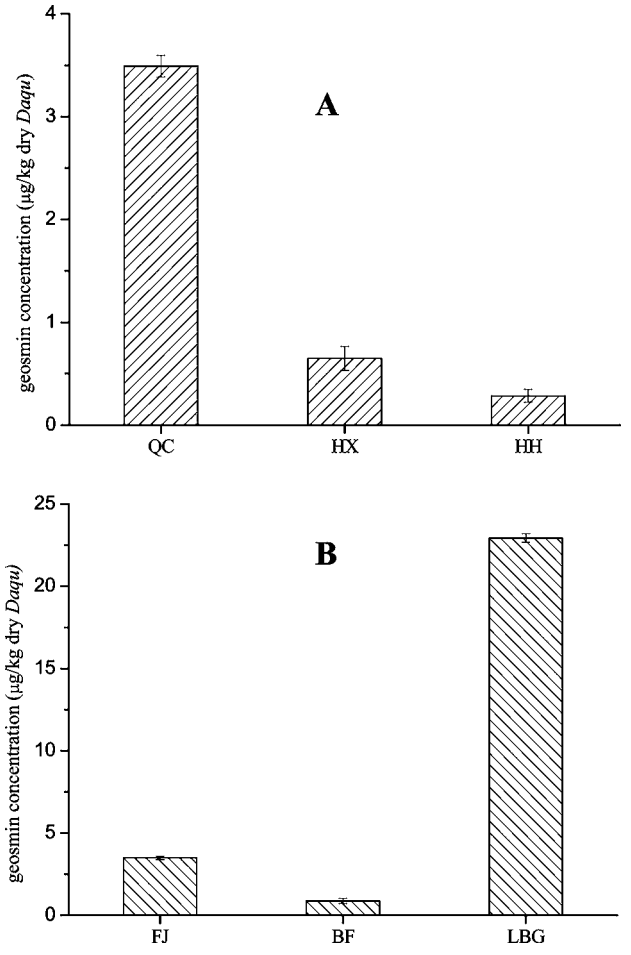

Figure 4. Comparison of geosmin content in three types of mature *Daqu* made by different technologies, *Qingcha* (QC), *Hongxin* (HX), and *Houhuo* (HH) (A), and in the mature *Qingcha Daqu* made by different light-aroma type liquor distilleries, *Fenjiu* (FJ), *Baofeng* (BF), and *Laobaigan* (LBG) (B).

Comparison of Geosmin Content in Different Types of *Daqu*. We analyzed the geosmin contents of three types of mature *Daqu*, *Qingcha* (QC), *Houhuo* (HH), and *Hongxin* (HX) via different technologies. All of the *Daqu* was collected from the *Fenjiu* distillery and randomly sampled among several batches. We found that geosmin presented the highest concentration in *Qingcha Daqu* (Figure 4A). The maximum incubation temperature (MIT) of QC is relatively low (44−46 °C), compared with that of HH and HX (47−48 °C for HH and 45−47 °C for HX). The number and type of microbes growing in *Qingcha Daqu* were relatively rich.[22] Moreover, we analyzed the mature *Qingcha Daqu* produced by different distilleries, *Fenjiu* (FJ), *Baofeng* (BF), and *Laobaigan* (LBG). The MIT of FJ, BF, and LBG are 45−46 °C, 48−49 °C, and 42−43 °C, respectively. Because of the differences in manufacturing practices, *Qingcha Daqu* produced by different distilleries presents diverse geosmin concentrations (Figure 4B). Interestingly, the *Daqu* from among the three distilleries, LBG with lowest MIT content had the highest geosmin concentration. Therefore, we infer that the lower MIT is more suitable for the growth of microbes including geosmin-producing microbes.

Isolation and Identification of the Microbes Causing the Synthesis of Geosmin in *Daqu*. In total, 572 strains were isolated from different types of *Daqu* collected from different liquor distilleries. Then all the strains were cultured and analyzed separately by the same method as that mentioned in Materials and Methods. Finally, 5 strains (LBG-FXJ, HX, QC-1, QC-2, and QC-3) were identified as geosmin producers (Table 1). Among them, LBG-FXJ was isolated from *Qingcha Daqu* of *Laobaigan* distillery. HX was isolated from *Hongxin Daqu* of Fenjiu distillery. QC-1, QC-2, and QC-3 were isolated from the *Qingcha Daqu* of *Fenjiu* distillery. The high incidence of geosmin-producing isolates from *Qingcha Daqu* corresponds with the high level of geosmin in *Qingcha Daqu*. It implies that the conditions for *Qingcha Daqu* making are favorable for geosmin-producing microbes.

Sequencing of the 16SrRNA gene fragment yielded good-quality sequences of approximately 1400 nucleotides that were used for identification purposes. In all cases, Blastn similarity values were more than 98%, matching those of previously cultured microorganisms, and then they were identified as *Streptomyces albus*, *Streptomyces fradiae*, *Streptomyces radiopugnans*, and *Streptomyces sampsonii*. Scholler et al. have reported that geosmin was the most frequently produced terpenoid compounds by streptomycetes.[25] This kind of microorganism is especially widespread in the soil and may well contaminate wheat, the main raw material of *Daqu*, at stages and under conditions that have yet to be determined.

The volatile profiles of the five isolates were also analyzed by HS-SPME-GC-MS. Interestingly, all of them produced only one earthy odorant compound, geosmin. While 2-methylisoborneol (2-MIB), the other earthy odorant compound usually produced by *Streptomyces* genus,[26] was not detected in the

Table 1. Identification and Capability of Geosmin Producing Strains Isolated from Three Different Types of *Daqu*

strain	closest cultivated strain	similarity (%)	geosmin (μg/L)[a]	origin isolate
LBG-FXJ	*Streptomyces albus* subsp. *albus* NRRL B-2365 (DQ026669.1)	99	75.28 ± 13.56	*Qingcha Daqu, Laobaigan*
HX	*Streptomyces fradiae* RMS4 (HQ267533.1)	99	31.20 ± 6.37	*Hongxin Daqu, Fenjiu*
QC-1	*Streptomyces radiopugnans* AN-15 (HQ202876.1)	99	42.44 ± 4.31	*Qingcha Daqu, Fenjiu*
QC-2	*Streptomyces sampsonii* HS5341 (HQ610448.1)	99	542.65 ± 43.46	*Qingcha Daqu, Fenjiu*
QC-3	*Streptomyces* sp. MTCC 8377. (EU523135.1)	99	102.41 ± 14.59	*Qingcha Daqu, Fenjiu*

[a]Geosmin production on solid-state bran medium simulating *Fuqu* (a starter based on a pure microbial culture).

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volatile profiles of all the five isolates. This finding could explain why only geosmin presents the earthy odor in Chinese liquor as we have reported previously.[12]

To the best of our knowledge, it was the first time to report that the *Streptomyces* genus isolated from *Daqu* was capable of synthesizing large quantities of geosmin. It is well known that most *Streptomyces* can produce antibiotics. The growth of *Streptomyces* is bound to affect the microbial ecology of *Daqu* and fermented grains, consequently influencing the enzyme and flavor of *Daqu*. Research about those issues are currently ongoing in our group.

AUTHOR INFORMATION

Corresponding Author

*Tel: +86 510 85918197. Fax: +86 510 85918201. E-mail: yxu@jiangnan.edu.cn.

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Notes

The authors declare no competing financial interest.

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