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Exploring the microbial origins of *p*-cresol and its co-occurrence pattern in the Chinese liquor-making process

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ABSTRACT

The compound *p*-cresol is the major off-odor and toxic component of strong aroma-type Chinese liquor. To trace its origin, the *p*-cresol contents in the liquor-making process were detected by gas chromatography-mass spectrometry. The prokaryotic communities involved in the process were revealed by 16S rRNA gene MiSeq sequencing. The results showed that the microbial diversity and concentration of *p*-cresol in pit mud significantly increased with increased pit depth. Canonical correspondence analysis further revealed that *p*-cresol was positively correlated with the genera *Aminobacterium*, *Clostridium*, *Sedimentibacter* and *Syntrophomonas*. On investigating potential *p*-cresol producers, we obtained 11 species from pit mud samples using selective culture media. *Clostridium butyricum*, *Clostridium tyrobutyricum*, *Clostridium aminovalericum* and *Eubacterium contortum* were confirmed to possess the capacity for *p*-cresol production. Moreover, based on volatile compounds data, these strains were assigned to the same clusters characterized by the high abundance of butanoic acid and *p*-cresol. Furthermore, 24 pairs of significant correlations were identified from 14 genera using co-occurrence network analysis. *Clostridium* was the hub in pit mud and could be inhibited by increasing levels of *Lactobacillus*. These findings represented a step forward for controlling *p*-cresol in a complex microbial community of Chinese liquor.

1. Introduction

p-cresol contributes to animal odors and destroys the original flavor of foods and beverages (Mo et al., 2010). More importantly, it is highly toxic even at low concentrations (recommended exposure limit issued by the National Institute of Occupational Safety and Health is 10 mg/m^3) and it has been classified as a possible carcinogen (pollutant of group C) by the Environmental Protection Agency. The World Health Organization (WHO) recommends a permissible concentration of *p*-cresol of 1 μg/L in potable waters (Singh et al., 2008). In the case of food hygiene, it has been detected as an off-odor substance in a variety of foods and beverages, such as fruit juices, coffee, wine and liquor, affecting food hygiene and safety. It has been reported that strong aroma type liquor, accounting for about 70% of total liquor production in China (Fan and Qian, 2006), contains more *p*-cresol than other aromatic liquors, reaching 1460 μg/L (Peng et al., 2013). Phenolic compounds are detoxified by glucuronidation in the liver and then excreted in urine (Wong et al., 2016). Excess alcohol consumption is the primary cause of liver-related mortality in the world, including conditions such as fatty liver, fibrosis and alcoholic hepatitis (Louvet and Mathurin, 2015). For the purposes of control, it is therefore important to identify the origin of *p*-cresol in strong aroma type liquor.

Compared with other aroma type Chinese liquors, the most characteristic process of making strong aroma type liquor is the fermentation, which is carried out in specialized rectangular soil pits that are coated by a special mixture of fermentation mud made largely from clay and grain (Jin et al., 2017). For the production of strong aroma type liquor, *Daqu* powder is mixed into the steamed grains, which were then moved to underground pits and fermented by microorganisms mainly from *Daqu* and pit mud. During this process of fermentation, the pit mud provides an anaerobic habitat for fermentation microbes that are considered to be important contributors to the flavor and taste of the liquor (Wu et al., 2009). The anaerobic microbes in the pit mud produce short chain fatty acids, such as caproic acid and butyric acid, which are the main precursors of ethyl hexanoate (Tao et al., 2014; Zheng et al., 2015). It has been noted that pit age and spatial position have a strong effect on the volatiles produced (Ding et al., 2016). However, the complex microbial community in pit mud could also produce some compounds that may threaten the quality of strong aroma type liquor. It is reported that phenolic compounds (such as 4-methyl phenol and 4-

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ethyl phenol) have been identified in pit mud (Ding et al., 2016), which induce the “horsy”, “medicinal” and “sweaty saddle”-like off-odor.

Recent research showed that bacteria were more dominant than fungi in pit mud (Zhao et al., 2012), accounting for 83.65% of the total microorganisms (Ding et al., 2014). Currently, bacterial communities are investigated using both culture-dependent and culture-independent methods (Hu et al., 2015). Among the complex anaerobic prokaryotic microbiota harbored in pit mud, the main species belong to the Classes Clostridia, Bacteroidia, Bacilli and other two archaeal classes Methanobacteria and Methanomicrobia (Ding et al., 2014; Hu et al., 2015; Tao et al., 2014). These microorganisms may be largely introduced from the fermentation starter (*Daqu*), pit mud, air and raw material. However, to the best of our knowledge, no direct relationship has been reported between *p*-cresol production and microbial taxa in the fermentation system.

In this study, our aim was to determine whether the origin and transfer process of *p*-cresol occurred during the production of strong aroma-type Chinese liquor. The microbial communities in pit mud were detected by 16S rRNA gene MiSeq sequencing to provide insight into the correlation between microbial species and *p*-cresol contents. Finally, *p*-cresol-producing microorganisms were isolated and identified by a culture-dependent method, and their liquor flavor contributions were evaluated.

2. Materials and methods

2.1. Chemicals

p-cresol (99%) and tyrosine were purchased from Sigma-Aldrich (Shanghai, China). 3,4-dimethyl phenol (98.0%, internal standard, IS) and absolute alcohol were purchased from TEDIA (Fairfield, OH, USA). IS spiking solutions were prepared in an alcohol solvent. All standard solutions were stored at -20 °C in the dark. Tryptone, yeast extract, dipotassium phosphate, monopotassium phosphate, sodium acetate, magnesium sulfate, ammonium sulfate, sodium chloride, glucose and beef extract were purchased from the China National Pharmaceutical Group Corporation (Shanghai, China).

2.2. Sample collection and storage

The fermentation vessel was a rectangular-shaped pit (2 m × 3 m × 2 m) under the ground, with the inside walls being covered with a layer (~10 cm thick) of fermentation pit mud. The pit mud, *Daqu* and saccharified grains used in this study were supplied by two well-known strong aroma type liquor distilleries (marked as J and L) in Southwest China. The pits marked as 50 yr-J1, 50 yr-L1 and 50 yr-L2 had been used for 50 years. The pits marked as 200 yr-L1 and 200 yr-L2 had been used for 200 years. The duplicate samples of pit mud were collected at depths of 0.2 m (upper pit mud, marked as U), and the triplicate samples were collected at depths of 1.0 m (middle pit mud, marked as M) and 2.0 m (bottom pit mud, marked as B). Triplicate subsamples at each position were collected and mixed immediately. The samples were transferred immediately to sterile anaerobic bags (MGC, Japan). Finally, the pit muds were divided and stored at -20 °C (for DNA extraction and gas chromatography-mass spectrometry (GC-MS) detection) and 4 °C (for strain culture and screening).

2.3. Illumina MiSeq sequencing of 16S rRNA genes

DNA extraction from the samples was performed using the CTAB (cetyltrimethylammonium bromide) method (Jara et al., 2008). The V3-V4 regions of the 16S rRNA genes were amplified using the universal primer set 338F (5′-GTACTCCTACGGGAGGCAGCA-3′) and 806R (5′-GTGGACACHVGGGTWTCTAAT-3′) (Cheung et al., 2010). PCR products were purified using the Qiagen Gel Extraction Kit (Qiagen, Hilden, Germany). The barcoded PCR amplicons were sequenced on a MiSeq Benchtop Sequencer for 300 bp pair-end sequencing (Illumina, San Diego, CA, USA).

Raw reads generated from MiSeq were filtered using Mothur according to the following criteria: (i) the sequences had a quality score < 30, (ii) they did not perfectly match the PCR primer, (iii) they contained an N base. The two pair-end reads were assembled into a contig using FLASH. After filtering the reads that were < 150 bp in length, chimera sequences were removed using the Uchime algorithm. The obtained high quality sequences were clustered into operational taxonomical units (OTUs) using QIIME (Version 1.9.1) at 97% sequence similarity. A representative sequence for each OTU was extracted and annotated using the Greengene database (Version 13.8). To explain bacterial richness and evenness, diversity indices including Chao1, Shannon, Simpson and ACE were calculated using QIIME. Moreover, the unifrac distance was used to assess the dissimilarity among bacterial communities.

2.4. Isolation and identification of p-cresol-producing microorganisms

Bottom pit mud (5 g) was mixed with 100 mL of sterile physiological saline (0.90% w/v sodium chloride) and vortexed for 10 min. Then, a 200-μL dilution was injected into three different media: beef extract peptone medium (BEPM) (Nilegaonkar et al., 1992), hexanoic acid bacterium medium (HABM) (Jeon et al., 2013) and enriched *Clostridium* medium (RCM) (Weenk et al., 1991). After incubation at 30 °C for 3 days, the *p*-cresol content in the fermentation broths was quantified using the head space-solid phase microextraction (HS-SPME)-GC-MS method. Finally, typical samples were selected for the further isolation and identification of *p*-cresol-producing microorganisms. The plate dilution method was used to isolate *p*-cresol-producing bacteria. Ten-fold serial dilutions of the samples with high *p*-cresol contents were spread onto the surface of the agar media, and a single clone was picked and used to inoculate an anaerobic tube containing medium for identification.

The cells of *p*-cresol-producing strains were collected from the anaerobic tube and genomic DNA was extracted using a modified phenol/chloroform method (Hoffman and Winston, 1987). PCR amplification was performed with the bacterial universal primer set 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) (Yu et al., 2013). Amplification reactions (25 μL total volume) comprised: 1 × PCR buffer (Mg^{2+} Plus), 0.8 mM dNTP mixture, 0.3 μM of each primer, 2.5 U of Taq DNA polymerase (EX *taq*, TAKALA) and 10–100 ng of DNA template. The sizes of the PCR products stained with GoldView were checked on TAE agarose gels (1%). The PCR samples were sent to Sangon Biotech (Shanghai, China) for sequencing. Species identification was performed using BLAST software (http://www.ncbi.nlm.nih.gov/BLAST/) and the NCBI NR database.

2.5. Sample preparation and head space-solid phase microextraction (HS-SPME) parameters

Two grams of pit mud were diluted in 15 mL of fresh redistilled deionized water and ultrasonically treated for 30 min at 30 °C. After centrifugation, the supernatants were extracted by SPME. For the SPME, an automatic headspace sampling system (MultiPurpose 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 analyses. A total of 8 mL of dilution was transferred to a screw-capped headspace vial with a 15 mL volume, and was spiked with 10 μL of internal standard solution containing 154 mg/L 3,4-dimethylphenol. The diluted solution was then saturated with NaCl. The vial was tightly capped with a Teflon-faced silicone septum.

2.6. GC-MS analysis

For the GC-MS analysis, an Agilent 6890 N GC coupled to an Agilent