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Effects of mixed fermentation on their volatile features of Sichuan Xiaoqu liquor by HS-SPME/GC-MS

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ABSTRACT

To clarify the impact of functional microorganisms on Sichuan xiaoqu liquor's flavor, during Xiaoqu preparation several pure functional microorganisms (a: alcohol active dry yeast (AADY) + Rhizoups oryzae; b: AADY + Rhizoups oryzae + Monascus; c: AADY + Rhizoups oryzae + Monascus + Mucor; d: AADY + Rhizoups oryzae + Monascus + Mucor + ester producing yeast, related to liquor a, b, c, d.) were used and the volatile components of the liquors evaluated by head space solid phase micro-extraction combined with gas chromatography-mass spectrometry (HS-SPME-GC-MS). In total 39, 31, 33, 36 compounds were detected in the liquor a, b, c, d respectively. Results revealed that the three microbes (Monascus, Mucor, ester producing yeast) have little impact on the liquor's total volatile compounds. Specific compounds, however, did have some distinction: On the basis of adding Rhizoups oryzae with AADY, Mucor could be positive for alcohol but negative for ester, Mucor and Monascus mixed were determined to be positive for the content of naphthalene and phenol, while Mucor, Monascus and ester producing yeast mixed together were positive for ester but negative for aldehyde, ketone and hydrocarbon level.

Key words: Sichuan Xiaoqu liquor, GC-MS, functional microorganisms, volatile components

INTRODUCTION

Sichuan Xiaoqu liquor, a famous traditional Chinese drink, is distilled from fermented grains (sorghum, especially) in the south province of China. It is of interest as one of the few examples of liquor distilled from the product of a fermentation using single microbe starter. Xiaoqu is the starter of this fermentation process. Despite Daqu (a starter too) liquor's various advantages such as excellent quality and wonderful taste, high cost and long term production cycle lead to a limited market [1]. Compared with Daqu liquor, Xiaoqu liquor requires lower cost and less yielding time, and is described as alcohol softly and refreshing, which is arousing people's interest [2]. As a result, it is significant to study how to produce Xiaoqu liquor with really good quality. The liquor hence the name because the most productive province of Xiaoqu liquor is Sichuan [3]. Sichuan liquor is a general call, which represents the production and technology of this kind of liquor. However, the brewing process has been following the five-day-solid-state fermentation with a single grain. The new roasted liquor was reserved only for two or three months at most, and then had a process of grouting and alcohol-degrading, filtering and filling by the time the end product were finished, without blending and a long time aging. The production end up with less highly technology and low additional value, being seemed as something low-end with low prices and thin profit. As a result, improving the quality of Sichuan Xiaoqu liquor has become a highly necessary task in order to struggle for a place in the competitive market.

Monascus has been found to improve liquor's flavor in the liquor-making industry [4]. Nevertheless, to date, very few studies have been conducted to learn if *Monascus* have any impact on Xiaoqu liquor's volatile compounds. *Mucor*, a fungi, can produce protease and have found application in flavor foods such as fermented bean curd, Laba bean, fermented blank beans [6]. The concern of studying *Mucor* would mostly center on the screening and cultivating of excellent strains [7-9] and development of traditional technology with pure-cultured *Mucor* [10-11], and found no application in liquor. Ester producing by yeast not only suitable for liquid-state culture but also suitable for solid-state culture and semi-solid-state culture. Since the 1960s, they have found application in liquor to remedy its deficiencies of insufficient flavor and inconspicuous aftertaste [12]. Nowadays, pure culture of *Rhizoups oryzae* and *Saccharomyces cerevisiae* are regularly used as saccharifying ferment for the production of Sichuan Xiaoqu liquor, with the subsequent production insufficient flavor. In that case, we prepared Xiaoqu with the three microorganisms respectively and added them to the Xiaoqu of *Rhizoups oryzae* and AADY, with the expectation of understanding their impact on the flavor of Sichuan Xiaoqu liquor.

Sorghum

↓

Moistened

↓

Initial steamed

↓

Water tightness

↓

Steamed again

↓

Remove the sorghum from the steamer

↓

Cooling

↓

Added Xiaoqu and mixed ← Activation the yeasts

↓

Cultivated the fungi and saccharifying

↓

Added grains

↓

Fermentation

↓

Distillation

↓

Analysis

Fig.1. Flow sheet of the production of the liquor

Solid phase micro extraction (SPME) has been widely used in the research of foods' flavor [13-19] and has potentially application in the study on starter and fermented grains. In the process of HS-SPME, volatile compounds of liquor will be affected by alcohol, liquid volume of the extraction flask, ionic strength; equilibrium temperature and extraction time [20-22]. In this study, parameters above were used at the level of optimized in these previous experiments [23].

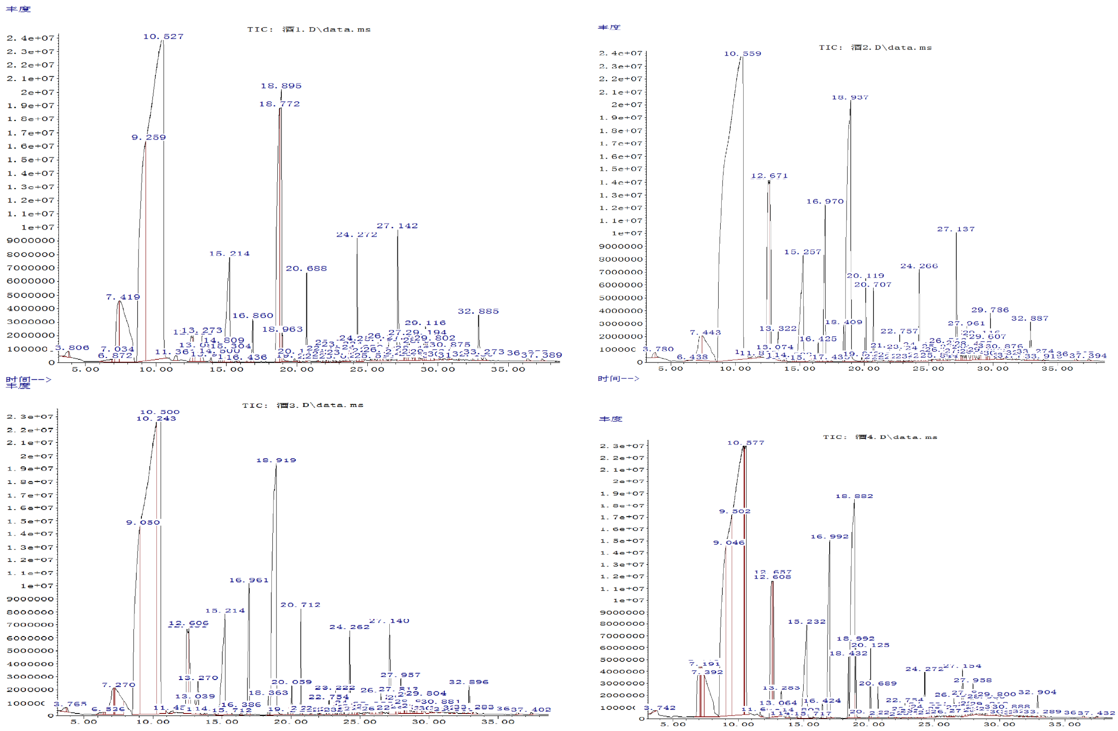

Fig.2. The GS-MS Total ion chromatograms of the liquor, Abscissa represents time(min), vertical coordinates represents abundance. The left-hand above is related to liquor a, the right-hand above b, the left-hand below c, the right-hand below d

MATERIALS AND METHODS

Raw materials and fungal strain

The Sorghum comes from north east of China. The strain *Rhizoups oryzae* (CICC40607) was bought from http://www.bnbio.com/; *Actinumucor ellegans* and ester producing yeast (*Hansenula polymorpha*) was from Industrial Microbial Culture Collection Center of China; and the *Monascus* was kindly provided by Zhejiang University of Technology; AADY was from ANGEL YEAST CO.LTD.

Chemicals and instrument

Angilent 6890N GC coupled to an 5975B MS mass spectrometry and equipped with a DB-WAX fused silica capillary column (60m × 0.25 mm i.d., 0.25 mm film thickness). The SPME manual device equipped with a 50/30UM DVB/CAR on PDMS fiber (Supelco, Bellfonte, PA, USA). GC-7860 (Shanghai appropriate electronic technology co., LTD, Shanghai, China). GX-300A/500A Nitrogen, hydrogen and air all-in-one (Beijing zte huili technology development co., LTD, Beijing, China). DB-WAX (60m × 250μm × 0.25μm) (Nanjing ng chromatography technology co., LTD, Nanjing, China). A 1-μLsyringe (Shanghai anting trace sampler factory, Shanghai, China). All standards used were of GC quality.

Starter preparation

The methods were used at the levels optimized in the previous experiments [24].

Rhizoups oryzae starter: Four 250-mL Erlenmeyer flasks, each containing 10g wheat bran and added 7.5mL distilled water, were autoclaved (121℃, 30min),after that, added 0.1mL *Rhizoups oryzae* suspension to each flask, and the flasks were incubated at 31℃for four days.

Monascus starter: Four 500-mL Erlenmeyer flasks, each containing 20g wheat bran and added 15mL distilled water, were autoclaved (121℃, 30min), after that, added 0.04mL lactic acid and 0.1mL *Monascus* suspension to each flask, and the flasks were incubated at 35℃ for five days.

Mucor starter: Four 250-mL Erlenmeyer flasks, each containing 10g wheat bran and added 10mL distilled water, were autoclaved (121℃, 30min), after that, added 0.2mL *Mucor* suspension to each flask,and the flasks were incubated at 28℃for four days.
The production of Sichuan Xiaoqu liquor

The process is following the traditional method[24] and shown in **Fig.1.** 2.5Kg sorghum was used in liquor a, b, c and d respectively, the dosage of Xiaoqu is used at the optimized level of Lin [23]. 5g, 5mL,16g, 17g, 10g to AADY, *Hansenula polymorpha*, *Rhizoups oryzae starter*, *Monascus starter*, *Actinumucor ellegans* starter, respectively.

SPME conditions
The extraction of the liquors volatile compounds was carried out with the following conditions: 9 mL sample in four glass vials, Then 1.6g NaCl were added, and the sample vial was closely capped with a PTFE-silicon stopper. Afterwards, the sample vial was equilibrated at 45°C for 30 min on a heating platform. Then, the extraction coating fibre of CAR/PDMS/DVB was placed in the headspace to extract the volatiles for 30 min with continuous heating. After extraction, the fibre was immediately desorbed into the GC injection port for 3 min [23].

GC-MS analysis

Table 1 The results of GC-MS of the four liquors

No.	Name	concentration（%）				No.	Name	concentration（%）			
		a	b	c	d			a	b	c	d
1	Ethyl alcohol	63.74	61.15	66.28	62.20	30	.beta.-Phenylethyl butyrate	—	0.18	—	0.15
2	1-Propanol	0.85	0.69	1.05	0.72	31	Phenethyl acetate	0.15	0.18	0.10	0.17
3	1-Propanol, 2-methyl	3.62	3.74	3.76	3.29	32	Undecanoic acid, ethyl ester	1.03	1.23	1.37	0.37
4	1-Butanol	0.47	2.67	2.11	4.21	33	Octadecanoic acid, ethyl ester	—	—	—	0.23
5	1-Hexanol	—	0.14	0.08	0.08	34	Butanedioic acid, diethyl ester	—	0.06	—	—
6	1-Butanol, 3-methyl	4.78	12.00	11.62	9.38	35	Eicosanoic acid, ethyl ester	—	—	0.36	—
7	2-Heptanol	—	0.07	0.01	—	36	Propanoic acid, 2-methyl-, 2phenylethyl ester	0.02	—	—	—
8	1-Octanol	0.13	0.11	—	0.03	37	Acetaldehyde	0.23	0.20	0.26	0.04
9	3-Buten-1-ol, 3-methyl-	0.02	—	—	0.02	38	Ethane, 1,1-diethoxy-	7.91	3.24	3.33	—
10	1-Tridecanol	—	—	0.04	—	39	Nonanal	0.10	0.04	0.05	0.03
11	Phenylethyl Alcohol	0.27	0.49	0.27	0.26	40	Furfural	—	—	0.04	0.07
12	Ethyl Acetate	0.15	—	—	7.11	41	Benzaldehyde	—	0.02	0.03	—
13	Butyl acetate	—	—	—	0.10	42	Nonane, 1,1-diethoxy-	0.20	—	—	—
14	Butanoic acid, ethyl ester	0.94	7.06	3.41	5.84	43	1, 1-2 - methyl 2- ethoxy butane	0.06	—	—	—
15	Butanoic acid, hexyl ester	—	0.34	0.17	0.65	44	Heptane, 1,1-diethoxy-1,1	—	—	0.01	—
16	Butanoic acid, pentyl ester	0.06	0.82	0.29	0.67	45	Styrene	0.05	0.05	0.03	—
17	1-Butanol, 3-methyl-, acetate	0.13	—	—	—	46	Benzoyl bromide	0.57	—	0.74	—
18	Butanoic acid, 2-methylpropyl ester	0.012	0.20	0.08	0.15	47	Propane, 1,1,3-triethoxy-1,1,3	0.01	—	—	—
19	Hexanoic acid, ethyl ester	0.28	—	—	0.89	48	2-Nonanone	—	0.02	—	—
20	Hexanoic acid, butyl ester	—	—	0.02	0.05	49	5,6-sebacic ketone	0.02	—	—	—
21	Butanoic acid, 2-methylpropylester	—	—	—	0.01	50	Phenol, 2-ethyl-2	—	—	—	0.02
22	Butanoic acid, 3-methyl-, 2-methylpropyl ester	0.08	—	—	—	51	Pentadecane	0.08	—	—	—
23	Propanoic acid, 2-hydroxy-, ethyl ester	0.02	0.03	—	0.02	52	Naphthalene	0.13	0.15	0.18	0.09
24	Formic acid, hexyl ester	0.09	—	—	—	53	Naphthalene, 2-methyl-1	—	—	0.06	—
25	Octanoic acid, ethyl ester	1.18	0.10	0.13	0.56	54	Naphthalene, 2-methyl-2	0.08	0.06	—	0.04
26	Nonanoic acid, ethyl ester	0.10	0.12	0.11	0.08	55	Biphenyl	0.017	—	0.02	0.01
27	Decanoic acid, ethyl ester	2.48	1.48	1.76	0.95	56	Acenaphthene	0.03	0.05	0.04	0.04
28	Benzoic acid, ethyl ester	—	—	—	0.02	57	6-Tetradecyne	0.06	—	—	—
29	Benzenepropanoic acid, ethyl ester3-	—	0.18	—	0.08	58	1-Pentadecyne	—	—	0.05	—

− represent there is no this compounds in that liquor.
Concentration means Relative areas normalized to n-amyl acetate

Injector temperature was 250℃. The column was held at 35℃ for 5 min and then increased from 35℃ to 100℃ at a rate of 5℃/min, then held at 100℃ for 2 min and finally increased to 230℃ at 15℃/min held for 10min, and helium (purity: 99.999%) carrier gas flow of 1mL/min. For mass spectrometry, the temperatures of the transfer line, quadruple, and ionization source were of 250℃, 150℃, 230℃, respectively. The mass spectra were generated in electron-impact (EI) mode at 70eV, and the detector was set to full-scan mode with a 300-500 amu range [23].

Table 2 Analysis of the volatile compounds

Sample	Volatile compounds	Ester		Alcohol		Hydrocarbon		Aldehyde,keetone		Other compounds	
		Qty.	concentration/%	Qty.	concentration/%	Qty.	Concentration/%	Qty.	concentration/%	Qty.	concentration/%
a	39	16	13.97	8	73.88	5	0.75	5	8.47	5	0.84
b	31	13	12.84	9	81.06	1	0.05	5	3.52	3	0.23
c	33	11	7.80	9	85.23	3	0.09	5	3.73	5	1.14
d	36	19	18.04	9	80.2	0	0	3	0.13	5	0.20

Table 3 Predominant volatiles of the four liquor

Sample	Compound 1		Compound 2		Compound 3		Compound 4	
	Name	concentration/%	Name	concentration/%	Name	concentration/%	Name	concentration/%
a	Ethane, 1,1-diethoxy-	7.91	1-Butanol, 3-methyl-	4.80	1-Propanol, 2-methyl-	3.62	Decanoic acid, ethyl ester	2.48
b	1-Butanol, 3-methyl-	12.00	Butanoic acid, ethyl ester	7.06	1-Propanol, 2-methyl-	3.74	1-Butanol	2.67
c	1-Butanol, 3-methyl-	11.62	1-Propanol, 2-methyl-	3.76	Butanoic acid, ethyl ester	3.41	Ethane, 1,1-diethoxy-	3.33
d	1-Butanol, 3-methyl-	9.38	Ethyl Acetate	7.11	Butanoic acid, ethyl ester	5.84	1-Butanol	4.21

Identification and Quantification of Volatile Compounds
The identification of volatile compounds was carried out by comparing their mass spectrum with the NIST05 spectrum database and a mixture of n-paraffins (C_6-C_{20}) as standards was used for calculating RIs. Semi-quantitative determinations were obtained by using n-amyl acetate as an internal standard. The contents of the volatile compounds were calculated from the GC peak areas relating to the GC-peak area of n-amyl acetate [25].

RESULTS AND DISCUSSION

The GS-MS total ion chromatogram of the liquor a, b, c and d were shown in **Fig.2.** The liquor a, b, c and d shared similar shape, but the peaks of the liquors were not kept the same altitude. The results of GC-MS of the four samples were shown in **Table 1**. In total of 39, 31,33 and 36 compounds were detected in the liquor a, b, c, and d respectively, which is similar to our previous study [21], in which 29, 31, 44, 45 volatile compounds were detected in Jiangjin, Yongchuan, Kaijiang, Zigong (four commercial available Xiaoqu liquors). The results suggest that *Monascus*, *Mucor* and ester producing yeast have little impact on the liquor's total compounds. No acids were detected in all of the samples which meant complete esterification. previously, it was found that Fen-flavor liquor has less total volatile compounds than Luzhou-flavor or Maotai-flavor [26]. Results of this study demonstrate that the volatile profile of Sichuan Xiaoqu liquor did have less total compounds.

Analysis of ester (concentration was shown by area percentage, the same below)
Concentration of sample a, b, c, and d were all different (**Table 2**). The sample a is close to b. But the c has the lowest concentration while d has the highest concentration. Like Yongchuan and Zigong, as shown in Table 3, the a has higher content (2.48%) about Decanoic acid, ethyl ester, whereas 1-Butanol, 3-methyl-butanol were considered to be predominant volatiles (7.11%) as well as Jiangjin. *Monascus* can produce ethyl caproate, but no ethyl caproate were detected in the four samples, suggesting *Monascus* can not work in this conditions. However, the highest total content of ester were observed in d compared to a, b and c which seemed to be the ester producing yeast played a key role in the brewing process.

Analysis of alcohol
Alcohol can improve aroma of liquor. Total compounds of alcohol were of similar in the four samples. But has the lowest content (73.88%) than other samples (Table 2). As b has higher ester content but lower alcohol content than c, *Mucor* seems to be negative to alcohol but positive to ester. Ethyl alcohol was considered to be the most predominant volatile in liquor, therefore it accounts for a large proportion in the four liquors. Besides, higher concentration of 1-Butanol, 3-methyl-butanol was observed in the samples, which is significant to liquor as a higher alcohol [27]

Analysis of aldehyde and ketone
As is shown in **Table 2**, 5, 5, 5, 3 total aldehydes and ketones were detected in a, b, c, and d respectively, the

highest content of aldehude and ketone was quantified in a (8.47%), higher than b, c, d about 5%, whereas Ethane, 1,1-diethoxy- was the key compound in a (**Table 3**). It seems that *Monascus* coupled with *Mucor* and ester producing yeast can decline the content of total concentration aldehyde and ketone.

Analysis of hydrocarbon

5, 1, 3, 0 total hydrocarbon were observed in a, b, c, d respectably (**Table 2**), and a has the highest concentration, suggesting that *Monascus* coupled with *Mucor* and ester producing yeast probably contributed to the decline of hydrocarbon content.

Analysis of other compounds (phenol, naphthalene and other compounds included)

There were 5, 3, 5, 5 total other compounds were quantified in a, b, c, d respectively. Obviously, c showed the highest concentration, the results may imply that *Monascus* cooperated with *Mucor* could increase the content of these components.

CONCLUSION

In this study, HS-SPME coupled with GC-MS were applied to identify and quantify the volatile compounds of four Sichuan Xiaoqu liquor whose starter included several different functional microorganisms, result showed that *Monascus*, *Mucor* and ester producing yeast have really rare impact on the total volatile compound Specific compounds, however, did have some distinction: On the basis of adding *Rhizoups oryzae* with AADY, *Mucor* could be positive for alcohol but negative for ester, *Mucor* and *Monascus* mixed were determined to be positive for the content of naphthalene and phenol, while *Mucor*, *Monascus* and ester producing yeast mixed together were positive for ester but negative for aldehyde, ketone and hydrocarbon level.

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