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Full Length Research Paper

Analysis of microorganisms and physicochemical properties in *Zaopei* during the fermentation of Chinese zhijiang-flavor liquor

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Zhijiang-flavor liquor, one of the most famous strong aroma style of Chinese liquor, is distilled from fermented grains in Hubei province of China. In order to know the diversity, the constitution, and the dynamic change of microflora and physicochemical components in *Zaopei* during the fermentation of Chinese Zhijiang-flavor liquor, a normal pit in Zhijiang Distillery in autumn (used as experimental pit) was studied. The analysis and comparison of microorganisms in *Zaopei* during the fermentation process showed that the microbial genera changed with increasing fermentation time. Knowledge of the microbial diversity provides a basis for understanding the role and the contribution of microbes in the liquor-production process. More than 200 colonies were isolated and characterized. The isolates were discriminated by phenotypic, conventional biochemical taxonomic methods and bioMerieux Vitek-32. The presence of bacteria, moulds, yeasts and antinomycetes were revealed. The results showed that bacteria, yeasts, moulds and antinomycetes counts increased significantly during the first 4 days of the fermentation, and the latter three decreased sharply in the following 3 days; the total microbial population decreased gradually during the 7 and 24 days; the proportion of *bacillus* to aerobiotic bacteria had a rise after 24 days fermentation. In this study, 5 *bacillus* isolates were identified with species by VITEK-32 system. The 5 *bacillus* isolates were *Bacillus cereus* group, *Bacillus amyloliquefaciens*, *Bacillus megaterium*, *Bacillus sphaericus* and *Bacillus pumilus*.

Key words: *Zaopei*, chinese zhijiang-flavor liquor, strong aroma style, microflora.

INTRODUCTION

Chinese liquor is well known worldwide and is very popular in China. Among Chinese liquors, there are five main categories, which are strong aroma, light aroma, soy sauce aroma, sweet honey, and miscellaneous styles. The first four styles are the basic styles. The manufacture of strong aroma style liquor is the highest in quantity produced of the five types and is also the most accepted distilled spirit in China. Zhijiang-flavor liquor, which belongs to the strong aroma style, is described as highly flavored, sweet and refreshing. It is produced from the distillation of *Zaopei,* which is a mixture of fermented grains such as sorghum, barley, wheat, rice and pease. Before fermentation in the pits, newly steamed grains are mixed with *Daqu*, which was also named as the starter culture. The microbes growing in *Zaopei* are mainly from pit mud, *Daqu* and the fermentation environment and their metabolic products are crucial for liquor quality. In order to clarify the fermentation mechanism, many researchers have focused on analyzing the microbial community in the pits of *Zaopei*. Although water and alcohol account for 97-98% of liquor, other components which were generated in fermentation anaphase determine the flavor and quality of liquor. Moreover, the microbial environment in pits changes during the fermentation process, together with raw material consumption and formation of metabolic products.

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Recently, molecular ecological methods such as denaturing/ temperature gradient gel electrophoresis (DGGE/TGGE) (Muyzer, 1999), restriction fragment length polymorphism (RFLP), and real-time fluorescent quantitative PCR have been widely used for analyzing microbial community in various complex environments (Wang et al., 2008a). Though these molecular ecological methods can reflect the microbes in their environmental community accurately, it has disadvantages in gaining the isolates, understanding the multiple morphological, physiological and biochemical traits, constructing and conserving special functional microbe. In this study, more than 200 colonies were isolated and characterized. The isolates were discriminated by phenotypic, conventional biochemical taxonomic methods and VITEK-32 system. The VITEK-32 system corresponds perfectly to current bacteriological constraints, both in the clinical field and in industrial quality controls. Automation provides more safety and eliminates repetitive manual operations and the response time means so that results can be provided more quickly than with manual techniques (Hu et al., 2001).

By taking *Zaopei* as the research object, dynamic changes of microflora and physicochemical properties during the fermentation process were studied. The relationship of microflora and physicochemical properties can provide basic roles of microflora in this complex solid fermentation process. The knowledge would help to improve the quality and alcohol productivity of Zhijiang-flavor liquor.

MATERIALS AND METHODS

Sample of *Zaopei*

Sampling was carried in the Zhijiang-flavor liquor production factory in Hubei province of China. Samples were taken from a pit at different times during the fermentation process (0d, 4d, 7d, 14d, 24d, 34d and 44d). A special sampler was used to collect the samples from different depths (the top layer, the middle layer and the bottom layer of the pit) and different points (the center and the edge of the same layer). All the samples were transferred to sterile polyethylene bags and transported to the laboratory immediately.

Determination of physicochemical index

A temperature measuring meter specially for observing the temperature of pit was used. Total acidity, pH, crude starch and moisture content were determined as the methods described by Shen (Shen, 2007a).

Isolation

Samples (10 g) were mixed with 90 mL sterile physiological saline (0.85% w/v sodium chloride) and homogenized at a low speed of 120 rpm for 30 min. For spread-plating, 0.2 mL of the dilution was spread on the surface of the dry plates. The dilutions were as follows: 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}. Duplicate counting plates were prepared using appropriate dilutions. Colonies that appeared on the selected plates were counted and colony forming units (c.f.u.) per gram fresh weight sample after incubation for a period calculated (Shen and Chen, 2007b).

Representative colonies of each type were picked up and diluted by streak plate method. After microscopic examination and micrography, the purified colonies were stored in 15% glycerol at -18℃.

For total microbial counting, serial dilutions of homogenate were surface plated on four differential medium including beef extract peptone medium, yeast peptone dextrose medium (YPD), Starch medium, and Gause's No.1 synthetic medium which are suitable for bacteria, yeast, mould, and actinomyces, respectively, and then incubated at 35℃ for 18 - 24 h, 28℃ for 2d, 28℃ for 3 – 5 days, and 28℃ for 5 - 7 days (Shen and Chen, 2007b).

In estimation of *bacillus*, 10% (w/v) sample suspension was suitably diluted and spread on beef extract peptone ager plates, after being heated at 80℃ for 10 min, followed by incubation at 35℃ for 2 - 3 days (Wang et al., 2008b). For facultative microbes and anaerobes, the dilutions spread on beef extract peptone ager plates were incubated in vacuum drying oven at 35℃ for 3 - 4 days. For spread-plating, the same dilution was spread on the surface of two dry plates for each sample.

Identification of microorganisms

The bacterial isolates were classified by comparing their characteristics described in Bergey's Manual of Determinative Bacteriology (Buchanan and Gibbons, 1984) and General Methods of Identifying Bacteria (Dong and Cai, 1991). The yeasts isolates were discriminated by comparing their colonial morphology, cellular characteristics, ascosporic formation, and vegetative reproduction according to the methods described by Barnett (Barnett et al., 1991).

Moulds were identified using 7-day-old cultures on starch agar medium, and classified by morphological and biochemical characteristics as the methods described by Wei (1979).

Actinomyces were identified mostly like the way taken by moulds, but using Gause's No.1 synthetic ager medium. Bacillus strains were identified by bioMerieux Vitek 32.

RESULTS

Morphological identification

All the bacterial isolates were confirmed on the basis of different colony morphology (diameter, colour, shape, texture and surface), gram staining and micrograph. These results allocated the isolates into the following phenotypic patterns: *Bacillus*, *Lactobacillus*, *Lactococcus*, *Sporolactobacillus*, *Streptococcus*, *Staphylococcus*, *Microbacterium*, *Clostridium*, *Arthrobacter*, *Acetobacter*, *Enterobacter*, *Pseudomonas*, *Xanthomonas*, *Halococcus*, and *Bacillus* strains belonging to the most frequently isolated bacteria.

Bacillus strains were identified by using BAC cards; the results are as shown (Table 1; Figure 1): *B. cereus* group (Z1), *B. amyloliquciens* (Z2), *B. megaterium* (Z3), *B. sphaericus* (Z4) and *B. spumilus* (Z5).

All the representative yeast isolates were classified by means of morphology, micrograph, ascospore formation and vegetative reproduction bacterial. The results allocated the isolates into following phenotypic patterns: *Saccharomyces*, *Brettanomyces*, *Hansenula*, *Dekkera*,

Table 1. Manual Vitek laboratory report.

	NEG	SUC	TZR	TAGI	GLU	INO	GAL	ARA	XYL	MAN	RAF	SAL	AGA	INU	RIB	MLT
Z1	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	+
Z2	-	+	-	-	+	-	-	-	-	+	-	-	-	+	-	-
Z3	-	+	-	-	+	-	+	-	+	+	+	-	-	+	-	+
Z4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Z5	-	+	+	+	+	-	-	+	-	+	-	+	-	-	-	-
	TRE	**PLA**	**SOR**	**NAG**	**AMY**	**KCN**	**NCL**	**MEN**	**OLD**	**NAA**	**ARB**	**PAS**	**NAE**	**ESE**	**THRM**	
Z1	+	-	-	+	+	+	-	+	-	-	-	+	+	+	-	
Z2	-	-	+	-	-	+	-	+	-	-	-	+	-	+	-	
Z3	+	-	-	+	-	+	-	+	-	-	-	-	-	+	-	
Z4	-	-	-	+	-	-	-	-	-	+	-	-	-	+	-	
Z5	+	-	-	-	-	+	-	+	-	+	-	+	-	+	-	

NEG: Negative control; SUC: sucrose; TZR: tetrazolium red; TAGI: tagatose; GLU: glucose; INO: inositol; GAL: galactose; ARA: galactose; XYL: xylose; MAN: mannitol; RAF: raffinose; SAL: salicin; AGA: amygdalin; INU: inulin; RIB: ribose; MLT: maltose; TRE: trehalose; PLA: palatal agarose; SOR: sorbitol; NAG: N- acetyl - D- glucosamine; AMY: amylopectin; KCN: potassium thiocyanate; NCL: 7% NaCl; MEN: mandelic Acid; OLD: oleandomycin; NAA: sodium acetate; ARB: arabitol; PAS: phytostreptin; NAE: nalidixic acid; ESE: aesculin; "+": positive; "□": negative; "+/_": have two results.

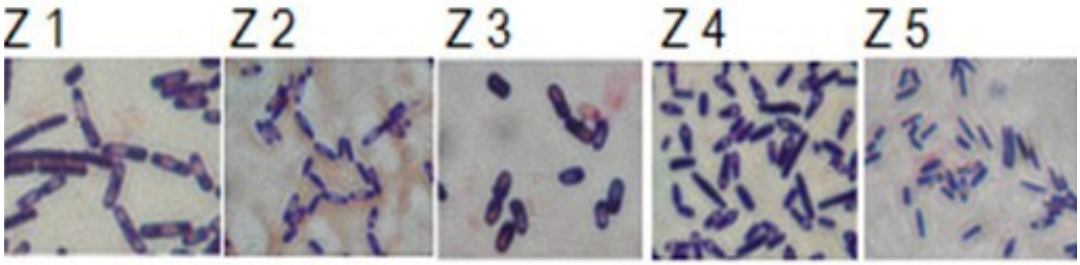

Figure 1. The micrographs of some *Bacillus.*

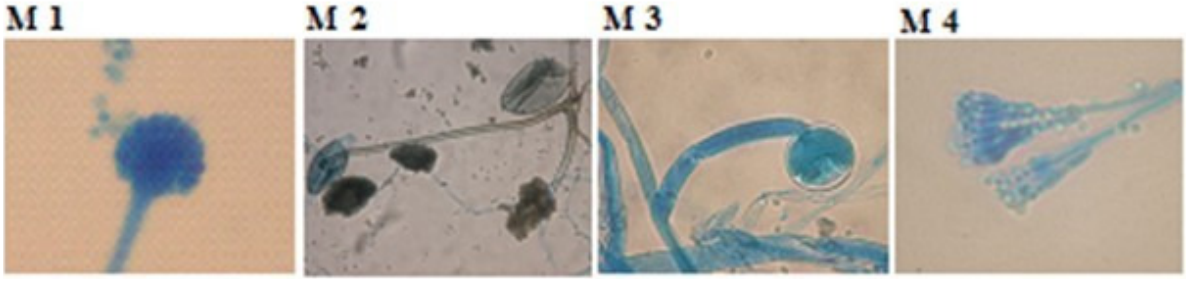

Figure 2. The micrographs of some moulds.

Issatchenkia, *Torulaspora*, *Rhodotorula*, *Candida*, *Pichia* and *Geotrichum candidum*, among which *Saccharomyces* and *Geotrichum candidum* were found to be predominant.

Mould isolates were identified by colony, cell morphology, and micrograph of conidiospore formation. These results allocated the isolates into five genera (Figure 2). *Aspergillus* (M1), *Rhizopus* (M2), *Mucor* (M3), *Penicillium* (M4), and *Aspergillus* and *mucor* were found to be predominant.

Antinomycetes were discriminated same way as the moulds; the result indicated that only *Streptomyces* were found in the fermentation process (Yan, 1992).

The dynamic change of microflora

The microbial community in *Zaopei* was studied by tracking the entire 44-day fermentation process. The shift

Table 2. The dynamic change of aerobiotic bacteria and Bacillus ($\times 10^4$ cfu/g).

Fermentation process	31		32		41		42		51		52	
	AB	B	AB	B	AB	B	AB	B	AB	B	AB	B
0d	5.15	2.7	5.15	2.7	3.88	1.24	3.88	1.24	4.2	0.3	4.2	0.3
4d	60	55	40	40	77.5	71	95	89	30	28	40	38
7d	15	4	13.5	13.5	20	18	28	15.4	9.5	8	30	1.5
14d	20	10.5	12.5	9.5	11	8.6	18	9.6	16.5	10.4	11	9.9
24d	7	6.5	13	12	4.5	4	14	8.4	9.5	9	12	11.4
34d	30	29	35	25	18	17	35	30	20.5	19	9.5	9.1
44d	1.9	1.9	1	1	0.45	0.45	2	2	0.65	0.65	1.45	1.35

31: The center of the top layer; 32: the edge of the top layer; 41: the center of the middle layer; 42: the edge of the middle layer; 51: the center of the bottom layer; 52: the edge of the bottom layer. AB: aerobiotic bacteria; B: *Bacillus*.

Table 3. The dynamic change of facultative anaerobes ($\times 10^4$ cfu/g).

Fermentation process	31	31	41	42	51	52
0d	—	—	—	—	—	—
4d	—	—	—	—	—	—
7d	89	34	72	66	45.5	75
14d	19.5	11	26	62.5	31.5	37.5
24d	1.75	13	1.35	21.5	1.25	2.25
34d	22.5	7.5	20	3.3	3.6	4.75
44d	0.6	4.4	0.3	2.25	0.2	0.5

Table 4. The dynamic change of yeasts ($\times 10^4$ cfu/g).

Fermentation process	31	31	41	42	51	52
0d	8.8	9	2.7	2.9	4.4	4.4
4d	1250	2050	955	855	260	160
7d	660	380	580	120	150	820
14d	19	12.5	50.5	7.5	21	25.5
24d	30	5	1.8	8.5	2.25	4.25
34d	20	11	8	7.5	1.25	0.9
44d	0.25	5	0	0.1	0	0

of microbial counts with the changes of fermentation process is shown in Tables 2, 3, 4, 5 and 6. The plant counts indicated that the total microbial population, bacteria, yeast, mould and antinomycete numbers were proliferated rapidly in the prophase and decreased in the anaphase. The increase in counts may be due to the favorable conditions such as temperature, pH and rich nutrition which availed the multiplication of the microorganisms.

It can be observed that the aerobiotic bacterial counts had a significant enhancement in the first 4 days of fermentation, and they reached a maximum (10^6cfu/g). They decreased suddenly in the following 3 days, and the counts kept at 10^5cfu/g during the 7 days and 34 days fermentation (Table 2). The increasing shift may be due to the developed amylolytic activity, causing degradation of the starch in the grains into glucose, disaccharide and so on, which were suitable for the growth of micro-organisms. Their subsequent decrease in number could owe to the death of some aerobiotic bacteria as a result of the increased temperature, the degradation of the starch, a fall in pH and the consumption of oxygen.

Bacillus was similar to aerobiotic bacteria, both in the count and the shift. The proportion of *Bacillus* to aerobiotic bacteria had a rise after 24 days fermentation. At the end of the fermentation process, esterification which is the formation of aromatic compounds replaced the production of alcohol. As a result, bacteria which can yield acid had a multiplication while the bacteria which were not acid-resistant dropped off. It attributed to the increased proportion of *Bacillus*.

Similarly, facultative anaerobes' counts proliferated fast

Table 4. The dynamic change of yeasts ($\times 10^4$ cfu/g).

Fermentation process	31	31	41	42	51	52
0d	8.8	9	2.7	2.9	4.4	4.4
4d	1250	2050	955	855	260	160
7d	660	380	580	120	150	820
14d	19	12.5	50.5	7.5	21	25.5
24d	30	5	1.8	8.5	2.25	4.25
34d	20	11	8	7.5	1.25	0.9
44d	0.25	5	0	0.1	0	0

Table 5. The dynamic change of moulds ($\times 10^2$ cfu/g).

Fermentation process	31	31	41	42	51	52
0d	5	5	7	7	3	3
4d	10	85	50	30	50	10
7d	1	8	0	5	0	0
14d	0	1	0	5	0	5
24d	0	5	0	0	0	0
34d	30	10	5	10	0	0
44d	3	5	0	0	0	0

Table 6. The dynamic change of antinomycetes ($\times 10^2$ cfu/g).

Fermentation process	31	31	41	42	51	52
0d	5	5	10	10	10	10
4d	85	100	12.5	25	50	2.5
7d	15	20	2.5	10	5.5	17.5
14d	2.7	2.1	1.1	13.1	6.6	5.1
24d	30	21	1	1.5	35	19
34d	44	60	3.5	4	8	16
44d	0.1	0.2	0	0	0	0

in the first 4 days fermentation, then reduced and the number maintained at 10^4 cfu/g after 14 days fermentation (Table 3). All the facultative anaerobe isolates were incubated both in aerobiotic conditions and a vacuum drying oven; the result turned out that the facultative anaerobes incubated in aerobiotic conditions proliferated faster then the latter. A conclusion could be drawn that most of the facultative anaerobes in *Zaopei* were preferred to be aerobic. The pit graduated into an anaerobic environment in the end of the fermentation process, which was not suitable for facultative anaerobes. This may be the reason for the death of aerobiotic bacteria and the low counts after 44 days fermentation (10^3cfu/g).

Yeasts in *Zaopei* were mainly from *Daqu* and the fermentation environment. The viable number of yeast increased tremendously in the first 4 days and remains high (10^7 cfu/g) in the latter 3 days (Table 4). This bloom of the fermentation process resulted from the high amylolytic activity caused by amylase, leading to degradation of the starch in the grains into sugars. The counts, which reduced to a low level after 14 days of fermentation, finally dropped to a non-detectable level after 44 days fermentation. In the end of the fermentation process, alcohol and acid compounds were increased compared to the decrease of sugars. These changes of substrates in *Zaopei* made the environment in pit unsuitable for yeasts surviving. Besides, yeasts cannot proliferate in the anaerobic condition. The facts above can be attributed to the few amounts of survivors after 44 days fermentation.

The amounts of moulds reached the peak at 4 days (10^3 cfu/g), following gradual decrease, even dropping to a non-detectable level. The aerobiotic moulds are mainly from *Daqu*, and they were not adapted to the anaerobic condition after 7 days fermentation. But moulds were detected at 34 days again. The moulds number decreased along with the increasing depth; this is because the middle layer and the bottom layer were more anaerobic

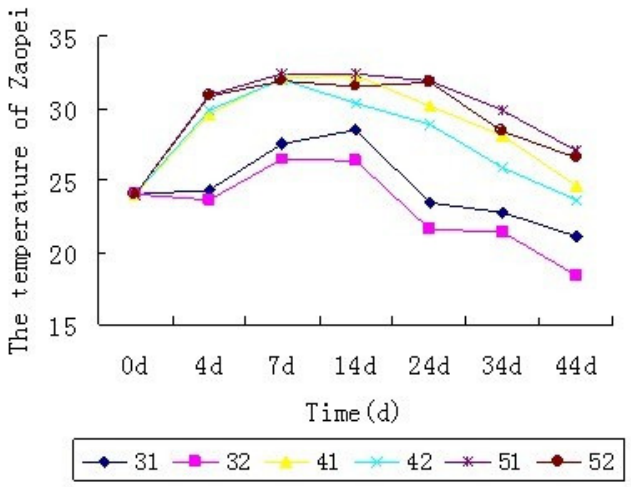

Figure 3. The change in temperature during the fermentation process.

compared with the top layer.

The shift of antinomycetes was similar to the moulds, this may due to the functions of antinomycetes and moulds which were the same. The rapid multiplication of antinomycetes in the beginning was displaced by bacteria in the end (Table 6).

The diversity of the microbial community

Bacillus, the dominant genus of the whole process, was discovered in the early days of the fermentation process. Most of the *Bacilli* were constituted by *B. cereus* group, *B. amyloliquefaciens*, *B. megaterium*, *B. subtilis, B. sphaericus* and *B. pumilus*. Among these *B. cereus* group and *B. amyloliquefaciens* belonged to the predominant genus.

Acetobacter and *Lactococcus* also appeared early, and their counts were not very big. They were mainly detected in the top and the middle layer. Other bacteria, including *Lactobacillus*, *Pseudomonas*, *Xanthomonas*, *Sporolactobacillus*, *Clostridium*, and *Staphylococcus* mainly developed in the middle and the end of the fermentation process. *Clostridia*, which were in the middle layer and the bottom layer, were more than the other bacteria.

Issatchenkia and *Saccharomyces*, the high frequently isolated yeasts, reached their maximum level (both 10^6 cfu/g) at 4 days. The amount in the top layer was more than the other two layers, and the edge was more than the center. The high concentration of alcohol during the 7 and 14 days resulted from these two predominant yeasts. *Geotrichum candidum*, another high frequently isolated yeast, obtained the peak at 14 days (10^5 cfu/g).

Mucor and *Aspergillus* were detected in the whole fermentation process. *Aspergillus* had high saccharifying and liquefying power. *Rhizopus* also had high saccharifying power, but its amount was very low, and so did *Penicillium*.

Streptomyces, the only genus of antinomycetes, were found in the fermentation process. The maximal number was 10^4 cfu/g. *Antinomycetes* came from the pit mud and the environment, contributed to the formation of aromatic compounds, and facilitated the degradation of the substrates.

Physical-chemical index in *Zaopei* during the fermentation

Temperature variation was shown in Figure 3. The temperature of the pit increased fast and reached maximum (32℃) after 7-14 days with the growth and reproduction of microbe, and then decreased. The bottom temperature reached maximum but the upper temperature reached minimum.

The change in pH and total acid between different spatial locations during the fermentation were shown in Figures 4 and 5. Organic acids, used as as carbon and energy source, decreased deeply while acidity increased between 7 and 21 days as a result of the reproduction of microbe and sugar supply deficiency. And the pH and total acid of the three layers appeared to have the similar changes.

Moisture change can be seen in Figure 6. The content of water in *Zaopei* increased and finally reached saturation due to the large amount of free water formulated by respiratory metabolism of microorganism. The excess water flowing to the lower fermented grains finally became the major components of yellow water (Zhang et al., 2005).

The change trend of the crude starch content during the fermentation was shown in Figure 7. With rich nutrition, microbes in the upper and mid layer had vigorous metabolism, starch were degraded, so the crude starch content of upper and mid layer decreased sharply at the beginning of the fermentation. But the starch in the bottom fermented grains decreased more slowly than the other two because of double bottom fermentation. The content of the crude starch finally reached about 12 - 13% in the upper fermented grains and about 8 - 10% in the middle and bottom fermented grains.

DISCUSSION

This study analysed the diversity, constitution, dynamic change of microbes and the physical and chemical components in fermented grains during the fermentation of Chinese Zhijiang-flavor liquor.

The plant counts indicated that the total microbial population, bacteria, yeast, moulds and antinomycetes numbers were proliferated rapidly in the first 4 days and decreased with the fermentation time. The total microbial population was influenced by physical and chemical components such as the pH, the total acid, moisture, temperature and the starch content.

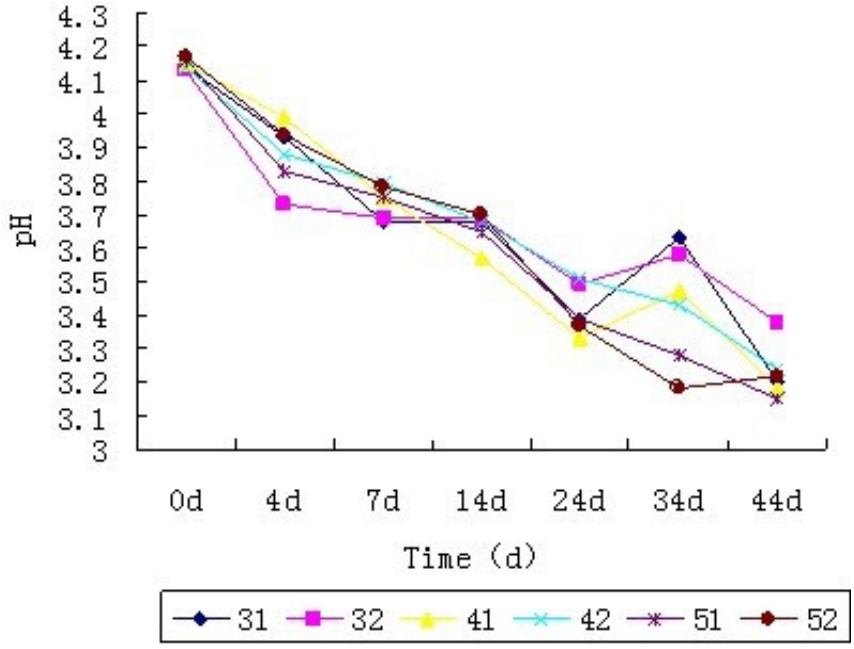

Figure 4. The change in pH during the fermentation process.

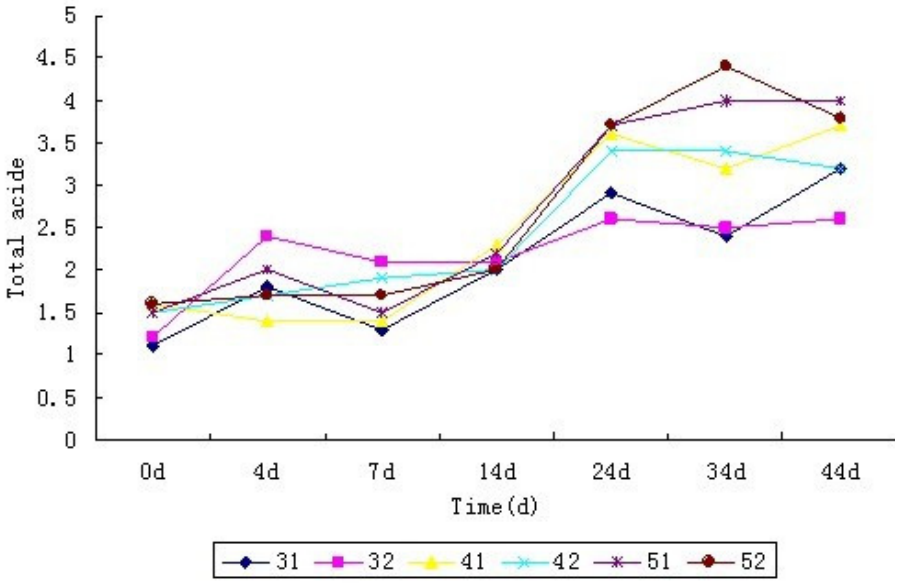

Figure 5. The change in total acid during the fermentation process.

Bacillus spp. was the most frequently isolated bacterium, which had a better ability than other bacteria to survive under the circumstance of high temperature and low pH. With the progressive increase of fermentation time, they gradually become the dominant microbes. *Bacillus* was the significant sources of amylases and proteases. The hydrolytic capabilities of these microorganisms, which depend on their possession of these two enzymes, can result in a precursor-rich environment, and that is vital for subsequent reactions leading to flavor liquor production (Beaumont, 2002).

Other bacteria such as *Acetobacter*, *Lactobacillus*, *Butyric acid* bacteria, and *Caproic acid* bacteria can produce acetic acid and lactic acid from glucose or other sugars. Esterification of ethanol and acetic acid, lactic acid, butyric acid and caproic acid, ester formation of and ethyl acetate, ethyl lactate, ethyl butyrate, and ethyl caproate were carried out during the fermentation process. *Butyric*

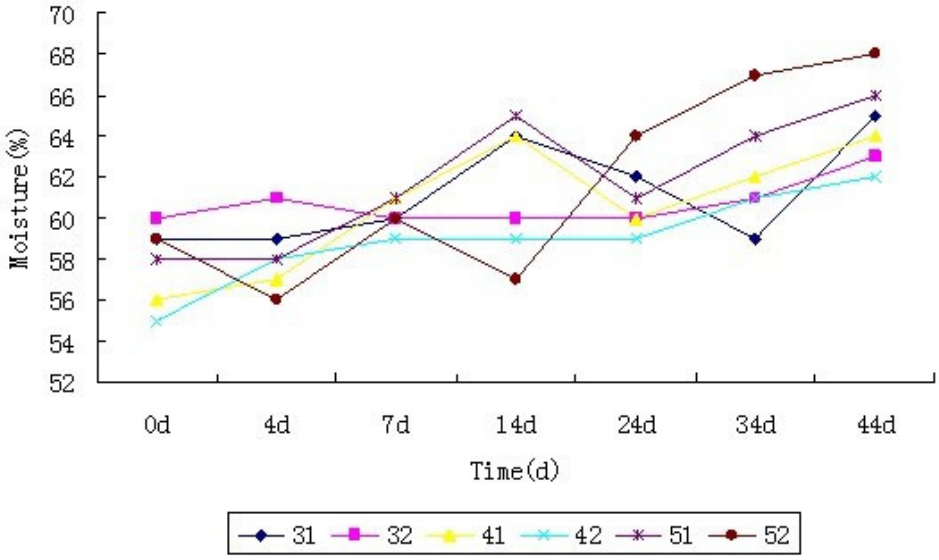

Figure 6. The change in moisture during the fermentation process.

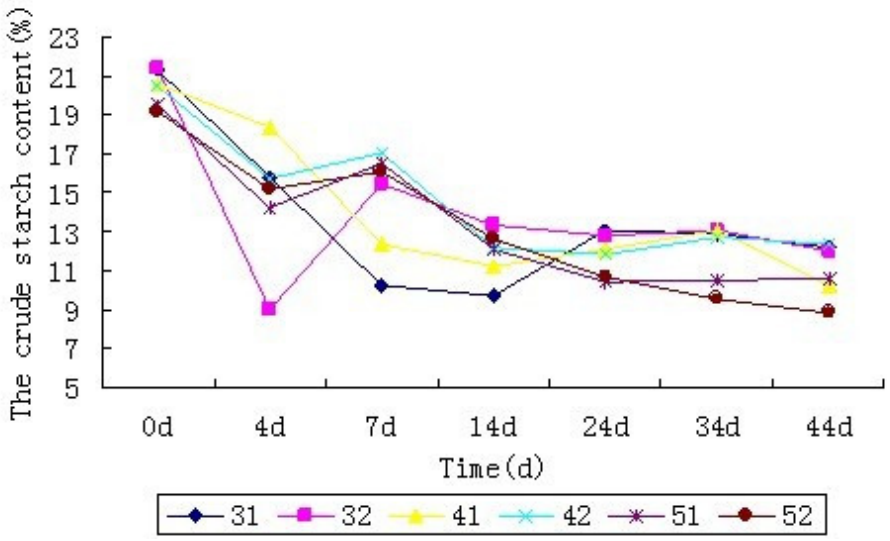

Figure 7. The change in the crude starch content during the fermentation process.

acid bacteria, and *Caproic acid* bacteria belong to the genus *Clostridium*, which play a crucial role in the formation of aromatic compounds in strong aroma distilled liquors.

Issatchenkia and *Saccharomyces*, the high frequently isolated yeasts, are often associated with alcoholic fermentation, and can produce esters and alcohols. In the end of the fermentation process, alcohol and acid compounds were increased. These changes of substrates in *Zaopei* and the anaerobic condition were no longer suitable for the multiplication of yeasts.

The amounts and the diversity of moulds and anti-nomycetes were lower than bacteria and yeasts. A frequent mould was the genus *Aspergillus*, which is probably due to the abundance of the organisms in nature. The genus has the ability to secrete large amounts of a wide range of different enzymes into its environment (Oda et al., 2006), which may contribute to the saccharification of the remaining starch.

A special micro-database of *Zaopei* was established by this research. *Pseudomonas* and *Halococcus* were gained while these bacteria were not found in *Zaopei* from other Distillery. The roles of them need further research. Compared with bacteria, the variety of yeasts and moulds were similar to others.

The analysis of microorganism community structure, the diversity and the dynamic change of microbes in *Zaopei* obtained by traditional microbial identication

based on culture and separation, and bioMerieux Vitek-32 will help to isolate and identify important functional microbes. Knowledge of the microbial functions of *Zaopei* provides a basis for microflora management and understanding of the role of microbes in the solid fermentation process, and will make the fermentation mechanisms clearer.

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