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Isolation and Identification of Aroma-producing Yeast Strain from Black Glutinous Rice Wine

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The important aroma-producing yeast strain from black glutinous rice (BGR) wine made in Guizhou was isolated through a primary and secondary screening method. The strain was then identified by morphological characteristics and gene sequence analysis. The results showed that the aroma-producing strain 27 aroma compounds in traditional fermentation broth and 32 aroma compounds in aroma-producing strains fermentation broth. Among these compounds, 16 of them were found in both aroma-producing strain traditional starter fermentation broths and more than half of the total detected aroma compounds in traditional starter. Based on the characteristics of colony and thallus appearance, the aroma-producing strain was identified as *Cyberlindnera jadinii (Pichia jadinii).*

Keywords: Black glutinous rice wine; aroma-producing strain; isolation; identification.

Black glutinous rice known as the black pearl rice, also called longevity rice, which has high nutritional and medicinal value (Song & Zhai, 2010; Sun, Xu, Chen & Chen, 2010). Tananuwong and Tewaruth (Tananuwong & Tewaruth, 2010) determined optimum extraction condition of BGR crude extract and antioxidants such as phenolic content (1878 ug gallic acid/g flour) and total monomeric anthocyanin content (288 ug cyaniding equivalent/g flour) .BGR wine, made from BGR, has also the advantages of both mellowness white wine and sweetness rice wine. BGR wine appearance is brownish red or amber, with tasty acidity and rich fragrance flavor. It contains a variety of nutrients needed by human, such as amino acids, vitamins, trace elements and natural pigment (Raozhou & Qinghua, 1995). BGR wine is a favor wine with a huge consumption population in China. In recent years, the microbial fermenters and aroma components of different types of wine has been an interesting research area. Baffi *et al*.(Baffi, Bezerra, Arevalo-Villena, Isabel Briones-Perez, Gomes & Da Silva, 2011) examined the diversity of yeast species isolated from grape skin and musts of two varieties of *Vitis labrusca* from a vineyard in the southeast region of Brazil. Eighty yeast samples were isolated from grapes and musts, and seven different species were identified. A total of 80 yeast strains from musts and wines on a selective medium were isolated during the entire wine fermentation. The *Saccharomyces cerevisiae* strains were tested for β-glucosidase activity with only one positive strain (Restuccia, Pulvirenti, Caggia & Giudici, 2002). About 50 local red wines including 27 fine wines (*V. vinifera*) and 23 table wines were analyzed (*V.labrusca*). The majority of

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isolates belonged to *Brettanomyces bruxelliensis*, followed by *Pichia guillermondii,* and more rarely *Candida wickerhamii and Trigonopsis cantarelli* (Hall, Zhou, Qian & Osborne, 2012). Ribosomal intergenic spacer analysis (RISA) and traditional culture-based methods were employed to examine the fungal community of Shaoxing rice wine wheat Qu. RISA profiles of total DNA were found that exhibited nine distinguishable bands. The RISA fingerprints recovered from enrichment media provided variable patterns containing fewer bands (Xie *et al*., 2007). For aroma components in wine, through a dehydration step followed by GC/MS, the wine aroma compounds were detected (Angioni, Pintore & Caboni, 2012) . Different headspace devices for the gas chromatography-olfactometric evaluation of wine aroma were also studied. A total of 23 volatile compounds of wine aroma added to a synthetic wine were purged by an inert gas and trapped in a solid-phase extraction (SPE) cartridge (San-Juan, Pet Ka, Cacho, Ferreira & Escudero, 2010). Besides, the aroma compounds in Chinese rice wine Qu were characterized by solvent-assisted flavor evaporation and headspace solid-phase microextraction(Mo, Xu & Fan, 2010) .The aroma-producing characteristics of *Saccharomyces cerevisiae* strains were evaluated by electronic nose and monitored quorum-sensing molecules during minifermentation studies on wine yeast (Liu, Ma & Liu, 2011) (Zupan, Avbelj, Butinar, Kosel, Sergan & Raspor, 2013). Although there are other reports about wines, such as molecular identification of the yeast strains associated with spontaneous wine fermentation of Kalecik Karasi and Emir grapes(Karasu-Yalcin, Senses-Ergul & Ozbas, 2013) and comparative analysis of Papaya wine from other fruit wine(Maragatham & Panneerselvam, 2011). However, the isolation and identification of BGR wine strains have not been well documented, especially for aroma-producing strains. Based on the research results of isolation and identification of BGR wine yeast strains in our laboratory (Xu, Mu, Chen, Lei & Su, 2012), a primary and secondary screening method was conducted to obtain some good aroma-producing strains. The fermented starter with the soft flavor and aroma was selected for analysis of the aroma components by GC /MS and molecular identification. The result of this study could provide a fundamental information for research and development of traditional BGR wine.

MATERIALS AND METHODS

Materials

BGR yeast,10 strains, numbered $Q_1 \sim Q_{10}$, were collected in Guizhou, China; BGR samples were purchased from local market of Guiyang city, China.

Brewing process of BGR wine

The black glutinous rice was cleaned twice or three times r. Then it was soaked in 60 °C water for 3 hours. During cooking at atmospheric pressure, 85°C hot water was added in order to ensure that the rice yield rate was 160% to 200%.The steamed BGR was cooled to 28 to 30°C. About 1.0% (w/w) starter was added and mixed. The steamed BGR was fermented for 6 to 7 days at 28 to 30°C. After fermentation, the wine was pressed in time. The pressed wine was clarified for 2 to 4 days. The BGR wine was obtained after filtration and sterilization.

Primary screening of aroma-producing strains

The primary screening medium consisted of soluble starch 12 g, yeast extract 8 g, NaCl 5 g, agar 15 g and water 1L.A reagent 2.2 g I_2 and 4.4 g KI, dissolved in l00 ml of distilled water was prepared. Preparation of isolates singlel spores (ISS) was by mixing sterile water into activation agarslantculture-medium .The spores or bacterial cells were scraped and beaten. The spores were diluted to 1×10^6 / ml after quantification using a hemocytometer. The isolates single spores were cultured at 30ⁱC for 1 ~ 3 d after diluted and coated at the primary screening medium. Then the iodine reagent was pour on the primary screening medium. The transparent circle around the colonies were observed and measured by the colony diameter (D) and transparent circle diameter (d) . The higher the size was, the stronger the force of glucoamylase production. The experiment was carried out by five plates. Relationship between the activity and transparent circle expressed by the formula (Xu & Jiang, 2001):

$$\log ([E]) / R) = k (d / D) (\Delta [C] / \log t)$$

(Formula: [E] - production enzyme concentration; R - cell volume; d - transparent circle diameter; D - diameter of the colony; Δ - agar concentration; [C] - concentration of substrate; t - incubation time; k – constant .)

Secondary bran screening was performed as described by Chen (Chen, 1996)

A total of 20 g fresh bran was weighed, and mixed with water (60% w/v). After mixed for 30 min, the mixture sample was added into 500 ml flask with a 2 -3 cm thick, stoppered with cotton wool tampon. The sample was autoclaved at 0.10 MPa for 40 min, and exhausted to lower pressure. When the temperature dropped to 25 ~ 30 °C, the second sterilization was repeated for 40 min. After sterilization, the flask was removed and shook well. When the bran medium was cooled to 30 °C, 3ml ISS was inoculated. The sample was cultured at 32 °C for 72 h. Then the sample was put into the sterile bag, and dried at 40 °C for 12 h. The liquefaction power and saccharifying power were measured. BGR wine based on the traditional brewing process was fermented. The sugar, total acid content and aroma of fermentation starter were measured through sensory evaluation by five wine professional trained sensory tasters. Aroma components in the fermented starter with soft flavor were analyzed by GC/MS.

Determination of aroma compounds by using GC/MS

The determination was performed as described by You *et al*.(You, Wang, Zhan & Huang, 2008). Fifty μm thickness of DVB/CAR/PDMS extraction head was selected. The sample was 120 ml/l alcoholicity with warm-up time 20min, extraction temperature 45 °C, extraction time 30 min.The concentration of electrolyte NaCl was 0.30 g/ml.

The analysis condition of GC was splitless mode,GC column HP-INNOWAX (30.0 m × 0.25 mm × 0.25 μm),programmed temperature 40 °C HELD for 8 min, then with 5 °C /min rate to 70 °C and held for 1 min, 5 °C / min rate to 185 °C, and then with 10 °C /min rate to 230 °C and held for 8 min,detector temperature 250 °C and inlet temperature was 280 °C. The MS condition was EI ionization source, with electron energy 70 eV, scan range 50 ~ 500 amu, ion source temperature 250 °C.

Aroma-producing strain ITS Sequence analysis method

Yeast ITS sequence analysis was performed using the methods of Lehtonen (Lehtonen & Patrikainen, 2012). Universal primers ITS5 (5'GGAAGTAAAAGTCGTAACAAGG-3 ') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') were used to amplify genomic DNA and ITS-5. 8S rDNA sequences were obtained. Five ul amplification product was analyzed by agarose gel electrophoresis.

RESULTS

Primary screening of starch transparent circle

A total of 28 *Saccharomycopsis* and 212 *Pichia* strains identified were inoculated on starch plates and cultured at 28 °C for 24 h in the previous studies. After coloration, the diameters of saccharification transparent circle and colonies were measured to screen 12 yeasts with strong saccharification. Strain Q_{10}-M_{62} was cultured at 28°C for 24 h. Saccharification transparent circles were shown in figure1. Traits of higher capacity of glucoamylase production strains were shown in Table 1.

According to the formula log ([E])/R) = k (d/D) (Δ [C]/logt), the ratio of glycated transparent circle diameter and colony diameter (d/D) and enzyme concentrations were positively correlated. Therefore, the larger d/D, the stronger the production of glucoamylase(Xu *et al*., 2001). On the basis of saccharification transparent circle of screening test, 12 strains with high glucoamylase production capacity such as Q_8-M_{27}, Q_9-M_{45}, Q_3-M_{24} and so on were selected for further screening.

Bran medium secondary screening and BGR test

Twelve strains selected by primary screening were screened by secondary screening through bran medium, including bran solid starter extraction enzyme solution. The enzyme solution liquefaction and saccharification power were determined to compare the strain enzyme production power, saccharification power, acid production capacity, and aroma-producing capacity.

As seen from Table 2, Q_{10}-M_{62} strain showed higher saccharification enzyme production capacity than others, and contained the highest sugar level in the final starter. It suggested that the strain had significant influences on BGR saccharification, but relatively lower acid production capacity. Its aroma score was the highest. As a result, Q_{10}-M_{62} is an excellent aroma-producing strain.

GC / MS analysis

Yeast is the key functional fungi of wine-

producing and aroma-producing. It directly affects the brewing process, thereby affects the wine production and aroma components (Hou, Wang, Li, Hu, Li & Gao, 2013). GC / MS has the abilities of efficient chromatographic separation and specific mass spectrometry identification and is commonly applied to the analysis of wine aroma components (Aznar, L O Pez, Cacho & Ferreira, 2001; D I Az-Maroto, S A Nchez-Palomo & P E Rez-Coello, 2004). From the GC / MS analysis results in Table 3, 27 and 32 aroma components were detected in traditional and aroma-producing strains fermentation broths. The variety was less than aroma detected in light aroma type liquors by means of gas chromatography"olfactometry coupled with mass spectrometry, which was a total of 66 aroma compounds (Gao, Fan & Xu, 2014). Besides, a total of 39 aroma compounds were characterized by GC-O in Chinese rice wine Qu (Mo *et al*., 2010). Because different microorganisms and enzymes can produce different aroma components.

As we can see, there was a big difference between the two content of aroma components, like composition and ratio. There were a lot of microorganisms in traditional fermentation broth .Because of antagonistic, these microorganisms may inhibit the growth of some other microbes, such as aroma-producing strains. As a result, aroma composition and ratio were different. The kinds of

Table 1. Saccharificating properties of 12 strains selected by starch transparent circle method

Number	Strains number	Diameter of saccharification transparent circles d (cm)	Diameter of	d/D colonies D (cm)
1	Q_8-M_{27}	1.70±0.14	0.50±0.00	3.40±0.28
2	Q_9-M_{45}	1.60±0.07	0.44±0.05	3.68±0.47
3	Q_6-M_{14}	1.48±0.04	0.40±0.00	3.70±0.11
4	Q_9-M_{43}	1.80±0.07	0.52±0.04	3.47±0.19
5	Q_3-M_{24}	1.72±0.08	0.50±0.00	3.44±0.17
6	Q_6-M_5	1.62±0.08	0.46±0.05	3.55±0.32
7	Q_{10}-M_{53}	1.66±0.09	0.50±0.00	3.32±0.18
8	Q_9-M_{27}	1.68±0.00	0.50±0.00	3.36±0.09
9	Q_{10}-M_{62}	1.86±0.05	0.56±0.05	3.34±0.24
10	Q_{10}-M_{45}	1.72±0.08	0.54±0.05	3.20±0.20
11	Q_{10}-M_{50}	1.94±0.05	0.56±0.05	3.49±0.29
12	Q_{10}-M_{25}	1.88±0.08	0.52±0.04	3.72±0.11

Table 2. Saccharificating property comparison of different strains

No.	Strains	Liquifying enzyme activity (U/g)	Saccharifying enzyme activity (U/g)	Reducing sugar (g/100g)	Black rice test Total acid (g/100g)	Aroma
1	Q_8-M_{27}	68.58±0.66	454.88±3.87	13.17±0.31	8.76±0.16	76±1
2	Q_9-M_{45}	69.40±0.71	419.61±5.24	11.75±0.21	8.01±0.05	76±2
3	Q_6-M_{14}	68.51±0.55	291.44±4.14	9.21±0.19	9.15±0.34	71±1
4	Q_9-M_{43}	68.57±0.58	407.66±2.59	14.21±0.20	9.56±0.05	74±2
5	Q_3-M_{24}	69.51±0.53	237.47±4.14	7.52±0.10	19.14±0.18	75±3
6	Q_6-M_5	69.45±0.59	288.30±3.27	8.85±0.08	6.30±0.20	74±2
7	Q_{10}-M_{53}	68.80±0.20	336.83±3.23	8.85±0.12	7.12±0.17	73±1
8	Q_9-M_{27}	69.43±0.50	430.75±5.14	12.22±0.20	9.75±0.05	75±3
9	Q_{10}-M_{62}	83.77±0.38	497.63±5.29	12.75±0.12	9.30±0.19	80±2
10	Q_{10}-M_{45}	68.51±0.55	264.30±2.07	7.43±0.38	9.11±0.14	75±2
11	Q_{10}-M_{50}	71.27±0.61	444.72±3.53	11.58±0.05	10.20±0.19	76±1
12	Q_{10}-M_{25}	70.16±0.18	345.84±2.57	8.35±0.09	8.90±0.09	72±1

aroma components in traditional fermentation broth were less than Q_{10}-M_{62} fermentation broth. Maybe there were more miscellaneous microorganisms in traditional fermentation broth and they may produce more methylalcohol. Methyl linolelaidate generated from methyl- esterification reaction of trans- linoleic acid with methylalcohol. The more methylalcohol benefited methyl- esterification

Table 3. GC/MS analysis result of aroma in traditional and aroma-producing fermentation starter

Aroma composition	Aroma content in traditional fermentation broth (%)	Aroma content in Q_{10}-M_{62} fermentation broth (%)
isopentyl alcohol *	10.679	3.551
acetylmethylcarbinol *	1.861	1.355
hydroxyacetone *	0.101	0.338
acetic acid *	10.745	6.739
methanoic *	0.319	1.709
2, 3 - butanediol *	1.835	22.675
isobutyric acid *	0.395	1.488
1, 3 - butanediol *	0.826	11.748
furfuryl alcohol *	0.29	1.017
isovaleric acid *	0.138	3.456
2 - hydroxy - 2 - ring pentene 1 - ketone *	0.157	0.375
phenethyl alcohol *	3.976	12.826
maltol *	0.101	0.368
4, 5 - dimethyl - 2 - formyl furan *	0.488	0.449
1, 3 - dihydroxyacetone *	0.626	5.882
2,4-di-tert-butylphenol*	0.801	1.646
n-propanol	7.177	—
isobutanol	7.875	—
n-butyl alcohol	0.14	—
ethyl lactate	0.758	—
diethyl succinate	0.573	—
azulene	0.575	—
trans - 1 - methoxy - 4 - (1 - allyl) benzene	0.216	—
guaiacol	0.58	—
5-hydroxymethylfurfural	8.405	—
mevalonic acid	2.221	—
methyl linolelaidate	37.025	—
sec-butyl alcohol	—	2.446
biose	—	2.743
methyl acrylate	—	0.345
furfuraldehyde	—	0.941
3 - methyl - 2 - (5 h) - furan ketone	—	0.149
butyrolactone	—	0.134
butyric acid	—	0.23
4 - hydroxy ethyl butyrate	—	0.563
4 - hydroxy - 5 - oxidation caproic acid lactone	—	0.772
^{3}-Heptalactone	—	0.461
ethyl tetradecanoate	—	1.106
succinaldehyde	—	0.486
pyranone	—	1.727
glycerol	—	0.762
ethyl palmitate	—	8.683
diethyl phthalate	—	3.458

Note :"—" means no date; "*" means common composition

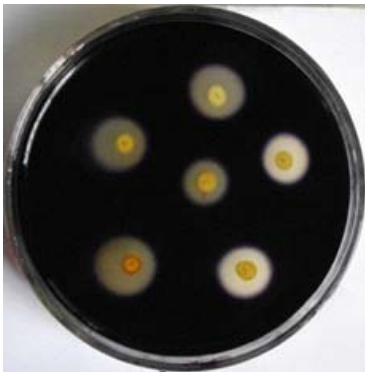

Fig. 1. Saccharification transparent circle of Q_{10}-M_{62}

Fig. 3. The colony photo of Q_{10}-M_{62}

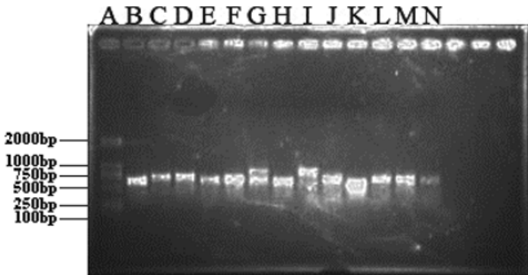

Fig. 2. PCR electrophoretogram of Q_{10}-M_{62} (A: Maker; J:ITS sequences of aroma-producing Q_{10}-M_{62})

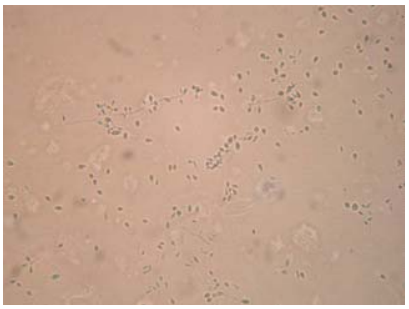

Fig. 4. The thallus photo of Q_{10}-M_{62}

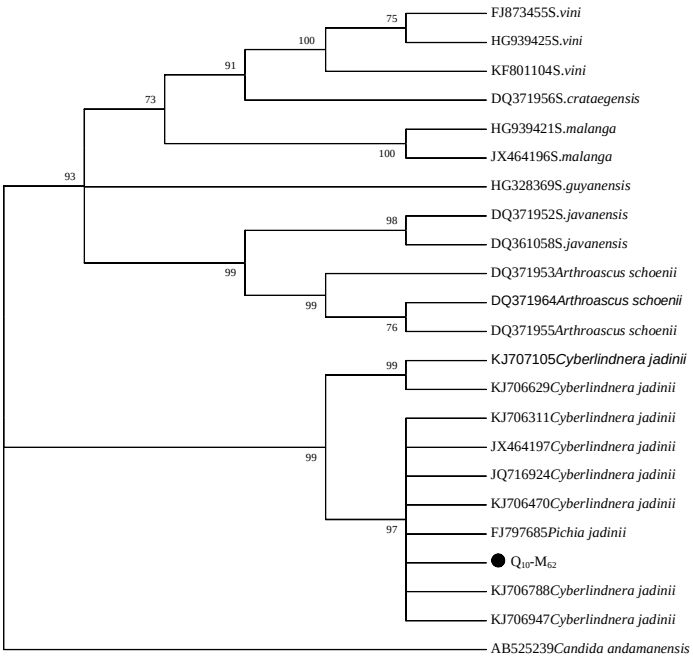

Fig. 5. Phylogenetic tree of Q_{10}-M_{62}

reaction. Therefore, the content of methyl linolelaidate accounted for about 37% of the total aroma in traditional fermentation broth, but it was not seen in Q_{10}-M_{62} broth.

From the types of aroma components, 16 existed in both of the broths, and were accounted for more than half of the total detected components in traditional starter. From the relative content, 16 kinds of aroma components in both accounted for 33.34% of the total aroma components in traditional fermentation broth, and 75.62% of the total strain Q_{10}-M_{62} fermentation broth. The reason was that the traditional starter contains a variety of microorganisms and enzymes, which contributed to the wine flavor profile. Aroma components produced by Q_{10}-M_{62} accounted for 33.34% of the total aroma components in traditional fermentation broth. As a result, Q_{10}-M_{62} was the major aroma-producing strain in BGR wine.

ITS sequence analysis of aroma-producing strains Q_{10}-M_{62}

The result of ITS sequence is shown in Figure 2. The amplification stripe of Q_{10}-M_{62} ITS-5.8S rDNA was clear and had good specificity .It indicated that Q_{10}-M_{62} ITS sequences of PCR amplification was successful. Compared with the mark band, the Q_{10}-M_{62} sequence length was about 750 bp.

Characteristics of colony and thallus

As shown in Figure 3, the colony was round and flat with a white and raised middle. The surrounding area was white and radial. The colony surface was dry and rough with short hypha. The colony edge was irregular and odontoid. As can be seen in Figure 4, most lemon-shaped thallus was big and round at one end, and small pointed at the other end. Of course, some were round. They formed pseudohyphae with some spores with about 4.2 × 2.8μm. The characteristics of colony and cell were very similar to *Cyberlindnera jadinii* (previously *Pichia jadinii*) (Fernández, Cabral, Delgado, Fariña & Figueroa, 2013).

Phylogenetic tree construction of Q_{10}-M_{62}

Q_{10}-M_{62} with fragment length 626 bp was amplified by ITS1-5.8S-ITS2 rDNA primers. ITS sequences were compared by Blast on NCBI. The results showed that Q_{10}-M_{62} had high homology with *Cyberlindnera jadinii* (*Pichia jadinii)* which was positive sequence similarity. MEGA5.1 maximum parsimony (MP) was used, with *Candida* as outgroup, to build phylogenetic tree (Numbers on the branch were obtained by 1000 Bootstrap Replications; CI = 0.757475 RI = 0.886115). As shown in figure 5, Q_{10}-M_{62} and *Cyberlindnera jadinii* were clustered into a group. Combined with characteristics of colony and thallus, Q_{10}-M_{62} was identified as *Cyberlindnera jadinii*, which was different from geotrichum candidum in white wine by (Zhou, Wang, Li, Hu, Hu & Wang, 2013).

CONCLUSION

The main aroma-producing yeast strain in BGR wine in Guizhou was identified as *Cyberlindnera jadinii* (previously *Pichia jadinii*). However, due to the limitation of time and other conditions, there are still many aspects that deserve our further study. For instance, the dynamic changes of microorganisms and substances content. How is the safety of pure fermentation? How to apply pure fermentation technology to the traditional process improvement of BGR wine made in Guizhou? Do functional ingredients such as pigment, polysaccharide and so on have influences on fermentation? All the problems urgently need to be studied.

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