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Effect of yeast species on the terpenoids profile of Chinese light-style liquor

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ABSTRACT

Terpenoids are important trace flavour constituents in Chinese light-style liquors, and are formed by the various yeast species present during fermentation of liquor from cereal and legume materials. *Saccharomyces cerevisiae*, *Pichia kudriavzevii* and *Wickerhamomyces anomalus* are three such yeast species, and we found *S. cerevisiae* capable of generating thirteen different terpenoids in cereal and legume extract fermentation, by both *de novo* and biotransformation pathways. We also found that cereals such as sorghum and barley, and legumes such as peas, contained different terpenoids precursors, which differentially affected the formation and profile of terpenoids mixtures. This work gives new insights into the role of yeast species in generating the various terpenoids mixtures found in Chinese light-style liquors.

1. Introduction

Terpenoids are a large group of natural compounds, present in plants and some animals, and are characterised by their strong and often pleasant smell. They have important biological roles (Pogorzelski & Wilkowska, 2007), and are key biosynthetic subunits in all organisms. Significant quantities of terpenoids exist in plant essential oils, and are also found in wine, beer and distilledalcoholic beverages.

Chinese light-style liquors are one of the oldest traditional alcoholic beverages in China, and are valued for their natural and refreshing flavour. A typical example is Fen liquor, with its renowned "light-fragrance" flavour. This liquor has been produced in Shanxi Province for over 1500 years, and won a gold medal at the 1915 Panama Pacific International Exposition (Li et al., 2011).

Terpenoids such as linalool, α-terpineol, nerolidol, geraniol, β-damascenone and farnesol are known to be key flavour constituents in Chinese light-style liquors (Gao, Fan, & Xu, 2014; Xu, Fan, & Wu, 2012), although little is known of their origin or mode of formation.

Terpenoids are also important flavour compounds in many other fermented beverages. For example, the predominant terpenoids contributing to wine flavour are linalool, geraniol, nerol, α-terpineol and β-citronellol (Dziadas & Jelen, 2010; Gamero, Manzanares, Querol, & Belloch, 2011b). The processes by which these terpenoids are formed have previously been investigated, and found to be influenced by main three factors: yeast species, grape variety and technical conditions of wine-making (Torrensa et al., 2008). Other research has shown that wine yeasts such as *Saccharomyces cerevisiae, Saccharomyces bayanus, Hanseniaspora uvarum, Kloeckera apiculata, Torulaspora delbrueckii, Debaryomyces carsonii,* and *Pichia anomala* (*Wickerhamomyces anomalus*) facilitate terpenoids formation from precursors in grapes (Hernandez-Orte et al., 2008; Swangkeaw, Vichitphan, Butzke, & Vichitphan, 2010). However, it has also been reported that terpenoids are produced in wine mainly by non-*Saccharomyces* yeasts, which is attributed to these species' high β-glycosidase activity (Ferreira1, Clímaco, & Faia, 2001; Manzanares, Rojas, Genovés, & Vallés, 2000). In addition, three terpenes were found in Tempranillo juice hydrolysates, citronellyl acetate, 3,7-dimethyloct-1-ene-3,7-diol and farnesol (López, Ezpeleta, Sánchez, Cacho, & Ferreira, 2004). β-Damascenone, as a C13-norisoprenoid compound, is an odour-active component of wines. It has been detected in many grape glycosidic fractions (Botelho, Mendes-Faia, & Clímaco, 2008, 2010).

Chinese light-style liquors are made by a unique spontaneous and solid-state fermentation process. As shown in Fig. 1, one of the characteristics of this process is the use of the starter culture *Daqu*, which is formed from a complex community of microorganisms via spontaneous fermentation. The other is the simultaneous saccharification and fermentation of the mixture, which plays an important role in regulating the liquor flavour. In addition, in contrast to many other beverages, both cereals (e.g., barley and

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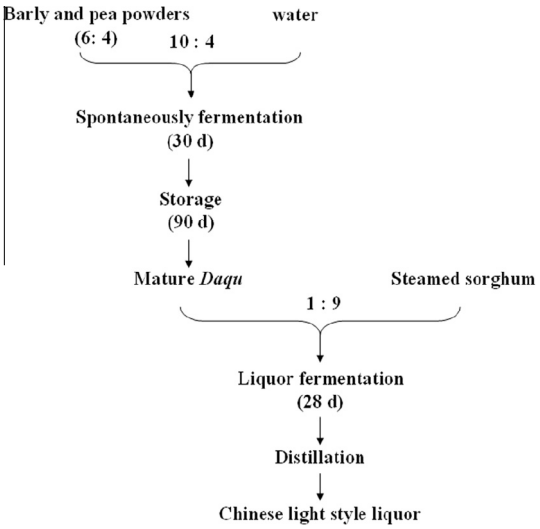

Fig. 1. Schematic diagram for Chinese light-style liquor production.

sorghum) and legumes (e.g., peas) are used as a fermentation material.

A specific community of microorganisms has been found to be the key to fermentation (Li et al., 2011), and comprises members of fifteen species of bacteria and six species of fungi. Sixty percent of these fungi are yeasts such as *Saccharomycetaceae* or related species, including *Saccharomycetaceae* sp., *S. bulderi*, *S. castellii*, *S. cerevisiae*, *W. anomalus, T. delbrueckii*, and *Pichia kudriavzevii*. It has thus been proposed that the fermentation of various Chinese light-style liquors is influenced by the particular mixture of microorganisms and the type of cereal and/or legume material used in the process (Li et al., 2011; Xu, Chen, Mo, & Zhou, 2009).

To explore the mechanism of terpenoids formation in Chinese light-style liquor fermentation, we have investigated the effect of various indigenous yeast species and different cereal and/or legume materials on the process.

2. Materials and methods

2.1. Reagents and standards

The reagents and standards used were purchased from Sigma–Aldrich (Shanghai, China), and included linalool (>95%), α-terpineol (>95%), β-citronellol (>95%), geraniol (>97%), β-damascenone (>95%), α-bisabolol (>95%), farnesol (>95%), nerolidol (>97%), 4-methyl-2-pentanol (>98%), and absolute ethanol.

2.2. Sampling

Sampling was carried out in a Fen liquor-making plant located in Shanxi Province, China. The procedure for preparation of Fen liquor is shown in Fig. 1. *Daqu* served as the starter culture for fermentation, and was spontaneously fermented for 30 d from a mixture of barley and pea powders with 40% (w/v) water. After fermentation, the solid *Daqu* mixture was stored for about 90 d, and then ground with nine volumes of sorghum [which was previously steamed for 30–40 min with water (w/v 1.0:1.1)], and then transferred to earthenware jars (at depth of 1.1 m and radius of 0.35 m) and fermented for about 28 d. The final ethanol content in solid-state fermented grain mixture (*Zaopei*) reached about 4–6% (w/w). The thus-formed *Zaopei* was put into a barrel, and distiled with steam to extract ethanol and other flavour compounds, then the liquor was collected. The distillation process was carried as previously reported (Li, Huang, Shen, & Yi, 2012). The average alcohol degree content of the final liquor is about 60% (v/v). After being aged for a period of time, these distillates are blended to yield the final product with the main alcohol degree content of 40–55% (v/v). The flavour of the distillate is primarily influenced by nature of the original fermented grains, with the distillation procedure itself having a minor effect.

Samples were collected from *Daqu* powders before liquor fermentation, and samples of *Zaopei* were collected from the centres of the earthenware fermentation jars at intervals of 5 d during the 28-d fermentation process. A total of eight samples were obtained.

2.3. Enumeration, isolation and identification of yeasts

Samples (10 g) were mixed with 90 mL sterile saline (0.85% NaCl), and soaked at 4 °C for 30 min. Portions (100 μL) of each of the resulting suspensions were spread on Wallerstein Laboratory Nutrient (WLN) medium, in triplicate, and these cultures were incubated at 30 °C for 5 d. Resulting colonies were examined for macroscopic features, (texture, surface, margin, elevation and colour) and thus classified into different types. Ten to twenty-five colonies of each type were selected and re-streaked and incubated as before, providing cultures of pure clones.

PCR products of the D1/D2 domain of the 26S rDNA of 5–8 randomly selected isolates per distinct restriction pattern with PCR-RFLP analysis were obtained. The 5.8S ITS rDNA region of yeast isolate was amplified by the literature method (Stringini, Comitini, Taccari, & Ciani, 2008), with the primers of ITSl (5′-TCCGTAGGTGAACCTGCGG-3′), ITS4 (5′-TCCTCCGCTTATTGATATGC-3′). Samples of PCR products (10 μL) were digested with the restriction enzymes *Hha* I, *Hae* III and *Hinf* I (Takara, Japan), which were mainly used for PCR-RFLP analysis of yeast species. For assignment of yeast species, comparisons were made with restriction profiles of isolates, reference strains and other published profiles (Clemente-Jimenez, Mingorance-Cazorla, Martínez-Rodríguez, Heras-Vázquez, & Rodríguez-Vico, 2004; Nisiotou, Spiropoulos, & Nychas, 2007; Stringini et al., 2008). The reference strains included *S. cerevisiae*, *P. fermentans*, *P. membranifaciens*, *W. anomalus*, *P. kudriavzevii*, *T. asahii* and *Clavispora lusitaniae*. To confirm yeast species, PCR products of the D1/D2 domain of the 26S rDNA of 1–7 randomly selected isolates per distinct restriction pattern were sequenced, and were compared with sequences available in the GenBank database (BLAST). The primers used for amplification of the D1/D2 domain of the 26S rDNA region were NL1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and NL4 (5′-GGTCCGTGTTTCAAGACGG-3′) as described previously.

2.4. Fermentation conditions

The fermentation medium was prepared from ground cereal (barley or sorghum) and legume (white pea) material, which were obtained from the Fen liquor-making plant. Two kilograms of each material was combined with 8 L of deionized water and steamed for 2 h. The mixture was then saccharified at 60 °C for 4 h, followed by the addition of glucoamylase to give a final concentration of 50 U/g. The resulting mixture was centrifuged at 8000×g for 15 min, and the supernatant separated. The total reducing sugar in the extract (initially ~90 to 95 g/L) was adjusted to 90 g/L by addition of deionized water. The original pH of ~6.0 was adjusted to 5.0 by addition of lactate prior to fermentation. The nitrogen in the extract was mainly protein and peptide, and the total free amino acid was about 1 g/L. This cereal- or legume-extract medium was autoclaved at 121 °C for 15 min.

A loopful of yeast cultures was inoculated into Yeast Nitrogen Base (YNB) medium (BD Difco™; 0.67% yeast nitrogen base without amino acids) supplemented with 2% glucose) at 30 °C for