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Performance of selected *P. fermentans* and its excellular enzyme in co-inoculation with *S. cerevisiae* for wine aroma enhancement

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Chemical compounds studied in this article:
β-damascenone (PubChem CID: 5366074)
1-hexanol (PubChem CID: 8103)
α-terpineol (PubChem CID: 17100)
ethyl isovalerate (PubChem CID: 7945)
isobutyl alcohol (PubChem CID: 6560)
isoamyl alcohol (PubChem CID: 31260)
phenylethyl acetate (PubChem CID: 7654)
ethyl acetate (PubChem CID: 8857)
ethyl isobutyrate (PubChem CID: 7342)
octanoic acid (PubChem CID: 379)

ABSTRACT

This study evaluated aroma enhancement in dry white wines made via a specific *Pichia fermentans* strain and its excellular enzyme in co-inoculation with *Saccharomyces cerevisiae*. The inoculation ratios of two yeasts ranged from 0.1 to 10 and fermentations with pure *S. cerevisiae* were utilized as control. Esterase activities that expressed as C2-C8 chain substrate specificity were surveyed during mixed fermentation. Finally, wine aroma was analyzed via GC-MS and a trained sensory panel. The results revealed that C2-C8 esterase activities were superior during mixed fermentation to the control. Mixed fermentations significantly increased the contents of acetates, ethyl esters, fatty acids, and numerous other fermentative volatiles; particularly those of medium-chain fatty acids and their corresponding esters, with an increased inoculation ratio of the isolate. Extracellular enzyme treatment highly improved the release of varietal aroma compounds, such as terpenols, C_{13}-norisoprenoids, and C_6 compounds. Sensory analysis indicated that the risk of negative earth odor emerged in the wine characterized by strong sweet and acid fruit trait. Therefore, mixed fermentations at yeasts inoculation ratios between 1:4 and 4:1 may provide better fermentation strategies for appropriate wine aroma enhancement.

1. Introduction

Wine aroma is an important aspect of the sensory quality of wine. The use of selected yeasts and the control of fermentation conditions can be considered to pose a significant influence on the quality of wine aroma. At present, *S. cerevisiae* has been commonly used in the wine industry due to its advantages of pure and complete fermentation as well as the high alcohol conversion rate (Lee, Kho, Yu, Curran, & Liu, 2013). However, fermentation by a single yeast strain may easily lead to the homogenization of wine flavor, which is detrimental to aroma complexity (Rainieri & Pretorius, 2000). Indeed, during grape maturation many types of yeasts can be found on the grape skin. These yeasts (most are non-*Saccharomyces*) can quickly initiate natural fermentation after berries are broken. It has been reported that non-*Saccharomyces* were able to secrete β-D-glucosidase with higher activity and stronger tolerance to winemaking conditions compared to *S. cerevisiae*; furthermore, glycosidase extraction from non-*Saccharomyces* could be used to increase the wine aroma (Cabaroglu, Selli, & Canbas, 2003; Wang, Kang, Xu, & Li, 2011). Several non-*Saccharomyces* may produce comparatively high contents of volatile fatty acids, which aid in the formation of fruity esters, although the resulting alcohol conversion rate is lower than for *S. cerevisiae*. Therefore, wine makers have begun to conduct mixed fermentations of non-*Saccharomyces* and

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S. cerevisiae. Several esters that are produced in the mixed fermentation of non-*Saccharomyces* have been shown to have a positive effect on wine quality, especially for wine of neutral grape varieties (Renault, Coulon, Revel, Barbe, & Bely, 2015; Wang et al., 2017). The ester accumulation in wine was determined via a balance of enzymatic synthesis and hydrolysis reactions involving esterase (Sumby, Grbin, & Jiranek, 2010); consequently, the activity of esterase from yeasts can influence the ester concentration in wine. However, few reports exist that link ester production and esterase activities with mixed fermentation.

The flavor of Chinese Liquor is based on ester aroma. Many types of yeasts exist in the liquor-brewing environment with biodiversity in ester producing capacities and characteristics, resulting in Chinese liquor with many different types of flavor. In a previous study, one yeast isolate (*P. fermentans*) with high activities of β-D-glucosidase and esterase was selected from the Sichuan liquor pit (China); the isolate was used in mixed fermentations with *S. cerevisiae* to produce Ecolly dry white wine for aroma enhancement. The aim was to evaluate mixed fermentations in several inoculation strategies for aroma improvement, with a particular focus on fruity esters. The revolution of esterase activities during fermentation was surveyed to confirm the formation of esters in winemaking. Furthermore, co-fermentation with extracellular enzyme and *S. cerevisiae* was used for comparison.

2. Materials and methods

2.1. Grape material

Approximately 200 kg of Ecolly grapes were harvested from the Guan Cun vineyard, Yangling, Shaanxi, China, during the August of 2014. Ecolly grapes had a titratable acidity of 3.9 g/L (expressed as tartaric acid) and 176 g/L of reducing sugars.

2.2. Yeasts and extracellular enzyme extract

Commercial *S. cerevisiae*: Actiflore F5 was purchased from Laffort France.

Yeast isolate from a Chinese Liquor pit: The yeast strain was selected from the Sichuan liquor pit in China through both Lysine medium and WLN medium due to its high activity of esterase and β-D-glucosidase. The isolate was identified as *Pichia fermentans* via sequence analysis of the 26S rDNA D1/D2 domain.

Extracellular enzyme extract: The enzyme extract was prepared according to Hu et al. (2016). Selected *P. fermentans* was incubated in fermentation medium (20 g/L peptone, 20 g/L glucose, 3 g/L NH_4NO_3, 4 g/L KH_2PO_4, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 10 mL/L Tween 80, and 10 mL/L yeast extract) on a shaking table for 72 h (28 °C, 150 rpm). Then, the supernatant was obtained via centrifugation (9338 × g, 4 °C, 15 min). Enzyme activities of different glycosidases were measured following the method proposed by Fia, Canuti, and Rosi (2014) with some modifications. Esterase activity was determined as described by Pérez-Martín, Seseña, Izquierdo, and Palop (2013). Substrate specificity of the esterase enzyme toward several *p*-nitrophenol esters with different acyl chain lengths was determined via the following different substrates: 4-nitrophenyl acetate ($pNPC_2$), 4-nitrophenyl butyrate ($pNPC_4$), 4-nitrophenyl hexanoate ($pNPC_6$), and 4-nitrophenyl caprylate ($pNPC_8$) (Sigma-Aldrich) in a standard assay. All assays were performed in duplicate.

2.3. Chemicals and reagents

Yeast extract powder, peptone, and tween 80 (chemically pure) were obtained from the Aoboxing Bio-Tech Co. Ltd (Beijing, China). Analytically pure ethanol, tartaric acid, sodium chloride (NaCl), D-glucose, ammonium nitrate (NH_4NO_3), magnesium sulphate ($MgSO_4 \cdot 7H_2O$), potassium dihydrogen phosphate (KH_2PO_4), sodium carbonate (Na_2CO_3), and sulphur dioxide (SO_2) were obtained from the Xilong Chemical Co. Ltd (Sichuan, China). *p*-nitrophenyl-β-D-glucopyranoside (98%), *p*-nitrophenyl-α-L-arabinofuranoside (98%), *p*-nitrophenyl-α-L-rhamnopyranoside (98%), *p*-nitrophenyl-β-D-galactopyranoside (98%), and *p*-nitrophenyl-β-D-xylopyranoside (98%) were obtained from the Yuan Ye Bio-Technology Co., Ltd (Shanghai, China). Thirty-nine analytically pure aroma references (≥97%) were obtained from Sigma-Aldrich (Shanghai, China).

2.4. Winemaking process

The activation and cultivation of *P. fermentans* and Actiflore F5 were implemented in YEPD medium and kept at 28 °C for 72 h. The inocula were *P. Fermentans*/Actiflore F5 at ratios of 1:10 (C1:10), 1:4 (C1:4), 1:1 (C1:1), 4:1 (C4:1), and 10:1 (C10:1). The number 1 referred to in the above ratios, indicated 10^6 CFU/mL. The treatments of the pure Actiflore F5 culture either with or without added extracellular enzyme extract before fermentation were used as extracellular enzyme treatment and control (CK), respectively.

Grapes were stemmed, crushed, and immediately pressed. The must was treated with sulfur dioxide (approximately 60 mg/L) and bentonite (1 g/L) for setting at 4 °C. After 24 h, the must was divided into 20 L glass jars, added with yeasts, and fermented at 18 °C. During vigorous fermentation, sucrose and tartaric acid were added to adjust the final alcohol level to 11% (v/v) and the total acid to 6.7 g/L (expressed as tartaric acid). Sulfur dioxide (60 mg/L) was added as soon as the sugar concentration fell below 2.0 g/L to end the fermentation. After two rounds of enclosed racking, wines were fined via 1 g/L bentonite, and then stored at 4 °C for two months. Wine stabilization lasted for six months prior to analysis. Each winemaking strategy was conducted in duplicate.

2.5. Survey of esterase activities during mixed fermentation

The mixed fermentation of yeasts at an inoculation ratio of 1:1 and the control fermentation of pure *S. cerevisiae* were sampled every 24 h to evaluate esterase activities during the fermentation process. Esterase activity was determined as described by Pérez-Martín et al. (2013). All assays were performed in duplicate. The esterase activities during fermentation were expressed as C2, C4, C6, and C8 substrate specificity. The total esterase activity was calculated as the sum of C2, C4, C6, and C8 activities. In this study, the cumulative values of esterase activities were calculated to show the cumulative impact of esterase during fermentation. The calculated formula was as following:

$$\sum_{i=1}^{i=n} X_i = (X_1 + X_2 + X_3 + \ldots + X_n)$$

where *Xi* indicates the esterase activity every 24 h.

2.6. Aroma analysis via GC-MS

Volatiles were extracted via solid phase micro-extraction (SPME) using DVB/CAR/PDMS fiber (50/30 μm film thickness, 20 mm stableflex), assembled with a 57330-U Holder (Supelco, Bellefonte PA, USA). Eight milliliter of a wine sample with internal standard solution was placed into a 15 mL headspace bottle with stirrer magnets; 1.0 g sodium chloride was added. Subsequently, the stirrer magnets were started, and conditioned for 15 min in 40 °C water. The extraction was performed for 30 min at 40 °C, after which volatiles were immediately desorbed in the GC injector for