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Regulating yeast flavor metabolism by controlling saccharification reaction rate in simultaneous saccharification and fermentation of Chinese *Maotai*-flavor liquor

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A B S T R A C T

Maotai-flavor liquor is produced by simultaneous saccharification and fermentation (SSF), in which filamentous fungi produce hydrolases to degrade the starch into fermentable sugar. *Saccharomyces cerevisiae* simultaneously transforms the sugars to ethanol and flavor compounds. The saccharification rate plays an important role in regulating the liquor yield and flavor profile. This work investigated the effect of saccharification rate on fermentation by regulating the inoculation ratio (1:0.1, 1:0.5, 1:1, 1:5, 1:10) of *S. cerevisiae* and *Aspergillus oryzae*, the main saccharification agent. We found no significant difference in reducing sugar content among the mixed cultures with different ratios. This indicated a balance of the saccharification rate and the sugar consumption rate, in which the former was controlled by the interaction between *A. oryzae* and *S. cerevisiae*, and the latter controlled the metabolism of the two species. The ethanol yield was the highest in ratios of 1:0.5, 1:1, and 1:5, while the total production of flavor compounds was the highest for the ratio of 1:0.5, which was mainly attributed to the vigorous metabolism of *S. cerevisiae*. The inoculum ratio of 1:10 produced the second highest content of flavor compounds in which a large number of alcohols and esters were derived from the vigorous metabolism of *A. oryzae*. This indicated that the saccharification rate significantly influenced the flavor metabolism. This study improves understanding of the interaction and cooperation between *A. oryzae* and *S. cerevisiae* in co-culture fermentation for Chinese liquor making.

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1. Introduction

Simultaneous saccharification and fermentation (SSF) is commonly used to produce many types of traditional fermented food and beverages in Asian countries (Chen et al., 2014; Furukawa et al., 2013; Li et al., 2013). The method combines the saccharification of starch to fermentable sugar and the sugar to different compounds, including ethanol and other compounds (Olofsson et al., 2008). The approach of SSF is different from the processes used to prepare some other alcohol beverages such as beer, which is made by separate hydrolysis and fermentation (SHF) processes (Farías et al., 2010; van Beek and Priest, 2002). During SSF, starch should be hydrolyzed to fermentable sugar by hydrolytic enzymes, such as α-amylase and glucoamylase, which could be produced by many fungal species, such as *Aspergillus oryzae*, *Paecilomyces varioti*, *Rhizopus* sp., *Monascus* sp., and *Penicillium* sp. Therefore, these filamentous fungi have been reported to serve as the saccharifying agents (Chen et al., 2014; Nahar et al., 2008; Lv et al., 2012), while *Saccharomyces cerevisiae* has served as a fermenting agent to convert fermentable substrates into ethanol. The main benefit of the SSF process, when compared with a SHF process, is less inhibition by reducing sugars in the enzymatic hydrolysis, which increases the efficiency of substrate utilization and improves the ethanol yield (Olofsson et al., 2008). Therefore, SSF methods have attracted considerable attention all over the world, although these processes have some drawbacks. It has been generally accepted that the main drawback of SSF is the different conditions (temperature and pH) that are required for optimization of the two reactions (Lee et al., 2012; Lin and Tanaka, 2006). However, the most important factor is the regulation of the coordination of the two reactions. This is because different saccharification rates will produce different sugar concentrations, thereby significantly influencing cell growth and the metabolism of ethanol and flavor compounds. This ultimately affects the final ethanol yield and quality of the food.

Flavor profile is the most important characteristic of fermented food, and it has been regulated by many methods. For example, flavor profile

Abbreviations: SSF, simultaneous saccharification and fermentation; SHF, separate hydrolysis and fermentation; FAN, free amino nitrogen.

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can be influenced by the nitrogen concentration of the substrate (Lage et al., 2014), while other studies have added several non-*Saccharomyces* to regulate the flavor profile (Medina et al., 2013). However, the effect of saccharification rate on the flavor metabolism of yeast has never been investigated, even though this process is important for flavor production in SSF processes.

The microbial interaction between fungi and yeast is important for regulation of the coordination of these two reactions. These microorganisms are considered to interact and cooperate with each other in various natural environments (Murado et al., 2008). There are a few studies concerning the interaction between filamentous fungi and yeasts in co-culture fermentation. Shin et al. (1998) reported that the biomass and pigment production of *Monascus* spp. increased significantly when co-cultured with *S. cerevisiae*. More recently, Zhou et al. (2011) showed that the polygalacturonase production of *Aspergillus niger* improved significantly when mixed with *S. cerevisiae* J-1. Ge et al. (2013) found that enzyme production of *S. cerevisiae* and the ethanol conversion rate increased significantly in co-culture with *A. oryzae*. In addition, it was found that *A. oryzae* was beneficial for flavor production in SSF of rice wine (Yang et al., 2013). These studies indicated that filamentous fungi and yeast have a positive interaction in co-culture fermentation.

Maotai-flavor liquor is a symbolic drink in China just as whisky in Scotland, and brandy in France, and is produced by a complicated spontaneous fermentation process (Wu et al., 2013). It includes *Daqu* (the starter) making, stacking fermentation and liquor fermentation stages. After *Daqu* making stages, the ground *Daqu* and steamed sorghum are mixed and piled up on the ground for stacking fermentation. When the temperature on the top of the stack reaches about 50 °C, the mixture is then put into the underground cubic pits, and sealed for liquor fermentation for about 30 days (Wu et al., 2013). It is also a typical SSF process, and accumulates a specific microorganism community, including filamentous fungi and yeasts. In stacking fermentation, the population of filamentous fungi and yeast increased from 3.4×10^3 to 5.8×10^3 CFU/g (Chen et al., 2014), and from 0.37 to 7.97×10^5 CFU/g at the top of the stack (Wu et al., 2013), respectively. While in the upper layer of liquor fermentation, the population of filamentous fungi continuously decreased and few fungal species survived after 10 days (Chen et al., 2014), while the yeast kept stable and quickly decreased after 10 days (Wu et al., 2013). The co-existence of filamentous fungi and yeast indicated that they could interact in the whole stage of stacking fermentation and the early stage of liquor fermentation, which might be important for the SSF process of *Maotai*-flavor liquor making.

In the whole liquor making process, eight and seven different fungal species were identified by using culture-dependent and culture-independent methods of analysis, respectively. Among them, *A. oryzae* was the predominant species; it exhibited high α-amylase and glucoamylase activities, indicating that it plays an important role in producing amylase for hydrolyzing the starch (Chen et al., 2014). *A. oryzae* is an important type of filamentous fungus that is widely used in east Asian foods and liquor fermentation (Furukawa et al., 2013).

This study investigated the interactions and cooperation between *A. oryzae* and *S. cerevisiae*, and the effect of the saccharification rate on ethanol and flavor metabolism. This work examines the metabolism processes of SSF, and furthers understanding of the fermentative mechanism for preparation of traditional fermented food.

2. Materials and methods

2.1. Microorganisms and medium

A. oryzae and *S. cerevisiae* were isolated from the *Maotai*-flavor liquor making process at a facility in Guizhou Province, by the method of Rose Bengal Agar medium and WLN medium as described in Chen et al. (2014), and Wu et al. (2013), respectively. They were deposited in the China General Microbiological Culture Collection Center with accession numbers CGMCC 6264 and CGMCC 4747, respectively.

2.2. Mixed-culture fermentations

For yeast starter culture, a loopful of yeast culture was inoculated into a 250-ml Erlenmeyer flask containing 50 ml of liquid sorghum extract, which is used as the main starting material for Chinese liquor production. Two hundred grams of sorghum powder was added with 800 ml of deionized water, steamed for 2 h and then saccharified at 60 °C for 4 h with the addition of glucoamylase at a final concentration of 50 U/g. The supernatant was selected as the final sorghum extract by centrifugation at 8000 *g* for 15 min. The final reducing sugar content of the liquid sorghum extract was about 90 mg/g, and was mainly glucose. No nitrogen was added, and the initial pH was kept unadjusted, which was about 6.0. Fermentations were conducted at 30 °C for 48 h with stirring at 150 rpm.

For filamentous fungi starter culture, the spores of *A. oryzae* were harvested after 3–5 days of culture on potato dextrose agar (PDA) medium at 28 °C. The PDA medium was prepared from 200 g washed and sliced potatoes, boiled in 500 ml deionized water and strained through gauze, then 20 g glucose, 20 g agar and another 500 ml deionized water were added.

After the preparation of these two starters, their cell numbers were calculated using a hemocytometer, and the inoculum concentrations were adjusted for the mixed culture. The population of yeast starter was about 5×10^8 CFU/ml, while the initial concentration of fungi spore suspension was about 5×10^9 CFU/ml, and the suspension was gradient diluted and added to the solid-fermentation medium, to keep the same volume of the added liquid.

The mixed culture was carried out in the solid-state fermentation medium, which was also prepared with sorghum. Sorghum was ground and soaked in hot water for 12 h. Then 200 g of sorghum containing 50% water was sealed in a flask and autoclaved for 30 min at 121 °C. To achieve limited aerobic conditions, flasks were fitted with a one-hole silicon stopper into which a cotton-plugged Pasteur pipette was inserted to vent CO_2 during fermentation.

To determine the effect of environmental factors on co-culture fermentation, *A. oryzae* (5×10^5 spores/g) and *S. cerevisiae* (5×10^5 CFU/g) were inoculated and fermented at different temperatures (25, 30, 35, and 40 °C) at pH 5.0, and at different pH (3.5, 4.0, 4.5, 5.0) at 30 °C. For the effect of reducing sugar content on single culture fermentation of *S. cerevisiae*, the initial sugar content in sorghum solid-state fermentation medium was controlled at 50 mg/g, and glucose was added to adjust the sugar content to different concentrations (50, 100, 150, and 200 mg/g). For the effect of microbial interaction of the two species, *S. cerevisiae* (5×10^5 cells/g) was mixed with *A. oryzae* spores at ratios of 1:0.1, 1:0.5, 1:1, 1:5, 1:10, at 30 °C and pH 4.5.

The fermentations were conducted for 15 days and sampling was carried out at 0, 2, 4, 6, 9, 12, and 15 days. The samples were stored at 4 °C for cell counting and −20 °C for chemical analysis. A non-inoculated sample of fermentation medium was prepared as the control. All experiments were performed in triplicate.

2.3. Chemical and biomass analysis of samples in liquor fermentation

Samples (10 g) were mixed with 90 ml of distilled water, ultrasonicated at 0 °C for 30 min, and centrifuged at 4 °C for 5 min. The obtained supernatant was used to determine the content of reducing sugars and ethanol. Reducing sugar was measured by the method of DNS (3,5-dinitrosalicylic acid) as described in Wang et al (2013). It was mainly glucose in this sorghum extract. The ethanol content was determined by HPLC (Agilent) using a column Aminex HPX-87H (Bio-Rad). The column was eluted at 60 °C with a degassed mobile phase containing 3 mM H_2SO_4 at a flow rate of 0.6 ml/min.

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