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Effect of gallic and protocatechuic acids on the metabolism of ethyl carbamate in Chinese yellow rice wine brewing

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ABSTRACT

It was studied that gallic and protocatechuic acids played important roles in ethyl carbamate (EC) forming. Gallic and protocatechuic acids can reduce the arginine consumption through inhibiting the arginine deiminase enzyme. Therefore, they are generally added to regulate EC catabolism in the course of yellow rice wine leavening at the third day. In this work, gallic and protocatechuic acids made little influence on the growth of *Saccharomyces cerevisiae*. Besides, the addition of 200 mg/L gallic or protocatechuic acid could prevent the transformation from urea/citrulline to EC. Gallic acid showed better inhibiting effect that the content of EC could be reduced by 91.9% at most. Furthermore, the production of amino acids and volatile flavor compounds are not markedly affected by phenolic compounds. The discoveries reveal that EC can be reduced by supplying gallic acid or protocatechuic acid while yellow rice wine leavening.

1. Introduction

Yellow rice wine is a noted conventional fermented alcoholic drink since ancient China, which can provide considerable benefits to human nutrition. There exists all kinds of microorganisms and complex conditions. However, toxic products can be produced because of metabolism and side reactions while yellow rice wine fermenting, notably ethyl carbamate (EC). EC can usually be found in fermented foods and drinks, like beer, wine, and yellow rice wine (Ancha, Marinda, & Hennie van, 2000; Wu, Pan, Wang, Shen, & Yang, 2012). It has been confirmed in some species, such as mice, rats, and monkeys, that EC can cause cancer (Mirvish, 1969; Salmon & Zeise, 1991; Thorgeirsson, Dalgard, Reeves, & Adamson, 1994). Furthermore, EC may cause multiple-site tumors which has been verified by later researches (Salaman & Roe, 1953; Tannenbaum, 1964). International agency for research on cancer (IRAC) has upgraded EC to Group 2A, a "probable human carcinogen" (Thorgeirsson et al., 1994). Additionally, earlier research has demonstrated that EC in yellow rice wine is twice that of other alcoholic drinks (Wu et al., 2012), which can be an underlying carcinogenic factor to mankind health.

EC can be effectively reduced through suppression of the generation of its precursors, including urea and citrulline. The most ordinary mechanism of EC formation is the reaction between urea and ethanol while citrulline is the lesser important precursor of EC (Uthurry, Lepe, Lombardero, & Del Hierro, 2006). During ethanol brewing, the cumulation of urea derives not only from the fermented sources such as rice, but also from the catabolism of arginine by *Saccharomyces cerevisiae* (Dahabieh, Husnik, & van Vuuren, 2010). And citrulline is produced by arginine catabolism through malolactic fermentation (MLF) of lactic acid bacteria (LAB) (Arena, Saguir, & de Nadra, 1999; Liu, Pritchard, Hardman, & Pilone, 1994). Arginine has been discovered to be one of the primary amino acids in grape juice and wine (Lehtonen, 1996). Earlier studies have proved that arginine is metabolized through arginine deiminase (ADI) pathway by LAB (Liu & Pilone, 1998; Liu, Pritchard, Hardman, & Pilone, 1995), in which mainly contains two enzymes: ADI and catabolic ornithine transcarbamylase (OTCase). Moreover, the strain which accumulated more citrulline showed higher ADI activity and lower OTCase activity (Spano, Massa, Arena, & de Nadra, 2007).

Over the past few years, many works have been conducted to reduce EC in alcoholic drinks: physical methods, chemical methods, enzymatic methods and metabolic engineering methods (Jiao, Dong, & Chen, 2014). EC content can also be regulated by adopting appropriate fermentation method or fermentation bacterium modification. However, the optimization of fermentation processing parameters are conducted as additional precautionary measure, and some of them are hard to accomplish in actual fermentation environments (Zhao et al., 2013). The operation of enzymatic method expends much money, such as using acid urease (Fidaleo, Esti, & Moresi, 2006). Besides, modifying fermentation bacterium by molecular techniques may cause unexpected problems.

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It has been confirmed that polyphenols in red wine are beneficial to prevention of cardiovascular heart disease (CHD) (Renaud & de Lorgeril, 1992). Yellow rice wine is also rich in phenolic compounds which mainly come from glutinous rice and wheat materials, including gallic acid and protocatechuic acids (Que, Mao, & Pan, 2006; Xie, Xu, & Fan, 2011). Interestingly, gallic and protocatechuic acids can inhibit the ADI enzyme in term of reducing arginine consumption which is associated with production of citrulline (Alberto, Manca de Nadra, & Arena, 2012). In this study, we develop a healthier and easier method to inhibit EC formation through addition of gallic acid and protocatechuic acid while yellow rice wine leavening at the third day. Consequently, here we also investigate the impact of gallic and protocatechuic acids on the growth of fermentation strain, and the formation of EC as well as its effect on fundamental quality.

2. Materials and methods

2.1. Reagents and standards chemicals

l-Citrulline (at least 98%) was purchased from Sangon Biotech Corporation (Shanghai, China). l-arginine (at least 98.5%) was obtained from BBI life sciences corporation (Shanghai, China). Protocatechuic acid (at least 97%) and *n*-propanol (HPLC grade) were provided by Aladdin Industrial Corporation (Shanghai, China). Gallic acid (98%) was purchased from J&K scientific Ltd. Corporation (Shanghai, China). 9-xanthydrol (98%) and EC (at least 99%) were provided by Sigma-Aldrich Chemical Corporation (St Louis, MO, USA). Methanol alcohol (HPLC grade) was provided by Tianjin Shield Specialty Chemical Ltd. Corporation (Tianjin, China). Besides, other reagents used in this work were of analytical grade. Chinese koji was obtained from Wuzhanmao yellow rice wine industry (Anji, Zhejiang).

2.2. Process of the cultivate of S. cerevisiae and L. brevis with different polyphenols

S. cerevisiae and *L. brevis* were grown in basal medium (BM). The BM consisted of 5 g/L peptone, 5 g/L l-arginine and 20 g/L glucose. Before high-pressure steam sterilization (121 °C, 20 min), the BM were adjusted to pH 6.0 by HCl (1 mM). Polyphenols were dissolved in ethanol. The eventual concentration of ethanol in media was 7% (v/v). These polyphenols were added to the germ free BM to meet the eventual content at 100 mg/L or 200 mg/L.

There exists all kinds of microorganisms in traditional Chinese yellow rice wine, especially *S. cerevisiae* and *L. brevis*. Two groups were divided in this test, namely the *S. cerevisiae* group (designated as S), the *S. cerevisiae* together with *L. brevis* group (designated as SL). During the incubation, five different groups were further divided both in group S and SL for this investigation: the control group was performed without additives (designated as SCK and SLCK), and the experimental group contained a total of four groups: 100 mg/L gallic acid (designated as SG100 and SLG100), 200 mg/L gallic acid (designated as SG200 and SLG200), 100 mg/L protocatechuic acid (designated as SP100 and SLP100), and 200 mg/L protocatechuic acid (designated as SP200 and SLP200). All these groups were statistically incubated at 28 °C for 48 h. The samples were obtained by timing sampling and stored at −20 °C for subsequent biological and chemical detection.

2.3. Process of yellow rice wine leavening

First, before cooking rice, 0.75 kg glutinous rice was mixed with 0.9 L water for 48 h at 28 °C. Second, the glutinous rice was steamed (20 min) after washing. Then, 1.5 g *S. cerevisiae* was added into cooled rice and incubated at 28 °C. After 48 h, it was the timing to add Chinese koji (75 g) as well as water (0.9 L) and then incubated at 28 °C for 2–3 days to complete the mashing process. Then the fermentation temperature was set to 18 °C at the time that the concentration of alcohol in brewing broth became stable, in other words, the process of rice wine making got into post-fermentation that lasted for 16–20 days. During the incubation, four different groups were divided for the determination: the control group was performed with no additives (designated as CK), and the experimental groups contained a total of three subgroups: 200 mg/L gallic acid (designated as GC200), 200 mg/L protocatechuic acid (designated as PC200), and 200 mg/L gallic acid together with 200 mg/L protocatechuic acid (designated as GP200). All these polyphenols were added at the third day of brewing process.

2.4. Determination of ethyl carbamate

The concentration of EC was determined by HPLC-FLD (Fu et al., 2010). The EC content in original samples was found to be low. Thus, it was worthwhile to concentrate samples until reaching to ten percent of original volume by vacuum rotary evaporator at 55 °C. Before the assay of EC by HPLC-FLD, the derivative reaction of sample was needed. The details were provided as follows: 1 mL concentrated sample (or standard EC) was mixed with 0.2 mL 9-xanthydrol (0.01 M). And then 0.1 mL HCl (1.5 M) was added to the reaction system. After fully mixing, the mixture had to react for 30 min without light and finally put into chromatographic vials.

The HPLC-FLD instrument used in this study was made up of two Shimadzu LC-20AD pumps (Shimadzu, Kyoto, Japan), a reversed phase Symmetry C18 (250 × 4.6 mm, 5 μm, Cosmosil) and a Shimadzu RF-20A fluorescence detector. The mobile phase consisted of methanol and water. The temperature of chromatographic column was 35 °C. The excitation wavelength was 233 nm and the emission wavelength was 600 nm. Besides, the injection volume of sample was 20 μL.

2.5. Determination of extracellular arginine, urea and citrulline concentration

The method of arginine detection was obtained from earlier research (He, Sun, & Chen, 2007). Briefly, one reagent was needed, consisting of 50 g 1-Naphthol, 2.5 μL 2, 3-butanedione and 1 L propanone. Then the procedure of detection is given as follows: 1 mL sample was mixed with 8 mL NaOH (15 g/L) and then reacted with 1 mL reagent by 30 °C water-bathing for 15 min. The absorbance value was measured by a microplate reader with the wavelength of 525 nm at the room temperature.

Extracellular urea and citrulline concentration were determined by a microplate reader using the method described by Fang et al. (Fang, Dong, Li, & Chen, 2015). Urea could react with acidic diacetyl monoxime in the presence of thiosemicarbazide by boiling water-bath heating for 15 min. Urea was measured at the wavelength of 526 nm. Citrulline could react with reagent prepared before use and was finally measured at the wavelength of 530 nm.

2.6. Determination of intracellular ornithine transcarbamylase (OTCase) activity

The way to detect OTCase activity was studied from the research reported previously (Carrasco, Perez-Ortin, & del Olmo, 2003). First, the crude extract was prepared. Incubated cells were obtained by centrifuging (4 °C, 3000 r.p.m.) which were then redissolved in 1.5 mL Tris–HCl (5 mM, pH 7.5) and 1.5 mL $MnCl_2$ (10 mM), and followed by 15 min of ultrasound to break cells. Crude extract was in the supernatant which got by centrifuging