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Monitoring the microbial community during solid-state acetic acid fermentation of Zhenjiang aromatic vinegar

Wei Xu [a], Zhiyong Huang [d], Xiaojun Zhang [c], Qi Li [b], Zhenming Lu [a], Jinsong Shi [f], Zhenghong Xu [a,b,*], Yanhe Ma [e]

[a] Laboratory of Pharmaceutical Engineering, School of Medicine and Pharmaceutics, Jiangnan University, Wuxi 214122, People's Republic of China
[b] The State Key Laboratory of Food Science and Technology, Jiangnan University, Wuxi 214122, People's Republic of China
[c] Laboratory of Molecular Microbial Ecology and Ecogenomics, College of Life Science and Biotechnology, Shanghai Jiao Tong University, Shanghai 200240, People's Republic of China
[d] Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences, Tianjin 300308, People's Republic of China
[e] Institute of Microbiology, Chinese Academy of Sciences, Beijing 100080, People's Republic of China
[f] Laboratory of Bioactive Products Process Engineering, School of Medicine and Pharmaceutics, Jiangnan University, Wuxi 214122, People's Republic of China

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ABSTRACT

Zhenjiang aromatic vinegar is one of the most famous Chinese traditional vinegars. In this study, change of the microbial community during its fermentation process was investigated. DGGE results showed that microbial community was comparatively stable, and the diversity has a disciplinary series of changes during the fermentation process. It was suggested that domestication of microbes and unique cycle-inoculation style used in the fermentation of Zhenjiang aromatic vinegar were responsible for comparatively stable of the microbial community. Furthermore, two clone libraries were constructed. The results showed that bacteria presented in the fermentation belonged to genus *Lactobacillus*, *Acetobacter*, *Gluconacetobacter*, *Staphylococcus*, *Enterobacter*, *Pseudomonas*, *Flavobacterium* and *Sinorhizobium*, while the fungi were genus *Saccharomyces*. DGGE combined with clone library analysis was an effective and credible technique for analyzing the microbial community during the fermentation process of Zhenjiang aromatic vinegar. Real-time PCR results suggested that the biomass showed a "system microbes self-domestication" process in the first 5 days, then reached a higher level at the 7th day before gradually decreasing until the fermentation ended at the 20th day. This is the first report to study the changes of microbial community during fermentation process of Chinese traditional solid-state fermentation of vinegar.

1. Introduction

Vinegar has over ten thousand years of historic record. Nowadays, a variety of popular vinegars are widely used around the world, for example, cereal vinegar in China and Japan, wine vinegar in France, malt vinegar in England, persimmon and pineapple vinegar in Southeast Asia, etc. Different from submerged pure-culture fermentation techniques for vinegar production in European countries (Tesfaye et al., 2002), semi-solid or solid mix-culture fermentation techniques are widely used in Asian countries. Vinegar fermentation usually includes three steps including starch saccharification, alcohol fermentation, and oxidation of ethanol to acetic acid. In this mixed-culture fermentation process (Haruta et al., 2006; Xu et al., 2007), the coexistence of different microbes could provide numerous enzymes for synthesis of flavor and functional materials, such as organic acids, amino acids, volatile components, and ligustrazine (He et al., 2004). Therefore, the constituents of Chinese vinegars are quite different from those of other vinegars, with unique aroma and many interesting physiological effects.

Chinese vinegars are mostly produced by a typical aerobic solid-state fermentation technique, and Zhenjiang aromatic vinegar is the representative product of this technique. It is made from glutinous rice, and the starch saccharification and alcohol fermentation steps are similar to the technique of rice wine in China (Li, 2005), while the solid-state acetic acid fermentation

* Corresponding author. Laboratory of Pharmaceutical Engineering, School of Medicine and Pharmaceutics, Jiangnan University, Wuxi 214122, People's Republic of China. Tel./fax: +86 510 8591 8206.
E-mail address: zhenghxu@jiangnan.edu.cn (Z. Xu).

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process is unique as shown in Fig. 1. After saccharification and alcohol fermentation through semi-solid culturing, solid-state acetic acid fermentation is conducted by mixing alcohol mash with the wheat bran and rice hull in different pots or ponds. This step generally lasts about 20 days, and the temperature and humidity of fermentation culture is retained at 40–46 °C and 60–70%, respectively. An unique cycle-inoculation style that the fermentation culture of the 7th day is used as seed (starter) for the next round inoculation is performed. Since no additional microbes are supplied during the fermentation process, the microbial community and quality of each fermentation circle is supposed to be comparatively stable. Finally, rice hull is mixed with fermentation culture, which is loose and has very large interspace, can hold enough air for the aerobic microbial growth and metabolic activities. The fermentation process of Zhenjiang aromatic vinegar is completed in 25–30 days, after which the fermented liquid is aged for over three months.

It is well known that the microbial diversity and its dynamic change affect the quality and characteristics of the fermentation products significantly. Recently, molecular fingerprinting techniques such as DGGE (TGGE) and clone library analysis were widely used to investigate the microbial diversity of plenty of traditional fermentation products, including Korean kimchi (Chang et al., 2008), Japanese rice black-vinegar (Haruta et al., 2006), Portuguese fermented sausage (Albano et al., 2008), Italian cheese (Bonetta et al., 2008; Fontana et al., 2010) and salami (Aquilanti et al., 2007; Silvestri et al., 2007), cocoa (Camu et al., 2007; Nielsen et al., 2008) and fermented seafood (Roh et al., 2010). These studies helped us to understand how a fermentation product was made and the relationship between the microbial diversity, its dynamic change and its special characteristics.

However, there is little reference concerning the microbial diversity of traditional Chinese vinegar fermentation. In the last decades, the microbial researches of traditional Chinese fermented vinegar were mainly based on culture-dependent approaches, with the purpose of isolating high-yielding acetic acid bacteria (Cui et al., 2008; Hao et al., 2008; Hu and Hao, 2004; Huang et al., 2006). On the other hand, molecular ecology techniques were introduced by some researchers to study Chinese traditional vinegars. Wu et al. (2010) used enterobacterial repetitive intergenic consensus (ERIC)-PCR fingerprinting to characterize 21 acetic acid bacterium (AAB) strains isolated from Chinese cereal vinegars produced by solid-state fermentation.

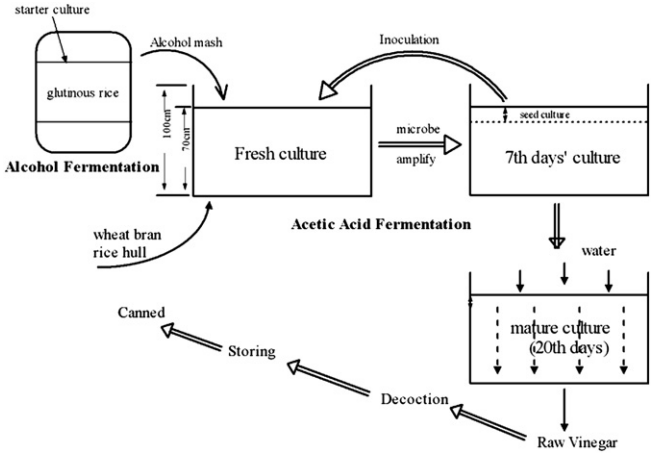

Fig. 1. Manufacture technique of Zhenjiang aromatic Vinegar. The production of Zhenjiang aromatic vinegar may be divided into three steps, namely alcohol fermentation, acetic acid fermentation, and storing. A unique circular inoculation technique is used during acetic acid fermentation process to keep the microbial flora stabilization.

This study aimed to investigate the structure and dynamic change of the microbial community during solid-state acetic acid fermentation process of Zhenjiang aromatic vinegar. Firstly, PCR-DGGE was applied to investigate the bacterial and fungal community existing in the fermentation process. Secondly, two clone libraries (bacteria and fungi) were constructed to qualitatively study the microbial structure. Thirdly, quantitative real-time PCR analysis was applied to investigate the change of biomass during the fermentation process.

2. Materials and methods

2.1. Sampling

The original cultures used in this study were collected in September, 2007 from Jiangsu Hengshun Vinegar Industry Co., located in the eastern coastal province of Jiangsu. Total 3 batches of sample which were always done at the same depth of the fermentation culture (approximately 20 cm from the upper surface) were collected, and every fresh sample (500 g) was laid in a sterile plastic bag on the 1st (humidity, 67.35%; pH, 4.24), 5th (humidity, 66.58%; pH, 4.52), 7th (humidity, 65.62%; pH, 4.13), 10th (humidity, 67.36%; pH, 3.89), 15th (humidity, 68.46%; pH, 3.77) and 20th (humidity, 68.10%; pH, 3.91) day of the solid-state acetic acid fermentation process. After sampling, samples were taken to the lab immediately and stored at −20 °C before further analysis.

2.2. The pH measurement and analysis of organic compounds

1 g sample was treated with 30 ml de-ionized water, and the pH of the solution was then measured by a pH meter. Total nine organic acids (oxalic acid, tartaric acid, pyruvic acid, malic acid, lactic acid, acetic acid, citric acid, fumaric acid and succinic acid) were analyzed by HPLC with described approaches by Versari et al. (2008). Amino acids were analyzed according to the methods previously described in detail (Fabiani et al., 2002).

2.3. DNA extraction and PCR amplification

1 g sample was treated with 30 ml de-ionized water, and total nucleic acids were extracted according to the described method (Zoetendal et al., 1998). All DNA concentrations were then determined by using DyNA quant 200 (Hoefer, San Francisco, CA, USA).

Two pairs of universal primer were used to amplify bacterial 16S rDNA and fungal 18S rDNA, respectively. Primers P_2 (ATT ACC GCG GCT GCT GG) and P_3-GC (CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG) were performed to amplify bacterial 16S rDNA (Muyzer et al., 1993). Meanwhile, primers NS_3-GC (CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GGC AAG TCT GGT GCC AGC AGC C) and YM_{951r} (TTG GCA AAT GCT TTC GC) were used to amplify fungal 18S rDNA (Haruta et al., 2006). The PCR mixture (25 μl) contained 1 U of rTaq polymerase (Takara, Dalian, China), 1× PCR buffer (Mg^{2+} free), 2 mM $MgCl_2$, 12.5 pM of each primer, and 10 ng of extracted total DNA. The samples were amplified using a PCR Express system (Hybaid Limited, Ashford, UK). For bacterial 16S rDNA and fungal 18S rDNA amplification, a 'touchdown PCR' process was applied (Muyzer et al., 1993; DiCello et al., 1997). The sizes of PCR products were assessed by electrophoresis on a 1.2% (wt/vol) agarose gel.

2.4. PCR-DGGE

DGGE was performed using a Dcode System apparatus (Bio-Rad, Hercules, CA, USA). Before DGGE, each PCR product was reconditioned for five cycles to reduce single-stranded and heteroduplex