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Screening of Key Species Involved in Baiyaojian Fermentation Based on Gallic Acid Content

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Abstract

The present study aimed to screen for dominant species involved in Baiyaojian fermentation based on the appearance and gallic acid content of Baiyaojian. The species were added via two routes: Jiuqu (dried fermentation starter) or distiller's grains. Baiyaojian was fermented with each individual species under the same fermentation conditions, and the dominant species was screened, isolated, and purified. Then, the dominant species from the two methods were compared to determine the optimal species for fermentation. One dominant species, each was isolated from Baiyaojian fermented with Jiuqu and distiller's grains. These species were subsequently identified as *Rhizopus oryzae BYJ.G-1* and *Aspergillus niger BYJ.H-1*, respectively. The gallic acid content in the fermentation products of *R. oryzae BYJ.G-1* was higher than that of *A. niger BYJ.H-1*, and the surface of *R. oryzae BYJ.G-1* was covered with white hyphae. Both *R. oryzae BYJ.G-1* and *A. niger BYJ.H-1* were dominant species involved in the fermentation of Baiyaojian. *R. oryzae BYJ.G-1* appeared to be more effective than *A. niger BYJ.H-1*. These findings suggested that single-species fermentation could be used to optimize the fermentation process of Baiyaojian and to improve its fermentation efficiency, thereby improving the quality of Baiyaojian as well as its safety for clinical use. © 2019 Friends Science Publishers

Key words: *Aspergillus niger BYJ.H-1*; Baiyaojian; Fermentation; Process optimization; *Rhizopus oryzae BYJ.G-1.*

Introduction

Baiyaojian is prepared by the fermentation of Chinese gall (Wubeizi) with tea leaves, which is shaped into blocks. As a type of traditional Chinese medicine (TCM), Baiyaojian is used to treat chronic cough and phlegm, sore throat, hematochezia, chronic dysentery and archoptosis, aphthous ulcers, tooth aches, carbuncles, and sores. In ancient times, Chinese gall was used for tanning and dyeing, and Baiyaojian was invented by leather workers, not for medical applications. Later, Baiyaojian was prepared for medicinal use, with the first report of medical application reported in the *Danxixinfa* (Danxi's Experiential Therapy). This TCM has been shown to moisten the lungs, remove phlegm, and quench thirst. Recent research has shown that Baiyaojian has anti-inflammatory and antibacterial effects (Peng *et al*., 2016).

The traditional processing method for Baiyaojian is natural fermentation, *i.e*., fermentation that occurs in the natural environment. Therefore, the fermentation temperature and humidity are affected by the weather, and the product is easily contaminated by pathogens, making it difficult to guarantee its safety for medical use (Shi *et al*., 2015). Modern methods for fermentation have improved the fermentation conditions, which involve the use of Jiuqu (dried fermentation starter) and distiller's grains for fermentation under constant temperature and humidity. The three major factors that affect the quality of fermentation are the fermentation species (Wang *et al*., 2017a), fermentation substrate (tea variety), and fermentation conditions (temperature, humidity). Our research group has determined the fermentation substrate and fermentation conditions for Baiyaojian through previous experiments; however, the fermentation species have not yet been confirmed. The key fermentation species for Baiyaojian is *Aspergillus niger*. Notably, according to the literature and the processing specifications from different provinces, Jiuqu and distiller's grains are currently the main sources of species used for the fermentation of Baiyaojian.

Due to the complexity of species in distiller's grains and the relative uniformity of species in Jiuqu, different methods are used to isolate and identify the dominant species from distiller's grains and Jiuqu (Li and Niu, 2013). For example, fungi are isolated with the streak plate method and hyphal tip purification for traditional Baiyaojian fermented with distiller's grains (Jin *et al*., 2006). Micromorphology identification is then carried out using a

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biological microscope, and molecular biology identification is performed using DNA sequencing (Wang *et al*., 2014). In this study, we used these methods to identify and characterize dominant microorganisms involved in Baiyaojian fermentation using the two approaches. Gallic acid is the main active substance in Baiyaojian, Single strain fermentation can increase tannin conversion and improve content of gallic acid in Baiyaojian. Furthermore, using pure bacteria, the fermentation process of Baiyaojian can be optimized. We aimed to screen for dominant species involved in Baiyaojian fermentation based on the appearance and gallic acid content of Baiyaojian, and for a better understanding and developing natural productions from Chinese herbal medicine.

Materials and Methods

Screening of Jiuqu for Baiyaojian Fermentation

Six types of Jiuqu were collected from three manufacturers: Haifeng Xinlong Jiuqu Plant (*Rhizopus* Jiuqu), Guangxi Quanzhou Shitang Baoshan Jiuqu Plant (Baoshan Jiuqu, Baoshan Jiuqu cake, TCM special Jiuqu, Sweet potato Jiuqu), and Angel Yeast Co., Ltd. (Angel Jiuqu). Chinese gall (Wubeizi) was purchased from Anhui Bozhou Chinese Medicine Decoction Pieces Factory (batch no.: 20150812) and was identified by Professor Chen Suiqing from the Henan University of Traditional Chinese Medicine as *Rhus chinensis* Mill of the Anacardiaceae family. Infestation of the tree by Chinese sumac aphids (*Schlechtendalia chinensis*) led to production of a gall that met the standards of the Chinese Pharmacopoeia 2015 edition: under the specifications of "Chinese gall". Baiyaojian was fermented under the same conditions, and the content of gallic acid was used as an indicator, which was combined with the appearance and shape to screen the fermentation Jiuqu.

Isolation and Purification of Species in Jiuqu

Dilution plating was performed. First, 10 g of the selected Jiuqu was weighed out, and 90 mL of sterile water was added. The mixture was placed in a constant temperature shaker and shaken at 150 rpm for 30 min. Gradient dilutions of 10^{-2} to 10^{-8} were prepared. Under sterile conditions, 200 μL of the diluent was pipetted and inoculated on potato dextrose agar (PDA) medium. Each gradient of diluent was inoculated onto three plates, spread gently and evenly with a sterile glass rod, and left to stand at room temperature for 5–10 min to allow the diluent to be adsorbed into the medium. The plate was then inverted, cultured in an incubator at 30°C for 3–5 days, and evaluated (Cui *et al.,* 2014).

Isolation and Purification of Species from Baiyaojian Fermented with Distiller's Grains

The dilution plating method was used. First, 10 g Baiyaojian fermented within 24 h was collected, and 90 mL sterile water was added. The mixture was placed in a constant temperature shaker and shaken at 150 rpm for 30 min. Gradient dilutions at 10^{-2}–10^{-8} were prepared (Fu, 2016). Under sterile conditions, 200 μL of the diluent was pipetted and inoculated on the PDA medium. Each gradient of diluent was inoculated onto three medium plates, spread gently and evenly with a sterile glass rod, and left at room temperature for 5–10 min to adsorb into the medium. The plates were then inverted, cultured in an incubator at 28°C for 3–5 days, and evaluated. After the colonies were cultured, the hyphae at the edges of each colony were picked and inoculated onto new medium using the streak plate method (Li and Wang, 2015). Samples were then isolated, cultured, and subjected to stepwise purification using hyphal tip purification. Individual species that were purified were individually numbered. An inoculating needle was used to obtain a small amount of spores, which were inoculated on potato dextrose agar (PDA), Sabouraud dextrose agar, and Rose Bengal agar using the three-point inoculation method. Samples were then cultured at 28°C in an incubator (Weselowski *et al.,* 2016). The colonies of the species in the culture medium were observed, and their morphology and growth status were characterized. Alternatively, the slide culture method was performed. The fungus was inoculated at the interface between the cover glass and medium, allowing the hyphae to adhere to the cover glass during growth. The specimens were cultured in an incubator at 28°C. Every day after the following day, the cover glass was removed with a pair of sterilized tweezers, stained with Cotton Blue in lactic acid, and placed under a microscope to observe the fungal structure. The isolated single species were stored in 30% aqueous glycerol solution at -20°C.

The fungal DNA extraction kit provided by Bioengineering (Shanghai) Co., Ltd. was used to extract the rDNA of the stored fungi described above. A pair of primers, ITS1 and ITS4, complementary to the ITS sequence was used to amplify the 5.8S-ITS segment. The amplified gene fragment was submitted to Shanghai Bioengineering Co., Ltd. for bidirectional sequencing. The results of 18S rDNA sequencing were submitted to the National Center for Biotechnology Information (NCBI) database for BLAST alignment search. When typical strain series were identified, ClustalX2.0 software was used to perform homology comparisons. Different lengths at the start and end of the sequence were trimmed to avoid uneven sequences, increasing the differences between the sequences, and files of aln format were generated (Yousef *et al*., 2018). The nucleotide differences among various groups were calculated using the kimura-2-parameter-distance in Mega 5.03 software. The phylogenetic tree was constructed by running 1000 bootstraps, and the molecular phylogenetic tree was constructed using Meta 5.03 software.

Screening of Dominant Species in Baiyaojian Fermented with Distiller's Grains

One gram of powdered tea leaves was boiled with 20 mL

water for 15 min and left to stand at room temperature. The tea was filtered to remove tea residue and decocted to 8 mL. Next, 3 mL tea was mixed evenly with tea residue and 10 g Chinese gall powder. The mixture was sealed with moist eight-layer gauze, sterilized at 115°C for 30 min, and left to cool. The individual species isolated in the laboratory were placed on PDA medium and activated for 3 days. Fifty milliliters of distilled water was then placed in a 200 mL conical flask, sterilized at 121°C for 30 min, and left to cool (Compant *et al*., 2011). Thereafter, 2–4 spores were picked with a sterile bamboo toothpick and placed in sterilized distilled water to produce a spore suspension at a concentration of more than 10^8 spores/mL. After being shaken at 180 rpm for 2 h, the spore suspension was obtained and added to the above materials (Liu *et al*., 2012) Fermentation commenced after the suspension was stirred with a sterilized glass rod to obtain Baiyaojian fermented with a single species. The dominant species of Baiyaojian fermentation were screened using gallic acid content as an indicator combined with their appearance and shape.

DNA Amplification and Sequencing

The DNA was isolated from strain through tphenol-chloroform method. The 18S-PCR sequences were amplified by using the primers of NS1 (5'-GTAGTCATATGCTTGTCTC-3') and NS8 (5'-TCCGCAGGTTCACCTACGGA-3'). Amplification of DNA was carried out in 50 μL reaction mixture, containing 2 μL DNA, 1 μL of each primer, 25 μL Ex Taq, 21 μL ddH_2O, PCR reaction was as follows: 32 cycles of 10 min at 94°C, 1 min at 94°C, 1 min at 55°C, 1.5 min at 72°C and ended with a final extension of 10 min at 72°C. The 26S-PCR sequences were amplified by using the primers of NL1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL4 (5'-GGTCCGTGTTTCAAGACGG-3'). Amplification of DNA was carried out in 50 μl reaction mixture, containing 2 μL DNA,1 μL of each primer, 25 μL Ex Taq, 21 μL ddH2O. PCR reaction was as follows: 34 cycles of 10 min at 94°C, 1 min at 94°C, 1 min at 55°C, 1.5 min at 72°C and ended with a final extension of 10 min at 72 °C. The ITS-PCR sequences were amplified by using the primers of ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and NS 8(5'-TCCTCCGCTTATTGATATGC-3'). Amplification of DNA was carried out in 50 μL reaction mixture, containing 2 μL DNA, 1 μL of each primer, 25 μL Ex Taq, 21 μL ddH2O, PCR reaction was as follows: 34 cycles of 10 min at 94°C, 1 min at 94°C, 1 min at 55°C, 1.5 min at 72°C and ended with a final extension of 10 min at 72°C (Dharmaprakash and Thomas, 2016).

Single-species Fermentation of Baiyaojian

The *A. niger BYJ.H-1* soft material was placed in a constant temperature and humidity chamber at 40°C and 85% relative humidity to ferment for 60 h. The *R. oryzae BYJ.G-1* soft material was placed in a constant temperature and humidity chamber at 32°C and 85% relative humidity to ferment for 66 h.

HPLC Method for the Determination of Gallic Acid

Ultrasoinic extraction method was used for extracting of Baiyaojian by KQ-500DV CNC ultrasonic cleaner (Kunshan Ultrasonic Instruments Co., Ltd.). The separation was performed on a Waters Symmmetry Shield^{TM} RP18 (4.60 mm × 250 mm, 5 μm) column at the column temperature of 30°C by high-performance liquid chromatograph (Waters 2489UV detector; Waters). The isometric elution system consisted of acetonitrile (HPLC/Spectro; TEDIA) and 0.1% phosphoric acid. The flow rate was 0.8 mL·min^{-1}, and the detection wavelength was set at 270 nm. Data processing was through Breeze 2 data analysis system (Wang *et al*., 2017b). Gallic acid (batch no: PS08121401, purity ⩾ 98%; Chengdu Pusi Biotechnology Co., Ltd.) was used as a reference substance.

Results

Screening Results of Jiuqu

Analysis of Baiyaojian fermented with six types of Jiuqu showed that the content of gallic acid in Baiyaojian by fermentation of Haifeng Xinlong Jiuqu (*Rhizopus* Koji) was the highest and its appearance was the best (Table 1).

It was observed that the same strain could only grow on PDA medium with different dilution gradients, named *BYJ.G-1*. They were sent to the China Typical Culture Preservation Center for identification of strains. Colony morphology and microscopic image of *BYJ.G-1* as shown in Fig. 1 and 2.

As shown in Fig. 1 and 2, *BYJ.G-1* colonies were round and had regular edges. The colonies were white floccules at the initial stage and were loose and large; the hyphae grew rapidly, and the creeping hyphae were transparent and developed. Subsequently, the colonies became dense and produced many black spores. Sporangia were formed at the tip and were nearly spherical, and the spores were spherical or nearly spherical (Šuranská *et al*., 2016). BLAST alignment was performed in the NCBI database, and the *BYJ.G-1* strain was identified as *Rhizopus*.

Species Identification in Baiyaojian Fermented with Distiller's Grains

Eight strains were isolated and purified through gradient dilution and streaking of naturally fermented Baiyaojian. Seven strains of mold and one strain of yeast were obtained. According to colony morphology, microscopic identification (Huang *et al.,* 2015), and The Manual for Fungal Identification (Wei, 1979) and Flora Fungorum Sinicorum (Qi, 1998), five strains of *Aspergillus* and one strain of *Saccharomyces* were identified (Fig. 3 and 4, Tables 2 and 3). The results of agarose gel electrophoresis of the

Table 1: Screening of Jiuqu for Baiyaojian fermentation

Selected Jiuqu*	Gallic acid content (%)	Appearance
Baoshan Jiuqu	10.21	Initial growth of a small number of white colonies on the surface of the soft material
Baoshan Jiuqu cake	13.73	Initial growth of a small number of white colonies on the surface of the soft material, light yellow in the center of the colonies, with a small number of black spores
Haifeng Xinlong Jiuqu	14.41	Hyphal growth and white colonies on the surface
Sweet potato Jiuqu	9.78	Surface of the soft material was covered with yellow hyphae and spores
TCM special Jiuqu	9.41	Surface covered with small white colonies
Angel Jiuqu	7.16	Surface covered with white hyphae Slightly yellow, with a small number of black spores

Note: Species identification in Baiyaojian fermented with Jiuqu

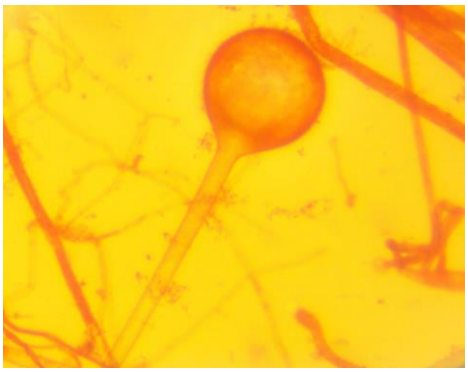
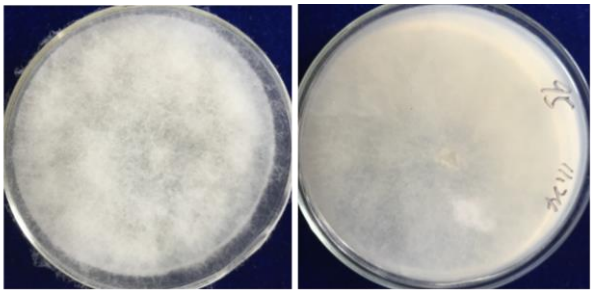
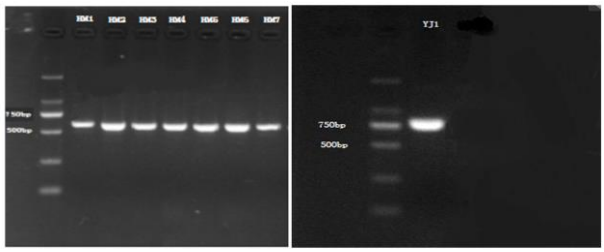


Fig. 1: Microscopic observations of *Rhizopus* (40×)



A B

Fig. 2: Front (**A**) and back (**B**) observations of *R.* colonies



A B

Fig. 3: PCR electrophoresis of *A. niger*

18S rRNA gene showed that the sequence was about 600 bp, which was consistent with the base sequencing results, indicating that the sequencing results were correct. Strains HM5 and HM6 showed a similarity of 99% in molecular BLAST alignment (Zhang *et al.*, 2011). When combined with selective medium and microscopic morphology, these two strains were suspected to be the same. The similarity of HM4 and HM5 was 100%, indicating that these three strains were the same. The results of agarose gel electrophoresis for the 18S rDNA sequence of the fungal strain YJ1 indicated that the sequence was about 750 bp, which was consistent with the results of nucleotide sequencing (728 bp). This indicated that the sequencing results were correct. The molds HM4, HM5, and HM6 were the same strain; thus a phylogenetic tree was not needed. The remaining strains were uploaded to the NCBI website for BLAST alignment, and similar sequences were downloaded for phylogenetic tree construction (Fig. 4). The alignment and phylogenetic tree showed that the three strains of *A. niger* may be different subspecies. See Tables 2 and 3 for molecular identification results (Table 2 and 3). The content of gallic acid in each single species was determined. The results showed that the gallic acid content of Baiyaojian fermented with HM3 was the highest and that this strain showed the best appearance. Therefore, this was found to be the dominant species for the fermentation of Baiyaojian and was renamed as *A. niger BYJ.H-1*. The sequence was submitted to the China Center for Type Culture Collection for identification and preservation with accession number: CCTCC M 2016376 (Table 4).

Comparison of Baiyaojian Fermented with *R. oryzae* BYJ.G-1 and A. niger BYJ.H-1

Frozen *R. oryzae BYJ.G-1* and *A. niger BYJ.H-1* were activated on PDA solid plates and then placed in a mold incubator at 30°C for 3–4 days until the spores covered the plate. The appropriate amount of spores was scraped off, placed in sterile water, and diluted to yield 1×10^8 mL^{-1} *R. oryzae BYJ.G-1* and *A. niger BYJ.H-1* spore suspensions. Chinese gall powder, tea dregs, and an optimal amount of tea were mixed to form the soft material, which was sterilized with high-pressure steam at 115°C for 30 min and placed on an ultra-clean work station to cool to room temperature. The two spore suspensions were mixed with the soft material. After the fermentation was completed, the gallic acid content of Baiyaojian fermented with *R. oryzae BYJ.G-1* was 26.0%, which was higher than that of the product fermented with *A. niger BYJ.H-1* (23.1%). This demonstrated that fermentation with *R. oryzae BYJ.G-1* was

Table 2: Molecular identification of molds

Isolates	Fungal species	Max identity %	Accession
HM1	*Aspergillus oryzae*	99	JN227035.1
HM2	*Aspergillus niger*	99	LN482469.1
HM3	*Aspergillus niger*	99	JN676109.1
HM5	Aspergillus	99	
HM7	*Aspergillus niger*	99	KJ639036.1

Table 3: Molecular identification of yeast

Isolates	Fungal species	Max identity %	Accession
YJ1	Saccharomyces cerevisiae	97	JX047334.1

Table 4: Appearance and gallic acid content of Baiyaojian fermented with a single species

Species number	Appearance	Gallic acid content (%)
HM1	Surface covered with brown or light brown spores, barely visible hypha, slightly hard texture after drying	13.46
HM2	Yellow-green hypha on the surface, a small number of spores, sparse pili, a large amount of soft material, slightly hard texture after drying	8.34
HM3 (*A. niger BYJ.H-1*)	Surface covered with light yellow and white hyphae, relatively thin double-layer, slightly hard texture after drying	15.41
HM5	Surface covered with brown spores, barely visible hyphae, hardened texture after drying	14.44
HM7	Covered with white, relatively long hyphae, slightly greenish, slightly hard texture after drying	12.37
YJ1	No characteristics of fermentation, no white hyphae, no spores, hardened texture after drying	4.64

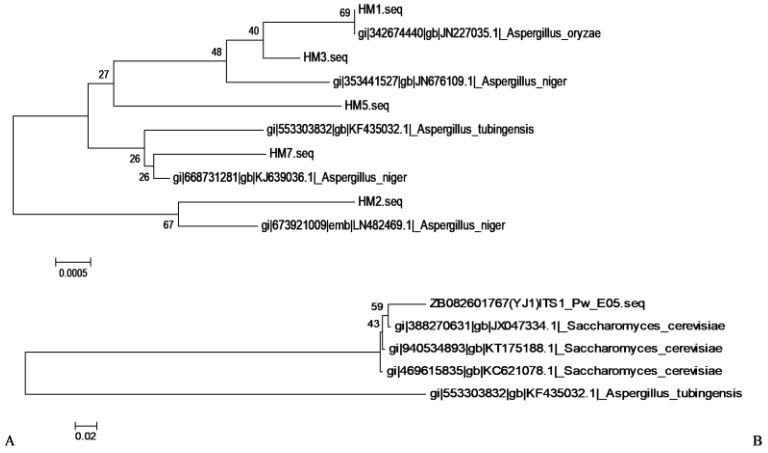


Fig. 4: Phylogenetic tree based on ITS sequences using the neighbor-joining (NJ) method

superior to that with *A. niger BYJ.H-1*. Therefore, we ultimately determined that *R. oryzae BYJ.G-1* was the best species for Baiyaojian fermentation.

Discussion

The tannin content in Galla chinensis is high, up to 70%. It is easy to combine with protein to form water-insoluble macromolecule sediment. A few people have adverse reactions such as loss of appetite because of the water-insoluble macromolecule sediment cause irritation of gastrointestinal mucosa. Tannins in gallnut can be hydrolyzed when they enter the body (Li *et al.*, 2008). Hydrolyzed tannins produced by gallnut are toxic to the liver and cause liver damage. *R. oryzae* and *A. niger* were selected as dominant strains of Baiyaojian fermented by Jiuqu and distiller's grains in this study. *A. niger* has many enzyme systems with strong activities and many other advantages, which makes it excellent for industrial fermentation (Tian *et al.*, 2018). *A. niger* can also be used for solid fermentation of Chinese gall to produce tannase. Tannase is an inducible enzyme (Jin *et al.*, 2013), also known as tannin esteryl hydrolase, which is synthesized by some microorganisms in the presence of inducers such as tannic acid. Tannase can hydrolyze the ester and carboxyl bonds of tannin gallate to produce gallic acid and glucose (Wu *et al.*, 2018), thus strengthening the antibacterial and expectorant effects of fermented Chinese gall (Hesham *et al.*, 2018). *R. oryzae* can promote the production of L-lysine, and then promote the gastrointestinal mucosa to absorb the protein in food, avoid the competitive consumption of tannic acid in the gastrointestinal tract, so that the convergence of gallnut fermentation Baiyaojian is weakened and the irritation is reduced. Baiyaojian is made prepared by the fermentation of

Chinese gall, which mainly contains gallic acid and tannins (Xing *et al.*, 2011). The fermentation process alters the types and contents of active ingredients. For example, lower tannin contents and higher gallic acid contents were observed after fermentation of Chinese gall (Shao *et al.*, 2014). Gallic acid has many biological activities, such as anti-inflammatory, anti-oxidative, antibacterial, and antiviral activities. Moreover, this compound also has preventive and therapeutic effects on cardiovascular system diseases, nervous system diseases, diabetes, liver fibrosis, and tumors (Zhu *et al.*, 2019).

Traditional Chinese medicine fermentation strains come from nature, which are diverse, unclear species, and the fermentation mechanism is not clear (Zhang *et al.*, 2015). The fermentation process has no strict aseptic control and is not easy to control. The stability of the product is poor, which is not conducive to the production of large industry. Nowadays, pure strain fermentation of microorganism is gradually applied to the fermentation of Chinese herbal pieces. The pure strain fermentation is single and controllable, the fermentation process is easy to control, the product is stable and has broad application prospects. For example, isoflavone content and enzyme activity of fermented light soybean sauce by single strain *A. oryzae* or *A. niger* were higher than those of naturally fermented light soybean sauce (Yang *et al.*, 2016). Compared with traditional Chinese medicine processing methods, modern fermentation technology of traditional Chinese medicine has better advantages. It combines traditional processing with modern microbiology, and produces effective Chinese medicine components by reacting enzymes secreted by microorganism growth, metabolism and chemical composition in traditional Chinese medicine. Fermentation objects have developed from the self-fermentation of single herbal pieces, such as *Ganoderma lucidum* and *Cordyceps sinensis* mycelium, to the two-way solid fermentation of traditional Chinese medicine and strains, and the fermentation of compound Chinese medicine. Through the combination of medicinal fungi and traditional Chinese medicine, it can change the original properties of medicines, improve the extraction rate of effective components, reduce or change toxic components, and expand the scope of medication. It provides new ideas and methods for the fermentation of modern Chinese medicinal pieces.

Microorganisms have a strong ability to decompose and transform substances, resulting in modification of the potency of medicines, improvement of clinical curative effects, and reduction of toxic side effects. This study proves that single strain fermentation can not only increase tannin conversion rate and gallic acid content in *Galla chinensis*, but also optimize the fermentation process of Baiyaojian, save costs, improve the efficacy of Baiyaojian and the safety of clinical medication, and provide scientific and reasonable guidance for Baiyaojian and related fermentation products. We also showed that fermentation with *R. oryzae BYJ.G-1* was more effective than that with *A. niger BYJ.H-1*. However, we did not determine whether the mechanism of fermentation with *R. oryzae BYJ.G-1* was the same as that of *A. niger BYJ.H-1*; thus, further experiments are required to verify these findings. In addition, some microbes with a relatively short growth cycle, fungi in the decline phase, or fungi that were less active and proliferated slowly could not be easily isolated due to the specificity of the growth conditions. If the value-added method or other culture methods are used to activate fresh Baiyaojian, the type and quantity of isolated fungi may be increased.

Conclusion

Both *R. oryzae BYJ.G-1* and *A. niger BYJ.H-1* were dominant species involved in the fermentation of Baiyaojian. They can obviously improve the tannin conversion rate of gallnut. *R. oryzae BYJ.G-1* appeared to be more effective than *A. niger BYJ.H-1*. These findings suggested that single-species fermentation could be used to optimize the fermentation process of Baiyaojian.

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