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Optimization of Dominant Strain Composite Fermentation for Jianqu and Dynamic Changes in Amylase Activity

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ABSTRACT

To ensure the safe production of Jianqu, the dominant strains —Absidia corymbifera, Lichtheimia ramosa, and Mucor circinelloides—identified through high-throughput sequencing analysis, were inoculated onto sterile Jianqu-building substrates for cultivation. Their appearance characteristics were observed, and the liquefying and saccharifying power of their amylase were measured. Subsequently, Saccharomycopsis fibuligera, Millerozyma farinose, Hyphopichia burtonii, Absidia corymbifera, Lichtheimia ramosa, Mucor circinelloides, and Monascus purpureus were co-inoculated onto Jianqu-building substrates in different proportions for composite fermentation. The fermentation phenomena were observed, and the liquefying and saccharifying power of amylase were detected to further evaluate the effects of restricted composite fermentation using different proportions of dominant strains. The results showed that when the ratio of yeast (Saccharomycopsis fibuligera, Millerozyma farinose, Hyphopichia burtonii were mixed in a ratio of 2: 1: 1): Absidia corymbifera :Lichtheimia ramosa :Mucor circinelloides :Monascus purpureus was 2:4:3:2:1, the fermentation product achieved the ideal state of "ubiquitous white mold with a fragrant alcoholic aroma." The changes in amylase liquefying and saccharifying power exhibited an "S"-shaped curve. At 84 hours of fermentation, the liquefying power reached 2.11 ± 0.08U, and the saccharifying power reached 398 ± 5U, significantly outperforming natural fermentation ($P < 0.05$). Conclusion: By adjusting the inoculation ratios of dominant strains, the liquefying and saccharifying power of amylase can be significantly enhanced, thereby shortening fermentation time and improving the quality of Jianqu fermentation.

KEYWORDS

Jianqu; Composite fermentation; Amylase; Liquefying power; Saccharifying power.

1. INTRODUCTION

Jianqu is a fermented product made from 23 traditional Chinese medicinal herbs, such as Polygonum hydropiper, Xanthium sibiricum, and Artemisia annua. It possesses various therapeutic effects, including strengthening the spleen, aiding digestion, relieving external symptoms, regulating the stomach, and promoting appetite [1]. As a type of fermented herbal preparation, its production method originates from the ancient " Massa Medicata Fermentata " and represents a traditional Chinese medicinal preparation with unique craftsmanship [2]. Currently, most manufacturers follow traditional methods, employing controlled temperature and humidity in an open natural state for fermentation, using "ubiquitous white mold with a fragrant alcoholic aroma" as the standard for completion. However, in open fermentation, the diverse environmental microorganisms involved are highly susceptible to environmental changes and contamination. This limits the potential of the microorganisms, leading to inconsistent quality and unreliable efficacy of Jianqu during the

fermentation process of traditional Chinese medicine [3-4]. In our previous studies, high-throughput sequencing and big data analysis revealed that the dominant microorganisms involved in the fermentation of Jianqu were mainly fungi such as Absidia corymbifera, Lichtheimia ramosa, and Mucor circinelloides [5]. These findings have laid the foundation for further improving the quality of Jianqu through restricted fermentation.

Jianqu has a complex composition and is rich in polysaccharides. During the fermentation process, microorganisms produce a variety of enzymes that can decompose and transform the medicinal components in Jianqu, thereby reducing the toxic side effects of the drugs and enhancing their therapeutic efficacy [6]. To improve the quality control and clinical application of fermented medicinal Jianqu, the detection methods and quality standards for digestive enzymes in medicinal Jianqu have been widely studied and emphasized [7]. Among these, amylase, as one of the key enzymes, can degrade starch to form substrates for other enzymatic reactions, further promoting microbial metabolism [8]. In the process of optimizing the fermentation technology of Massa Medicata Fermentata, the activities of amylase and protease produced by different fermentation methods can serve as important references for establishing quality standards [9]. Similarly, the activity of amylase also significantly impacts the quality control of Jianqu. Currently, the quality control and improvement of Jianqu still require in-depth research. This study attempts to provide an effective method for Jianqu quality control by detecting the activities of liquefying and saccharifying amylases.

In preliminary studies, dominant strains such as Absidia corymbifera, Lichtheimia ramosa, and Mucor circinelloides were screened using high-throughput sequencing and strain isolation techniques. In this study, these dominant strains were inoculated onto Jianqu raw material blocks, and the changes in the liquefying and saccharifying power of their amylases were analyzed. Designing restricted composite fermentation with different ratios of Saccharomycopsis fibuligera, Millerozyma farinose, Hyphopichia burtonii, Absidia corymbifera, Lichtheimia ramosa, Mucor circinelloides, and Monascus purpureus, and comparing the “white mold” phenomenon, analyzing the changes in liquefying and saccharifying power of amylase, to explore restricted fermentation and provide beneficial references for the safe production of Jianqu.

2. MATERIAL

Reagents: Tween-80: Wenzhou Qingming Chemical Factory; Anhydrous glucose: Shanghai Macklin Biochemical Technology Co., Ltd; Iodine, potassium iodide, sodium hydroxide, glacial acetic acid, soluble starch, sodium acetate anhydrous, copper sulfate pentahydrate, and sodium potassium tartrate: ChengDu Chron Chemicals Co,.Ltd.

Instruments: Electronic balance (DG-2245), Nanjing Suchu Measuring Instruments Co., Ltd.; Intelligent electric thermostatic incubator (DHP-9160B), Shanghai Langxuan Experimental Equipment Co., Ltd.; Vertical pressure steam sterilizer (LX-C35L), Hefei Huatai Medical Equipment Co.,Ltd.; Oven: 400T, Shanghai Xingye Vacuum Equipment Factory; Mini centrifuge (5418-R, Eppendorf), Eppendorf; Intelligent thermostatic shaker (HT-26X3C-W), HerryTech Co.,Ltd.; Vacuum freeze dryer (LGJ-18S), Shenzhen Sanli Chemical Co., Ltd.; Grinder (YB-700B), Yongkang Shufeng Industry and Trade Co., Ltd.; Constant temperature and humidity incubator (SPX-150B), Beijing Hengnuolixing Technology Co., Ltd.

3. METHOD

3.1. Preparation of Fungal Spore Suspension

The dominant strains identified in previous experiments—*Saccharomycopsis fibuligera*, *Millerozyma farinose*, *Hyphopichia burtonii*, *Absidia corymbifera*, *Lichtheimia ramosa*, *Mucor circinelloides*, and

Monascus purpureus [5]—were inoculated onto PDA medium and cultured in a constant temperature incubator at 28°C. After the fungal spores matured, they were washed with sterile water containing Tween-80 (0.2%, v/v) to prepare a spore suspension. The concentration was adjusted to 2×10^8 spores/mL using a hemocytometer, and the suspension was stored at 4°C for later use.

3.2. Composite Fermentation of Jianqu

The raw medicinal powder of Jianqu was dry-heat sterilized in an oven at 120°C for 60 minutes and then transferred to a sterile beaker. An appropriate amount of sterile water was added, and the spore suspension was precisely pipetted, with a total inoculation volume of 8%. Sterile water was used to supplement the inoculation solution to match the weight of the dried Jianqu powder. The spore suspension was mixed with the Jianqu powder, and the mixture was pressed into 30g Jianqu blocks using a pre-sterilized mold. The blocks were fermented indoors at 28°C and 85% relative humidity. Three groups were set up, with 10 blocks in each group, labeled as MF1, MF2, and MF3 (MF1: *Absidia corymbifera*; MF2: *Lichtheimia ramosa*; MF3: *Mucor circinelloides*).

Different fungi were selected for composite fermentation in varying proportions, designed as follows: The composite fermentation groups (JF1, JF2, JF3) were composed of *yeast*(*Saccharomycopsis fibuligera*, *Millerozyma farinose*, *Hyphopichia burtonii* were mixed in a ratio of 2: 1: 1), *Absidia corymbifera*, *Lichtheimia ramosa*, *Mucor circinelloides*, and *Monascus purpureus* in different ratios, specifically 4:3:2:2:1, 2:4:3:2:1, and 2:3:4:2:1, respectively, with an inoculation rate of 8%. The natural fermentation group (NF group) used non-sterilized Jianqu powder mixed with an equal proportion of clean tap water and fermented under natural conditions. Fermentation was carried out for 96 hours, with observations and sampling every 12 hours to record the apparent characteristics and measure amylase activity. The samples were dried in an oven at 80°C for 2 hours and stored at room temperature for further use.

3.3. Determination of Liquefying and Saccharifying Power during Jianqu Fermentation

The liquefying power was determined based on the characteristic blue reaction between starch and iodine. The sample extract was enzymatically hydrolyzed in a solution at 35°C and pH 4.6 until the blue-violet reaction with iodine disappeared. The liquefying power was calculated as the grams of starch liquefied per gram of dry Jianqu per hour under these conditions, denoted by the symbol "U" and expressed as "g/g·h." The detailed method for each sample group is referred to in QB/T 4257-2011.

The saccharifying power was determined based on the ability of saccharifying amylase in Jianqu to hydrolyze starch into glucose. The sample was hydrolyzed under specified conditions, sequentially cleaving α-1,4-glucosidic bonds from the non-reducing ends of starch to produce glucose. The amount of glucose generated was measured using the Fehling's method, which represents the saccharifying power. The detailed method is referred to in QB/T 4257-2011.

3.4. Statistical Analysis

The experimental data were analyzed using SPSS 17.0 software, and the results are expressed as "mean ± standard deviation." Statistical analysis was performed using GraphPad Prism software. One-way analysis of variance (ANOVA) was used to analyze the significance of differences between groups, and the LSD method was applied for post hoc comparisons. A P-value of less than 0.05 was considered statistically significant.

4. RESULTS

4.1. Appearance Characteristics of Composite Fermentation in Jianqu

Absidia corymbifera, *Lichtheimia ramosa*, and *Mucor circinelloides* were selected for fermentation on sterile Jianqu raw material blocks, respectively. The group inoculated with *Absidia corymbifera* showed slight white mycelium at 24 hours of fermentation, while the groups inoculated with *Lichtheimia ramosa* and *Mucor circinelloides* were slightly delayed. At 36 hours, all three groups exhibited distinct white mycelium. By 48 hours, the white mycelium had completely covered the surface of the Jianqu blocks, and as fermentation time increased, the mycelium continued to thicken. No significant changes were observed in the Jianqu blocks from 72 to 96 hours (Figure 1A), indicating that all three *molds* can grow on the Jianqu substrate.

Saccharomycopsis fibuligera, *Millerozyma farinose*, *Hyphopichia burtonii*, *Absidia corymbifera*, *Lichtheimia ramosa*, *Mucor circinelloides*, and *Monascus purpureus* were combined in different proportions to form the JF1, JF2, and JF3 groups for fermentation on Jianqu blocks. In the JF1 group, the surface of the Jianqu was covered with a large amount of *yeast* during the early and middle stages of fermentation, while the mycelium of *molds* was sparse. In the late stage, the white mycelium appeared flocculent, lying flat on the surface of the Jianqu block with fewer mycelial growths. In the JF2 group, the white mycelium completely enveloped the Jianqu block by the end of fermentation, with dense mycelial growth. In the JF3 group, the white mycelium also completely covered the Jianqu block in the late stage, but the mycelial density was lower than that of the JF2 group (Figure 1B). As fermentation progressed, the alcoholic aroma of the composite fermentation groups intensified with the growth of mold mycelium. However, in the NF group, the growth of molds and yeast was slower, and only a faint alcoholic aroma could be detected at the end of fermentation.

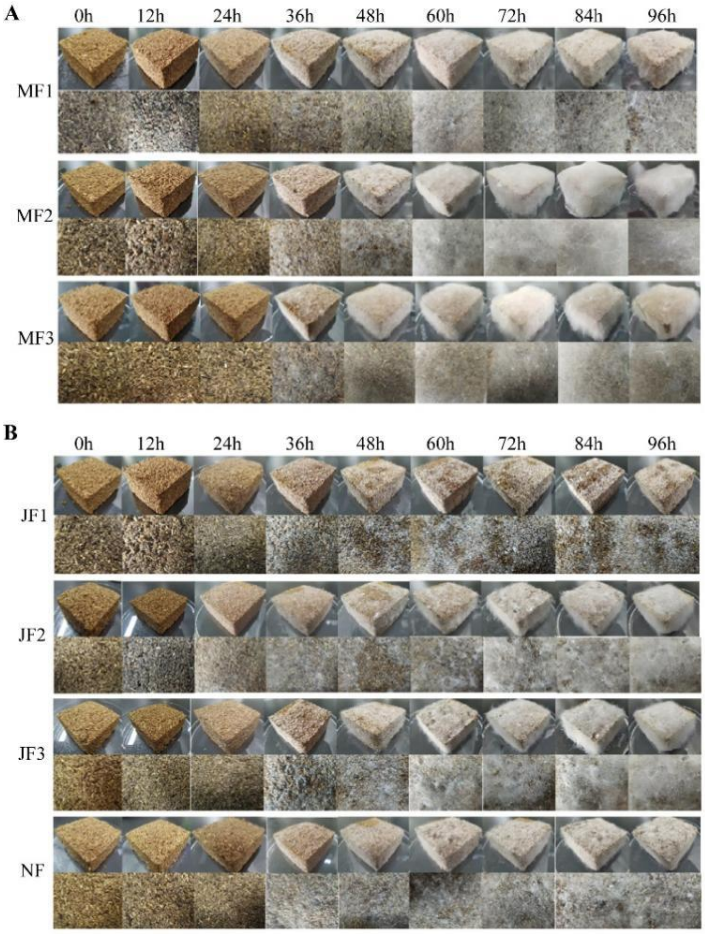

Figure 1. Appearance characteristics of Jianqu in different fermentation periods

(A) Appearance characteristics of Jianqu fermented by single molds at different fermentation periods. (B) Appearance characteristics of Jianqu fermented by composite strains at different fermentation periods.MF1, MF2, and MF3 represent fermentations inoculated with *Absidia corymbifera*, *Lichtheimia ramosa*, and *Mucor circinelloides*, respectively.JF1, JF2, and JF3, represent directed fermentation with three different combinations. The combined proportions of isolated strains, including *yeast* (*Saccharomycopsis fibuligera*, *Millerozyma farinose*, *Hyphopichia burtonii* were mixed in a ratio of 2: 1: 1), *Absidia corymbifera*, *Lichtheimia ramosa*, *Mucor circinelloides*, and *Monascus purpureus*, were 4:3:2:2:1 for JF1, 2:4:3:2:1 for JF2 and 2:3:4:2:1 for JF3, respectively. NF, the natural fermentation group.

4.2. Changes in Liquefying Power

In the fermentation of Jianqu by single fungal species, the liquefying power of the amylase was measured. For the individual fermentations using *Absidia corymbifera* (MF1), *Lichtheimia ramosa* (MF2), and *Mucor circinelloides* (MF3), the liquefying power of the amylase exhibited an "S"-shaped curve, initially increasing slowly and then rising rapidly after 48 hours of fermentation. The liquefying power of the MF2 group showed significant differences ($p < 0.05$) compared to the MF1 and MF3 groups after 48 hours. After 84 hours, the liquefying power stabilized, with values of 1.76 ± 0.07 U for MF1, 1.99 ± 0.1 U for MF2, and 1.51 ± 0.07 U for MF3 (Figure 2A). The activity of liquefying amylase followed the order MF2 > MF1 > MF3, with significant differences observed between MF2 and the other two groups ($p < 0.05$), as well as between MF1 and MF3 ($p < 0.05$).

In the composite fermentation of Jianqu, the composite fermentation groups (JF1, JF2, and JF3) were composed of *yeast*, *Absidia corymbifera*, *Lichtheimia ramosa*, *Mucor circinelloides*, and *Monascus purpureus* in ratios of 4:3:2:2:1, 2:4:3:2:1, and 2:3:4:2:1, respectively. The *yeast* component was a mixture of *Saccharomycopsis fibuligera*, *Millerozyma farinosa*, and *Hyphopichia burtonii* in a ratio of 2:1:1. The natural fermentation group was labeled as NF. Liquefying power was measured starting at 12 hours. The JF1, JF3, and NF groups showed a rapid increase after 60 hours, stabilizing by 84 hours. The JF2 group exhibited significant differences ($p < 0.05$) in liquefying power compared to the JF1, JF3, and NF groups after 36 hours. At 84 hours, the liquefying power stabilized at 1.34 ± 0.06 U for JF1, 2.11 ± 0.08 U for JF2, 1.81 ± 0.1 U for JF3, and 1.89 ± 0.07 U for NF (Figure 2B). Overall, the liquefying power followed an "S"-shaped curve with the order JF2 > NF > JF3 > JF1. Significant differences were observed between JF2 and the other groups ($p < 0.05$), as well as between JF1 and the other groups ($p < 0.05$).

4.3. Changes in Saccharifying Power

In the fermentation of Jianqu by single fungal species, the saccharifying power of the amylase from *Absidia corymbifera* (MF1), *Lichtheimia ramosa* (MF2), and *Mucor circinelloides* (MF3) exhibited an overall "S"-shaped curve. The saccharifying power was measured starting at 12 hours of fermentation. A rapid increase in activity was observed after 48 hours, with the MF2 group showing significant differences ($p < 0.05$) compared to the MF1 and MF3 groups after 72 hours. By 84 hours, the saccharifying power stabilized at 331 ± 9 U for MF1, 390 ± 13 U for MF2, and 293 ± 11 U for MF3 (Figure 2C), indicating the order MF2 > MF1 > MF3. Significant differences were observed between MF2 and the other two groups ($p < 0.05$), as well as between MF1 and MF3 ($p < 0.05$).

In the composite fermentation of Jianqu, composite fermentation groups (JF1, JF2, and JF3) and a natural fermentation group (NF group) were set up. The ratios of *yeast*, *Absidia corymbifera*, *Lichtheimia ramosa*, *Mucor circinelloides*, and *Monascus purpureus* in the composite fermentation groups were as follows: JF1 group, 4:3:2:2:1; JF2 group, 2:4:3:2:1; JF3 group, 2:3:4:2:1. The yeast component was a mixture of three different yeasts. Saccharifying power could be detected after 12 hours, increased rapidly after 48 hours, and stabilized by 84 hours. The saccharifying power of JF1, JF2, JF3, and NF were 296±8 U, 398±5 U, 372±7 U, and 366±8 U, respectively (Figure 2D). Overall,

an "S"-shaped curve was observed, with the order JF2 > JF3 > NF > JF1. Significant differences in saccharifying power were observed between JF2 and the other groups ($P<0.05$), as well as between JF1 and the other groups ($P<0.05$).

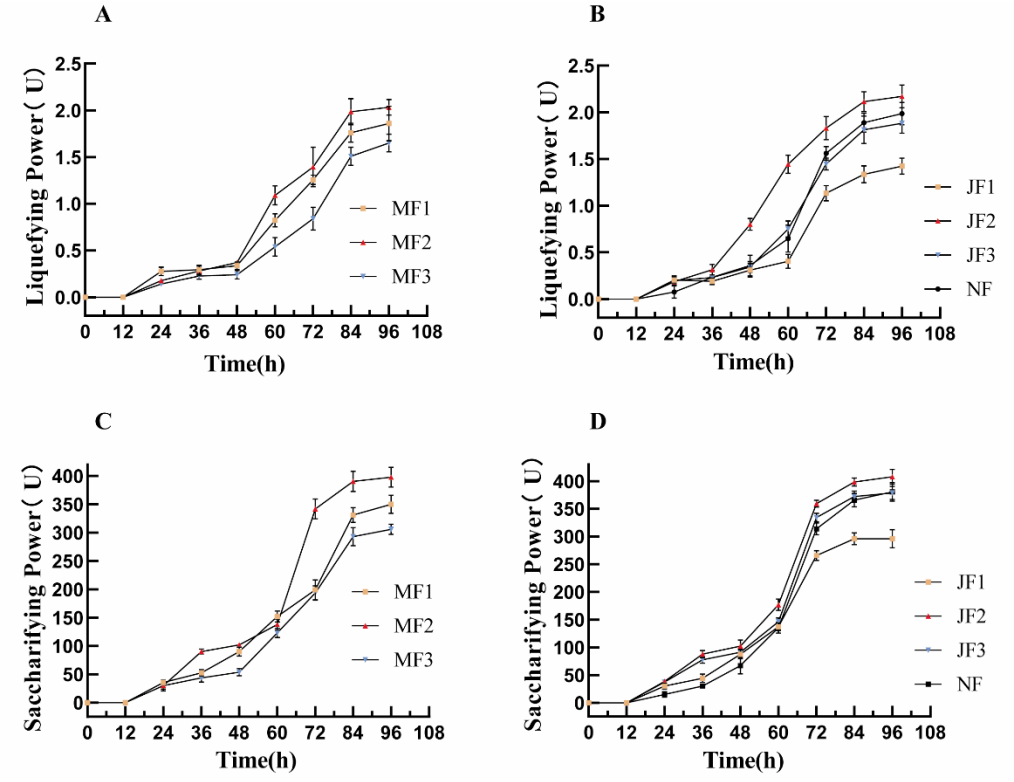

Figure 2. Changes in liquefied and saccharified amylase activities in different fermentation groups

(A) Liquefying Power changes in single-strain fermentation.; (B) Changes in liquefying Power during dominant strains directed fermentation; (C) Changes in saccharifying Power during single-strain fermentation; (D) Changes in saccharifying Power during dominant strains directed fermentation. MF1, MF2, and MF3 represent fermentations inoculated with *Absidia corymbifera*, *Lichtheimia ramosa*, and *Mucor circinelloides*, respectively.JF1, JF2, and JF3, represent directed fermentation with three different combinations. The combined proportions of isolated strains, including *yeast* (*Saccharomycopsis fibuligera*, *Millerozyma farinose*, *Hyphopichia burtonii* were mixed in a ratio of 2: 1: 1), *Absidia corymbifera*, *Lichtheimia ramosa*, *Mucor circinelloides*, and *Monascus purpureus*, were 4:3:2:2:1 for JF1, 2:4:3:2:1 for JF2 and 2:3:4:2:1 for JF3, respectively. NF, the natural fermentation group.

5. DISCUSSION

The fermentation of Jiuqu (fermentation starter) has a history of thousands of years, characterized by exquisite fermentation techniques and a diverse range of fungal species that interact and influence each other [10]. The enzymes and microorganisms involved in fermentation [11-14] are regarded as the "soul of liquor" [15]. Jianqu is a further development based on Jiuqu [16]. Compared to Jiuqu, Jianqu incorporates various Chinese medicinal herbs during fermentation, not only retaining the fermentation characteristics of Jiuqu but also endowing it with unique properties and certain medicinal effects. The optimal fermentation time and temperature for Jianqu can be determined by monitoring the activity changes of amylase, protease, and lipase [17]. Therefore, this study focuses on detecting the liquefying and saccharifying amylase activities in Jianqu. Liquefying amylase, also known as α-amylase or 4-dextrinase, is systematically named 1,4-α-D-glucan glucanohydrolase (1,4-α-D-Glucan maltohydrolase). Liquefying amylase can hydrolyze the internal α-1,4-glycosidic bonds

of starch, producing dextrins, oligosaccharides, and monosaccharides as hydrolysis products. After enzymatic action, the viscosity of gelatinized starch rapidly decreases, converting it into liquefied starch. The ability to liquefy starch is referred to as liquefying power [18]. Saccharifying amylase, also known as glucoamylase, is conventionally named and scientifically referred to as α-1,4-glucan glucohydrolase (α-1,4-Glucan glucohydrolace). It generally has no toxic or side effects [19] and is widely used in industries such as alcohol production, starch sugar, monosodium glutamate, antibiotics, citric acid, beer, as well as in the brewing of baijiu, huangjiu, and qujiu. Given the importance of saccharifying and liquefying amylases in Jianqu, we can determine the fermentation quality standards and identify the fermentation endpoint by monitoring the changes in the activities of liquefying and saccharifying amylases.

Jianqu is composed of 23 Chinese medicinal herbs as the substrate and undergoes fermentation under open natural conditions without restricting microbial participation. During the fermentation process, the product exhibits the characteristic of "ubiquitous white mold with a fragrant alcoholic aroma," but there is a lack of clear qualitative standards for determining the fermentation endpoint. Xiong Huan [20] and colleagues discovered the potential risk of aflatoxin contamination during the open fermentation of Jianqu. Therefore, based on preliminary high-throughput sequencing, this study reinoculated the dominant strains from natural fermentation—*Absidia corymbifera*, *Lichtheimia ramosa*, and *Mucor circinatus*—onto sterile Jianqu substrates to observe mold growth and amylase activity. The results showed that these strains grew well, and the enzymatic reaction exhibited an "S"-shaped curve. This study investigated the effects of restricted fermentation by inoculating dominant strains in different proportions. The results showed that the optimal fermentation effect was achieved when the ratio of *yeast*, *Absidia corymbifera*, *Lichtheimia ramosa*, *Mucor circinatus*, and *Monascus purpureus* was 2:4:3:2:1. Specifically, the *yeast* component consisted of *Saccharomycopsis fibuligera*, *Millerozyma farinosa*, and *Hyphopichia burtonii* in a ratio of 2:1:1. The enzymatic reaction of this combination exhibited an "S"-shaped curve [5], and the activities of liquefying and saccharifying amylases were significantly superior to those of natural fermentation. This study demonstrates that restricted composite fermentation effectively optimizes the fermentation of Jianqu, laying an important foundation for shortening fermentation time and improving product quality.

6. CONCLUSIONS

The fermentation process of Jianqu can be conducted in a closed and controlled environment. By utilizing the restricted composite fermentation of *yeast* and *molds*, the directional transformation of fermentation products can be achieved. The determination of the fermentation endpoint is primarily based on the following two aspects: first, observing the apparent characteristics of the fermentation product, such as "ubiquitous white mold with a fragrant alcoholic aroma"; second, detecting comprehensive indicators such as the liquefying and saccharifying power of amylase. This judgment method, which combines apparent characteristics with biochemical indicators, not only accurately controls the fermentation process but also provides an important basis for shortening fermentation time and ensuring fermentation safety.

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