Analysis of Saccharification Products of High-Concentration Glutinous Rice Fermentation by *Rhizopus nigricans* Q3 and Alcoholic Fermentation of *Saccharomyces cerevisiae* GY-1

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**ABSTRACT:** A two-stage process was used to prepare rice alcohol, i.e., saccharification of glutinous rice by *Rhizopus nigricans* Q3, followed by *Saccharomyces cerevisiae*’s fermentation. *Rhizopus nigricans* Q3 was cultured during the saccharification fermentation, and *Saccharomyces cerevisiae* GY-1 was added in the fermentation stage. Total sugar content and reducing sugar content in these two stages were analyzed. The relationship between the production proportion and consumption of the reducing sugar in the saccharification interval was analyzed using reducing sugar indices. It is an important rule that the high-concentration syrup and oligosaccharides prepared by glutinous rice could reach 42°Bx and 250 mg/mL by high-concentration fermentation in the growth stage of *R. nigricans* Q3.

1. INTRODUCTION

Rice alcohol\(^1\)\(^−\)\(^3\) is a kind of traditional fen-flavor liquor in China formed by the fermentation of *Rhizopus* and yeast. Two typical traditional manufacturing modes can be used: (a) cultivation and saccharification of the raw material followed by fermentation and (b) simultaneous fermentation and saccharification.\(^4\) Given that starch materials are the primary raw materials, sugar preparation is a relatively crucial production stage. The concentration of reducing sugar (RS) in the fermented mash influences the growth and reproduction of microorganisms. A large number of fungi have been used in functional screening continuously. The typical microorganisms in rice wine are molds and saccharomyceses.\(^5\)\(^,\)\(^6\) Molds often use *Rhizopus*, *Mucor*, *Aspergillus flavus*, *A. niger*, and *Absidia orchidis* for saccharification.\(^7\)\(^,\)\(^8\) *Rhizopus* can secrete a large amount of amylase and glucoamylase for hydrolyzing starch polysaccharides.\(^9\)\(^,\)\(^10\) *Rhizopus* can cut α-1,4 and α-1,6 bonds in raw starch, thereby transforming starch into fermentable sugar. *Rhizopus* can grow in culture media with low water content because it simultaneously undergoes growth and saccharification.\(^11\)\(^,\)\(^12\) It is used to prepare rice liquor and syrup\(^11\) via solid-state and semi-solid-state methods. Moreover, *Saccharomyces cerevisiae* can also produce a series of zymases to hydrolyze micromolecular monosaccharides into alcohol.\(^13\)\(^,\)\(^14\)

Solid-state fermentation (SSF)\(^15\)\(^,\)\(^16\) refers to the biological reaction of one or multiple microorganisms in a water-insoluble matrix with absolute humidity when no or hardly any free-flowing water is present.\(^18\)\(^−\)\(^20\) SSF is a critical process for *Rhizopus*’ growth, and it can produce many fermentable sugars.\(^21\)\(^,\)\(^22\) The content of the produced reducing sugar is high, which is beneficial for the preparation of alcohol.

Glucoamylase was used in the preparation stage of reducing sugar for amylolysis into micromolecular sugars,\(^23\) and a large amount of reducing sugar is related to the starch concentration in raw materials.\(^24\) When the material–water ratio was higher than 1:1 and the water content in materials was lower than 50%, no free water was observed in the whole reaction system, restricting the enzymatic activity.

Based on the characteristics of SSF, high-concentration syrup was prepared from the growth characteristics of *Rhizopus*. Obtaining high-concentration alcohol fermentation liquor from the fermentation of high-concentration syrup can decrease energy consumption and increase its quality. In this study, glutinous rice was used as a raw material for the saccharification and alcohol fermentation stages. The saccharification capability of *Rhizopus nigricans* Q3 and the alcohol production capability of *S. cerevisiae* GY-1 were analyzed. Saccharifying power, hydrolysis capacity of oligosaccharides,
and alcohol production were analyzed to provide a theoretical reference for preparing high-concentration alcohol and syrup mash based on the SSF of rice liquor.

2. MATERIALS AND METHODS

2.1. Experimental Materials. 2.1.1. Microorganism. Test-tube agar slant of R. nigricans Q3 and S. cerevisiae GY-1 were stored in the Microorganism Engineering Research Center of Yunnan Normal University.

2.1.2. Raw Materials. The raw materials were glutinous rice and potato. The glutinous rice purchased from a supermarket and the potato from the Chenggong Wujiaying Market in Kunming City.

2.2. Experimental Methods. 2.2.1. Determination of Reducing Sugar (RS). Each RS sample was collected and placed into three test tubes with pistons. Filters of the centrifuged fermented glutinous rice liquid (0.2 mL) and distilled water (1.8 mL) were added into the test tubes and evenly shaken. The determination method of reducing sugar was the DNS method.\textsuperscript{25} Data were substituted into the standard curve to calculate the content of the reducing sugar of glutinous rice liquid.

2.2.2. Determination of Soluble Total Sugar (STS). Sugar liquor in the culture solution was centrifuged, and liquid supernatant was used to test the sugar degree with an Abbé refractometer, and the readings were recorded.

2.2.3. Determination of Nonreducing Oligosaccharides (NRO). The content of nonreducing oligosaccharides was equal to STS content minus the reducing sugar content

\[ \text{NRS (mg/mL)} = \text{STS} \times 10 - \text{RS} \]

2.2.4. Determination of Alcohol. Liquid R. nigricans Q3 seeds were inoculated in a glutinous rice solid medium and cultured at 30 °C for 72 h, after which the fermented rice formed a certain concentration of the sugar solution by saccharification. Yeast inoculated into the mash for alcoholic fermentation for 60 h. First, 200 mL of the alcohol fermentation liquid was placed into a small glass distillation unit, 100 mL of distilled liquid was collected, the alcohol concentration was measured with a specific gravity alcoholometer, and the readings were recorded.

\[ \text{alcohol content} \% = \frac{\text{alcohol reading}}{2} \]

2.2.5. Preparation of Liquid R. nigricans Q3 Seeds. Potato (150 g, peeled and ground), maltose (10 g), and distilled water (500 mL) were mixed and stirred into serous fluid. Next, 200 mL of liquid was collected and placed in a 500 mL triangular flask. The flask was sealed for sterilization at 121 °C for 30 min and cooled to 30 °C, and inoculated test-tube agar slants of R. nigricans Q3, hypha, and spores were added into the flask and then placed on a 30 °C shaking table and cultured for 32 h at 180 rpm.

2.2.6. Preparation of the S. cerevisiae Seed Solution. Glucose (10 g), peptone (10 g), and yeast extract (5 g) were evenly mixed with 500 mL of distilled water, and the pH adjusted to 6.0. Next, the solution was transferred to 250 mL triangular flasks, with 100 mL in each flask. The flasks were sealed for sterilization and cooled to inoculate the test-tube agar slant of S. cerevisiae GY-1 and then placed on a 30 °C plate and cultured for 48 h at 180 rpm.

2.3. Analysis of R. nigricans Q3 in the Saccharification Stage. Eight stainless-steel pots (v = 2 L) were used, to which 30, 60, 90, 120, 150, 180, 210, and 240 g of glutinous rice were added. In addition, 300 mL of single distilled water evenly mixed into each pot, and glutinous rice formed at concentrations of 9, 16.7, 23, 28.6, 33.3, 37.5, 41.2, and 44.4%, respectively. All pots were covered, marked, and then sterilized for 45 min in a 118 °C pressure steam autoclave. Next, the mixtures were cooled, and a 10% (mass fraction of glutinous rice) R. nigricans Q3 seed solution (3, 6, 9, 12, 15, 18, 21, and 24 mL) was inoculated. Glutinous rice and R. nigricans Q3 were mixed completely, and the mixtures were sealed with a plastic film. The mixtures were cultured in a 30 °C constant-temperature incubator. The samples were collected at 18, 30, 41, 45, 49, and 53 h to test the pH, STS, and RS in the fermented liquid.

2.4. Analysis of S. cerevisiae GY-1 in the Fermentation Stage. After 53 h of saccharification, the pH of glutinous rice hydrolysate was adjusted to 6.0. The yeast seed solution was inoculated to 5% of the total weight of the liquid. The mixture was mixed evenly to allow complete contact between the glutinous rice mash and S. cerevisiae GY-1. The mixture was sealed with a piece of plastic film and cultured continuously in a 30 °C constant-temperature incubator. The pH, reducing sugar content, and total sugar content were measured at 0, 12, 18, 24, 36, 42, 48, 60, 66, 72, and 84 h, and the alcohol content in the fermentation liquor was measured after 84 h.

2.5. Statistical Analysis. The results are the means of three biological replicates, and the error bars indicate standard deviations. The statistical significance of the results was evaluated using Student’s t-test. Correlation analysis was performed using Spearman’s correlation analysis (SPSS 19.0). Origin 8.5 software was used for plotting and data processing.

3. RESULTS AND DISCUSSION

3.1. Changes in the pH of the Fermentation Liquor. There is a traditional method in China to prepare baijiu with rice. Since different raw materials are used, the flavor substances of the alcohol produced are different. The quantities of flavor substances formed by solid fermentation are higher than those formed during liquid fermentation of glutinous rice. Glutinous rice contains a large amount of amylpectin, having the characteristics of high viscosity after steaming. Glutinous rice starch amylpectin increases the viscosity of the liquid during liquid fermentation. Thus, low-moisture-content solid-state methods are often used initially, during which a considerable amount of glutinous rice is consumed. The hydrolysis of soluble polysaccharides causes the small molecular sugars’ ratio to increase gradually. With the consumption of a large amount of glutinous rice, the hydrolysis of soluble polysaccharides and the water molecule ratio’s metabolism increased gradually, and the solid stage changed to the liquid stage. As a result, the Rhizopus and yeast metabolic process produced a large amount of organic acid.\textsuperscript{26–28} Consequently, the higher acid concentration increased the [H⁺] proportion gradually, which was determined by the fermented liquid’s acid content.

pH monitoring at the saccharification stage helps to understand the growth status of Rhizopus and its ability to produce organic acids. In the fermentation process of yeast, the attention to pH is conducive to analyzing the strength of yeast-making alcohol and further understanding the analysis of alcohol and organic acid esterification analysis to determine the
strength of flavor substances in the fermentation broth. Because of the production of organic acids, ethanol can carry on the esterification reaction to produce ester substances.\textsuperscript{29,30} pH monitoring can directly judge the utilization of glutinous rice and indirectly evaluate \textit{Rhizopus}' and yeast’s growth status.

Figure 1 shows that 0–53 h is the saccharification stage and 54–140 h is the alcohol fermentation stage. The pH gradually declined as the different concentrations of glutinous rice were cultured. A large amount of organic acid was produced during the saccharification of glutinous rice by \textit{R. nigricans} Q3, resulting in a reduction in the pH in the fermentation liquor of glutinous rice. Meanwhile, high free water content was observed during fermentation with a low material concentration, which was beneficial for the catalysis of glucoamylase and the diffusion of [H\textsuperscript{+}]. A large number of studies have reported that the activity of glucoamylase is optimal under the condition of pH 4–6.\textsuperscript{31,32} The fermentation liquor’s pH values with 37.5, 41.2, and 44.4% glutinous rice contents became stable at approximately 4.0. Based on comparing the different glutinous rice concentrations in the saccharification stage, the acid production ability of \textit{R. nigricans} Q3 with 9% glutinous rice was the highest, and the pH reached 2.6. In the study of the two-stage process, yeast’s acid production ability on the reducing sugar was analyzed, and \textit{S. cerevisiae} GY-1 inoculated after 53 h. As time passed, the pH gradually declined. The decreases in the pH at different concentrations of glutinous rice fermentation liquor slowed and became stable after 120 h. In the whole alcohol fermentation stage, the fermentation liquor’s pH values with 10, 20, and 30% glutinous rice rapidly decreased and were lower than 4.0. The fermentation liquor’s pH with 9% glutinous rice was 3.77, and that of the fermentation liquor with over 28.6% glutinous rice was higher than 4.0. \textit{S. cerevisiae} GY-1 with a low water content was still beneficial for the development of \textit{R. nigricans} Q3. After saccharification for 53 h, the contents of RS at 37.5, 41.2, and 44.4% concentrations of glutinous rice rapidly decreased and were lower than 4.0. The fermentation liquor’s pH with 9% glutinous rice was 3.77, and that of the fermentation liquor with over 28.6% glutinous rice was higher than 4.0. \textit{S. cerevisiae} GY-1 with a low water content was still beneficial for the development of \textit{R. nigricans} Q3. After saccharification for 53 h, the contents of RS at 37.5, 41.2, and 44.4% concentrations of glutinous rice still increased. This phenomenon indicated that \textit{Rhizopus} did not reduce the hydrolysis of starch due to the inhibition of RS concentration in the fermentation process. Therefore, it is feasible to prepare high-concentration syrup by \textit{Rhizopus}.

\textit{S. cerevisiae} GY-1 was added into the saccharifying glutinous rice liquid at 53 h. RS in the glutinous rice was consumed by \textit{S. cerevisiae} GY-1, thereby gradually decreasing its content. The content of RS at 28.6–44.4% glutinous rice concentrations increased to different extents 12 h after the addition of \textit{S.cerevisiae} GY-1. The consumption of reducing sugar by yeast stage. The saccharification stage is a process during which \textit{R. nigricans} Q3 produces saccharifying enzymes and hydrolyzed glutinous starch. As saccharification continued, the content of RS exhibited an increasing trend. With an increase in the concentration of glutinous starch, RS also increased. From Figure 2, the range of RS at a 9% glutinous rice concentration was the lowest, whereas the content of RS at a continuously 33.3–44.4% glutinous rice concentration was the highest. \textit{R. nigricans} Q3 initiated the simultaneous growth and saccharification by secreting glucoamylase from growing hypha cells. Therefore, a high concentration of glutinous rice at a low water content was still beneficial for the development of \textit{R. nigricans} Q3. After saccharification for 53 h, the contents of RS at 37.5, 41.2, and 44.4% concentrations of glutinous rice still increased. This phenomenon indicated that \textit{Rhizopus} did not reduce the hydrolysis of starch due to the inhibition of RS concentration in the fermentation process. Therefore, it is feasible to prepare high-concentration syrup by \textit{Rhizopus}.

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was lower than the production of RS from the saccharification of R. nigricans. The RS at 9–23% glutinous rice concentrations reached 0.38 mg/mL at 84 h, whereas the reducing sugar content at 33.3–44.4% glutinous rice concentrations was 149.7 mg/mL.

3.3. Changes in STS and NRO. The STS in this experiment refers to the soluble, fermentable sugars in
glutinous rice. TSS content includes monosaccharides and macromolecular sugar. In this study, a saccharimeter was used for determining STS. The solid-state model’s efficiency for preparative oligosaccharides was analyzed based on the STS content variations.

Figure 3 shows that the STS content at different concentrations of glutinous rice continuously increased in the saccharification stage (0–53 h). However, STS’s variation curves at 9 and 16.7% glutinous rice concentrations were relatively flat, whereas the slope of the variation curve of the STS at 23–44.4% glutinous rice concentrations increased gradually. These results showed that glutinous rice starch was continuously decomposed into RS and oligosaccharides in the saccharification stage. Moreover, inadequate water content at high concentrations of glutinous rice is disadvantageous for enzyme reactions in the brewing process of glutinous rice, which refers to the alcohol fermentation stage from 53 to 150 h. Given that abundant reducing sugar was decomposed and utilized to form alcohol by yeast, the total sugar concentration decreased continuously, and the nonreducing oligosaccharides (NRO) were decomposed accordingly due to the exhaustion of the reducing sugar. NRO content in the solution was high during the 0–53 h period (Figure 4). In the yeast’s fermentation stage (54–150 h), the soluble oligosaccharides decomposed significantly.

3.4. Analysis of Alcohol Production Capacity. Figure 5 shows that at 84 h of S. cerevisiae GY-1 fermentation, the hydrolyzed glutinous rice’s alcohol concentration with glutinous rice concentrations of 23.1 and 28.6% was higher than that of the other hydrolyzed rice, i.e., 9.5 and 8.7%, respectively. By comparing RS, it was found that RS consumption is related to the generation of alcohol. RS consumption in Figure 5 influences the yield of alcohol and is positively correlated. After comparing the production rate of reducing sugar to produce alcohol, it was found that the output of alcohol increased with increasing glutinous rice concentration. When the glutinous rice concentration is 44.4%, a downward trend mainly attributed to the high sugar content formed effect in yeast’s alcohol metabolism is shown. With increasing glutinous rice concentration, Rhizopus’s growth mode goes through the liquid and solid states. The raw materials were saccharified during the growth process, leading to an increase in reducing sugar with an increasing glutinous rice concentration.

The culture medium’s water content will strongly affect the alcohol yield in liquid fermentation and solid-state fermentation. In solid-state fermentation mode, the glutinous rice concentration is 41.2% (the water content of 58.8%), which can produce a significant amount of RS and oligosaccharide in the Rhizopus growth saccharifying stage; inoculated yeast and a large amount of alcohol are prepared by fermenting reducing sugar with yeast growth. In liquid fermentation, a glutinous rice concentration of 28.6% can produce 220 mg/mL RS; the yeast can convert this RS into alcohol in more than 80 h, well below the solid-state fermentation time. In Figure 6, the conversion degree of RS at a 9% concentration of glutinous rice was the highest, i.e., a 73% conversion degree of glutinous rice. The RS could fully ferment with yeast in a 91% aqueous solution. With the increase in the glutinous rice concentration, the water content in the reaction system decreased. Much of the glutinous rice formed a viscous liquid, which reduced the saccharification efficiency and weakened the glutinous rice’s transformation ability into RS. The production capacity of RS was negatively correlated with glutinous rice concentration.

Experiments on glutinous rice for alcohol production using R. nigricans Q3 and S. cerevisiae GY-1 in the SSF study were carried out using the two-stage method, namely, the saccharification and alcohol fermentation stages. The SSF of R. nigricans Q3 growth in high-concentration thick glutinous rice was carried out. Given that the hypha growth inhibited to some extent, it accelerated glucoamylase’s secretion, which degraded the glutinous rice starch into more useful sugar.

4. Conclusions

In this study, R. nigricans Q3 was used to degrade the glutinous rice to prepare soluble oligosaccharides, and S. cerevisiae GY-1 was used to test the alcohol-producing capacity of the sugar solution. The variation trends of the reducing sugar, soluble total sugar, and oligosaccharide produced by R. nigricans Q3 were analyzed. According to Figures 2–4 and 6, the contents of RS, oligosaccharide, and STS formed in the glutinous rice solution increased with the increase of the glutinous rice concentration. However, in terms of the raw materials’ utilization rate, the conversion degree of low-concentration glutinous rice was higher. The conversion degree of a 9% glutinous rice concentration was much higher than those of 16.7–44.4% concentrations. The results demonstrated that the utilization of raw materials and the alcohol production capacity were the highest when the material concentration was 16–23%. Under this concentration range, R. nigricans Q3 saccharifies to form total soluble sugar and reducing sugar amounts of 150–200 and 125–175 mg/mL, respectively.

The SSF of Rhizopus for preparing high-concentration soluble syrup is effortless and led to a sugar degree higher than 42°Bx. It is also a technique used in sugar refineries that can reduce environmental pollution. In some studies, for the rice liquor fermentation industry, the osmotic pressure capacity and production requirements of S. cerevisiae GY-1 in high-concentration sugar liquor were considered. Thus, in liquid fermentation processes, the high-concentration sugar liquid must be diluted with water into 20–25°Bx syrup suspensions. Next, S. cerevisiae GY-1 was added for alcohol fermentation to prepare sweet rice wine or distilled baijiu.
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Notes
The authors declare no competing financial interest.

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