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FOOD MICROBIOLOGY

Synergistic Effect of Multiple Saccharifying Enzymes on Alcoholic Fermentation for Chinese Baijiu Production

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ABSTRACT Chinese *Jiuqu* (fermentation starter) provides saccharifying enzymes for baijiu (Chinese liquor) fermentation, which undergoes a simultaneous saccharification and fermentation process. However, the key saccharifying enzymes associated with alcoholic fermentation from *Jiuqu* and their effects on ethanol production remain poorly understood. In this study, we identified 51 carbohydrate hydrolases in baijiu fermentation by metaproteomics analysis. Through source-tracking analysis, approximately 80% of carbohydrate hydrolases in the baijiu fermentation were provided by *Jiuqu*. Among these enzymes, alpha-amylase (EC 3.2.1.1) and glucoamylase (EC 3.2.1.3), from *Aspergillus*, *Rhizomucor*, and *Rhizopus*, were positively related to starch hydrolysis and ethanol production, indicating that they were the key saccharifying enzymes associated with alcoholic fermentation in the baijiu fermentation. Moreover, a combined mixture of alpha-amylase and glucoamylase (in a ratio of 1:6, wt/wt) enhanced ethanol production in a simulative baijiu fermentation under laboratory conditions. This result revealed a synergistic effect of multiple saccharifying enzymes on ethanol production in baijiu fermentation. Our study provides a potential approach to improve the efficiency of saccharification and alcoholic fermentation by optimizing the profile of saccharifying enzymes for fermentation of baijiu and other beverages.

IMPORTANCE *Jiuqu* starter provides enzymes to the simultaneous saccharification and fermentation process of baijiu (Chinese liquor) production; however, the key saccharifying enzymes associated with alcoholic fermentation from *Jiuqu* and their effects on ethanol production remain unclear. We confirmed that *Jiuqu* was the main source of carbohydrate hydrolases for baijiu fermentation and identified two types of saccharifying enzymes from multiple microbes as the key enzymes associated with alcoholic fermentation. Moreover, a proper combination of multiple saccharifying enzymes could enhance ethanol production in baijiu fermentation. This combination provides an approach to optimize the profile of saccharifying enzymes for enhancing ethanol production in baijiu and other food fermentations.

KEYWORDS *Jiuqu*, saccharifying enzyme, synergistic effect, ethanol, baijiu, Chinese liquor

Baijiu (Chinese liquor), one of the oldest distilled liquors in the world, is generated by simultaneous saccharification and fermentation of cereals (sorghum, wheat, rice, corn, etc.) (1–3). This unique, complex process always selects *Jiuqu* (a mixed starter including *Daqu*, *Xiaoqu*, and *Fuqu*) as the saccharifying and fermenting agent (4). *Jiuqu*, composed of abundant microorganisms and enzymes, determines the hydrolysis of cereal-derived macromolecular nutrients in baijiu fermentation (1–3). This hydrolytic process determines the acquisition of available nutrients (like fermentable sugars) and

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subsequently influences microbial metabolism and the formation of flavor in baijiu (5–10). Therefore, it is important to identify the key saccharifying enzymes associated with alcoholic fermentation in *Jiuqu* and study their effects on ethanol production for improving baijiu fermentation (11, 12).

Jiuqu provides microorganisms and enzymes to baijiu fermentation and significantly contributes to ethanol and flavor compound generation (13, 14). Many studies revealed the structure and composition of microbial communities in *Jiuqu* (15–20). Furthermore, source-tracking analysis indicates that *Jiuqu* provides approximately 10% to 20% of the bacterial communities and 60% to 80% of the fungal communities to the baijiu fermentation (5, 21). Meanwhile, *Jiuqu* also provides abundant enzymes (starch-hydrolyzing enzymes, proteinases, cellulases, phases, and so on), accumulated from *Jiuqu* preparations, to the baijiu fermentation (15–20). However, the key saccharifying enzymes associated with alcoholic fermentation from *Jiuqu* remain unclear. In addition, starch hydrolysis determines the acquisition of fermentable sugars in baijiu fermentation and requires the sequential catalysis of multiple enzymes as a result of complex physical structure and modifications (22). Previous studies revealed the profile of saccharifying enzymes in *Jiuqu* and further indicate that the saccharifying activity of *Jiuqu* influences starch hydrolysis and metabolite formation in baijiu fermentation (6, 7, 19). However, the most efficient mode of saccharifying enzymes for enhancing ethanol production remains poorly understood. As a result, it is important to reveal the key saccharifying enzymes and study their effects on ethanol production for improving baijiu fermentation.

Here, we selected a typical *Jiuqu* (classified as *Xiaoqu*) as the subject and used enzymatic source-tracking analysis and multivariate statistical analysis to identify the key saccharifying enzymes associated with alcoholic fermentation from *Jiuqu*. Moreover, we revealed the metabolic features of the key saccharifying enzymes on ethanol production in a simulative baijiu fermentation under laboratory conditions. Our study sheds new light on the effects of multiple saccharifying enzymes on ethanol production. Furthermore, our study also provides a potential approach to optimize the profile of saccharifying enzymes for enhancing ethanol production in baijiu fermentation in particular and food fermentations in general.

RESULTS

Enzyme profile in baijiu fermentation. Saccharifying activity increased from 1,910 $\pm$ 58.02 to 2,031 $\pm$ 51.18 U/g dry weight (DW) in the baijiu fermentation from days 0 to 3 and then decreased to 1,649 $\pm$ 69.20 U/g DW at the end of fermentation. (Fig. 1a).

We used a label-free quantitative proteomics approach to study the enzyme profile in the baijiu fermentation (*Jiuqu*; samples from the baijiu fermentation at days 0 and 3). Among 499,255 spectra obtained, 97,198 (19.47%) identified spectra were assigned to 15,228 peptides and 5,311 proteins. After filtering, we retained 2,373 potential nonredundant protein groups (see Tables S1 and S2 in the supplemental material).

According to Clusters of Orthologous Groups (COG) annotations, 1,819 proteins were classified into 21 categories, covering information storage and processing, cellular processes and signaling, metabolism, and so on (Fig. 1b and Table S3). Approximately 60% of all proteins were related to metabolism, including carbohydrate transport and metabolism, energy production and conversion, amino acid transport and metabolism, and so on. Three hundred thirty-eight proteins were related to carbohydrate transport and metabolism, the most active category in the baijiu fermentation (Fig. 1b and Table S3). Furthermore, we identified 89 carbohydrate hydrolases in *Jiuqu* and the baijiu fermentation, including 30 glycoside hydrolases (GHs), 15 glycosyltransferases (GTs), 11 carbohydrate esterases (CEs), 15 auxiliary activity enzymes (AAs), and 18 carbohydrate-binding module enzymes (CBMs) (Fig. 1c and Table S4).

Source-tracking analysis of carbohydrate hydrolases and their affiliated microbes in baijiu fermentation. We identified 51 carbohydrate hydrolases in the baijiu fermentation at day 3 (Fig. 2a and Table S4). Among them, up to 40 enzymes appeared

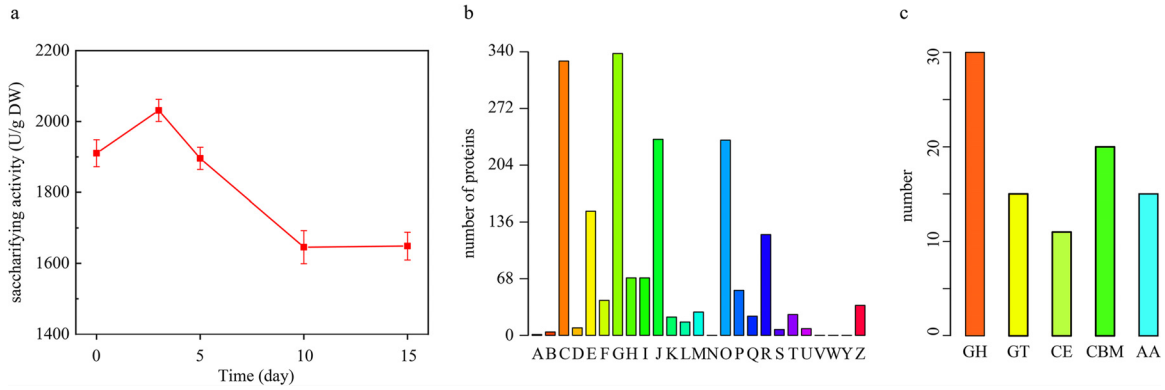

FIG 1 Enzyme profile in baijiu fermentation. (a) Dynamic of saccharifying activity in baijiu fermentation. (b) Identified protein functions in COG database annotation. A, RNA processing and modification; B, chromatin structure and dynamics; C, energy production and conversion; D, cell cycle control, cell division, and chromosome partitioning; E, amino acid transport and metabolism; F, nucleotide transport and metabolism; G, carbohydrate transport and metabolism; H, coenzyme transport and metabolism; I, lipid transport and metabolism; J, translation, ribosomal structure, and biogenesis; K, transcription; L, replication, recombination, and repair; M, cell wall/membrane/envelope biogenesis; N, cell motility; O, posttranslational modification, protein turnover, and chaperon functions; P, inorganic ion transport and metabolism; Q, secondary metabolites biosynthesis, transport, and catabolism; R, general function prediction only; S, function unknown; T, signal transduction mechanisms; U, intracellular trafficking, secretion, and vesicular transport; V, defense mechanisms; W, extracellular structures; Y, nuclear structure; Z, cytoskeleton. (c) Category of identified carbohydrate hydrolases in the baijiu fermentation. GH, glycoside hydrolase families; GT, glycosyltransferase families; CE, carbohydrate esterase families; AA, auxiliary activity families; CBM, carbohydrate-binding module families. Bar, numbers of proteins.

in both *Jiuqu* and the baijiu fermentation, including 6 AAs, 5 CBMs, 4 CEs, 16 GHs, and 9 GTs. Eight enzymes (six glycoside hydrolase and two glycosyltransferase families) appeared only in the baijiu fermentation, and the other three glycoside hydrolase families appeared only in the baijiu fermentation at day 3 (Fig. 2a). These enzymes were mainly affiliated with five genera, including *Lactobacillus*, *Aspergillus*, *Rhizomucor*, *Rhizopus*, and *Saccharomyces* (Fig. 2b and Table S4).

Furthermore, we used high-throughput sequencing analysis to trace the key microbes (identified as the main microbial source of enzymes by metaproteomics analysis) in the baijiu fermentation. We obtained 450,408 and 535,304 high-quality reads from bacterial 16S rRNA gene sequences and fungal internal transcribed spacer (ITS) regions in all 18 samples. We found an average of 25,023 reads for bacteria and 29,739 reads for fungi per sample. Bacterial and fungal diversity was analyzed with the sequences normalized to 14,624 and 18,753 reads (Table S5). Chao1 richness and Shannon index showed that bacterial and fungal diversity decreased gradually in the baijiu fermentation (Table 1). Furthermore, eight bacterial and seven fungal genera were abundant in *Jiuqu* or the baijiu fermentation (with relative abundance above 1%) (Table S6). Seven bacterial genera (*Acetobacter*, *Acinetobacter*, *Klebsiella*, *Lactobacillus*, *Pantoea*, *Pediococcus*, and *Weissella*) and six fungal genera (*Aspergillus*, *Candida*, *Pichia*, *Rhizopus*, *Saccharomyces*, and *Saccharomycopsis*) were dominant in the baijiu fermentation. Among them, *Lactobacillus*, *Aspergillus*, *Rhizomucor*, *Rhizopus*, and *Saccharomyces*, which were the main microbial sources of enzymes in the metaproteomics analysis, were also dominant in *Jiuqu* (Fig. 2c and Table S6).

Identification of key carbohydrate hydrolases in baijiu fermentation. Thirty glycoside hydrolases were classified into GH4, GH5, GH13, GH15, GH16, GH17, GH43, GH47, and GH109 families and were related to polysaccharide hydrolysis (Table S4). GH13 (alpha-amylase, EC 3.2.1.1) and GH15 (glucoamylase, EC 3.2.1.3) were related to starch hydrolysis and affiliated with *Rhizopus*, *Aspergillus*, and *Rhizomucor*. GH5 (cellulase, EC 3.2.1.4) was related to cellulose hydrolysis and affiliated with *Rhizopus*. GH16 [endo-1,3(4)-beta-glucanase, EC 3.2.1.6], GH43 (beta-xylosidase, EC 3.2.1.37), and GH47 (alpha-mannosidase, EC 3.2.1.113), affiliated with *Rhizopus*, were related to hemicellulose hydrolysis (23–25). Besides extracellular glycoside hydrolases, several intracellular glycosyltransferases were also related to polysaccharide hydrolysis. For example, GT35 (alpha-1,4 glucan phosphorylase, EC 2.4.1.1) was related to stored glycogen or oligo-

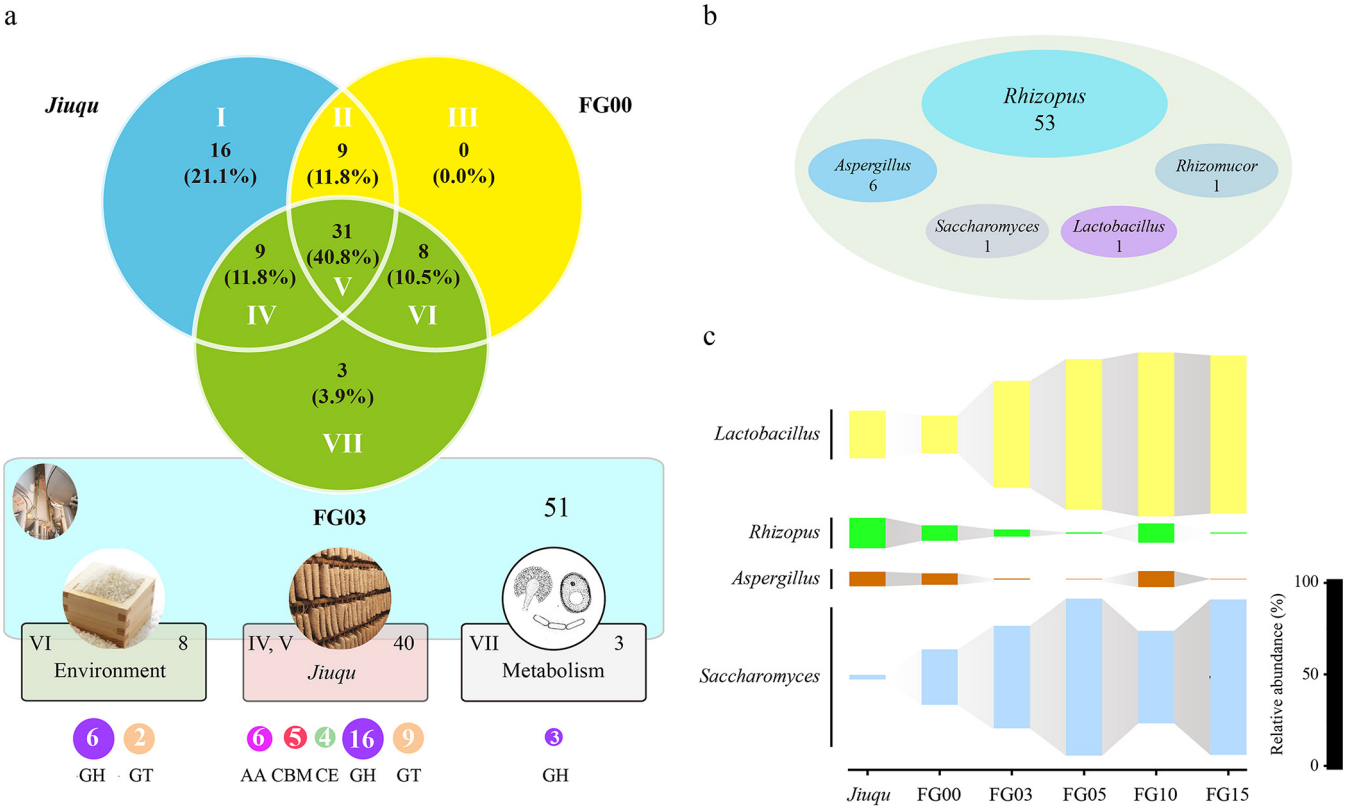

FIG 2 Overview of carbohydrate hydrolases in baijiu fermentation. (a) Source of carbohydrate hydrolases in the baijiu fermentation at day 3. Part I, numbers of proteins only in *Jiuqu*; part II, numbers of proteins in *Jiuqu* and fermented samples at day 0; part III, numbers of proteins only in fermented samples at day 0; part IV, numbers of proteins in *Jiuqu* and fermented samples at day 3; part V, numbers of proteins in *Jiuqu* and fermented samples at days 0 and 3; part VI, numbers of proteins in fermented samples at days 0 and 3; part VII, numbers of proteins only in fermented samples at day 3. Part VI of the fermented samples at day 3 was from the local environment (raw materials and others). Parts IV and V of fermented samples at day 3 was from *Jiuqu*. Part VII of fermented samples at day 3 was from microbial metabolism. (b) Microbial source of carbohydrate hydrolases. Numbers are statistics of proteins from specific genera. (c) Dynamic of microbes across *Jiuqu* and baijiu fermentation. Bar, relative abundance of specific genus across samples.

saccharide hydrolysis and was affiliated with *Rhizopus*. In addition, we also found several intracellular enzymes related to oligosaccharide or disaccharide hydrolysis. For example, GH4 (maltose-6-phosphate hydrolase, EC 3.2.1.122) was related to maltose hydrolysis and affiliated with *Lactobacillus* (Fig. 3a and Table S4).

We identified the key carbohydrate hydrolases in the baijiu fermentation, combined with the interactions among enzymes, microbes, and metabolites (based on Spear-

TABLE 1 Bacterial and fungal microbiota diversity index based on 16S rRNA and ITS amplicon sequencing across samples

Sample	No. of clean reads	*P* value	No. of OTUs	*P* value	Goods' coverage	*P* value	Chao1 richness	*P* value	Shannon diversity index	*P* value
Bacteria										
Jiuqu	33,649 ± 2,301	0.07	236 ± 6	<0.001	0.9942 ± 0.0002	0.04	312.00 ± 8.99	<0.001	3.56 ± 0.25	<0.001
FG00	29,540 ± 8,984		236 ± 11		0.9943 ± 0.0004		299.76 ± 8.67		4.08 ± 0.03	
FG03	25,931 ± 11,564		130 ± 32		0.9956 ± 0.0012		213.1 ± 30.28		3 ± 0.14	
FG05	18,131 ± 1,901		133 ± 2		0.9959 ± 0.0004		207.24 ± 41.49		2.1 ± 0.08	
FG10	19,965 ± 1,478		117 ± 14		0.9962 ± 0.0004		171.32 ± 18.26		1.69 ± 0.12	
FG15	22,921 ± 2,524		188 ± 63		0.9939 ± 0.0018		312.69 ± 49.05		2.51 ± 0.36	
Fungi										
Jiuqu	41,605 ± 2,501	0.02	57 ± 8	<0.001	0.999 ± 0.0001	<0.001	80.28 ± 15.3	<0.001	2.22 ± 0.09	<0.001
FG00	29,513 ± 2,957		79 ± 8		0.9989 ± 0.0002		92.91 ± 11.73		2.75 ± 0.27	
FG03	29,845 ± 3,834		46 ± 5		0.9991 ± 0.0002		70.8 ± 16.07		1.51 ± 0.07	
FG05	30,491 ± 11,378		32 ± 5		0.9995 ± 0		37.3 ± 3.72		0.66 ± 0.08	
FG10	24,952 ± 2,830		54 ± 16		0.9992 ± 0.0001		66.8 ± 10.61		2.25 ± 0.12	
FG15	22,029 ± 4,779		37 ± 2		0.9992 ± 0		52.63 ± 5.21		0.71 ± 0.11	

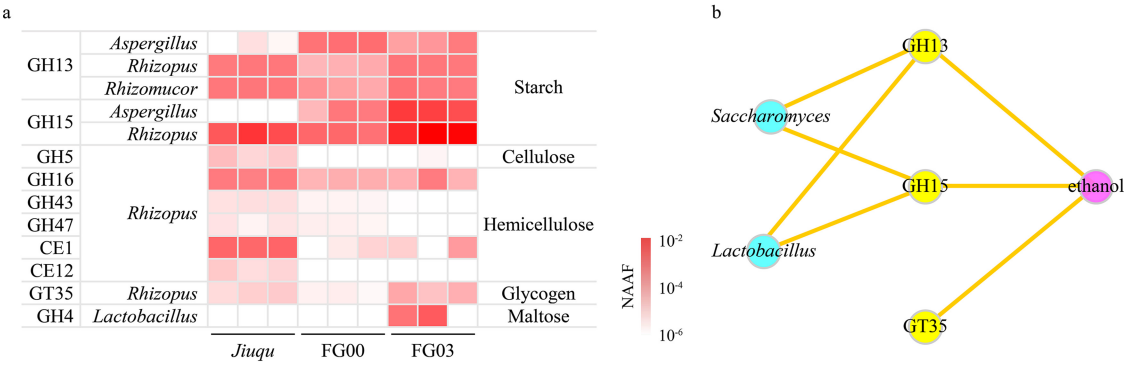

FIG 3 Identification of key carbohydrate hydrolases in baijiu fermentation. (a) Carbohydrate hydrolases associated with saccharides hydrolysis and their microbial sources. Heatmap indicates the expression of proteins across samples. (b) Relationships between enzymes and microbes or metabolites, based on the connection for a significant ($P < 0.05$) and strong correlation (Spearman's rho of >0.6). Yellow spots, enzymes; blue spots, microbes; purple spots, metabolites. Line, strong correlations between objects.

man's rank correlations of >0.6, $P < 0.05$). Here, GH13 and GH15 were positively correlated with the growth of *Lactobacillus* and *Saccharomyces* (Fig. 3b). Meanwhile, GH13, GH15, and GT35 were also positively correlated with ethanol production (Fig. 3b and Table S7).

Enzyme profile of isolated strains. We isolated functional strains from samples and studied their enzyme profiles under culture-dependent conditions (Fig. S4). A *Rhizopus microsporus* strain grew from 6.00 ± 0.75 to 8.81 ± 0.13 $\log_{10}$ (CFU/g DW), and the saccharifying activity increased from $1{,}962 \pm 112.8$ to $20{,}031 \pm 498.2$ U/g DW in the 8-day fermentation (Fig. S4a and b). A *Lactobacillus fermentum* strain grew from 6.06 ± 0.11 to 9.00 ± 0.16 $\log_{10}$ (CFU/ml), whereas a *Lactobacillus helveticus* strain grew from 6.17 ± 0.11 to 9.00 ± 0.15 $\log_{10}$ (CFU/ml) in the 72-h fermentation (Fig. S4c). The pH decreased from 6.21 ± 0.05 to 5.00 ± 0.04 in the fermentation of *L. fermentum*, whereas it decreased from 6.35 ± 0.05 to 5.02 ± 0.04 in the fermentation of *L. helveticus* (Fig. S4d).

Effects of key carbohydrate hydrolases on ethanol production. We assessed the effects of two starch hydrolases (GH13 and GH15) on ethanol production by statistical analysis. The two-component experiment had 13 enzyme mixtures according to a central composite design model (Fig. 4 and Tables S8 and S9). Analysis of variance (ANOVA) revealed that the quadratic mixture model was significant ($F = 12.3$, $P < 0.01$). A predicted R^2 of 0.62 was reasonable, with an adjusted R^2 of 0.65. Adequate precision was 9.71, and it indicated an adequate signal (Fig. 4) (26). As a result, GH15 was the most important factor influencing ethanol production ($P < 0.01$). The best enzyme profile (combination 2) produced 478.5 ± 7.98 mg/liter ethanol (Fig. 4, Table S8). A mixture of GH13 and GH15 (in a ratio of 1:6, wt/wt) had the highest predicted ethanol yield, corresponding to 439.9 mg/liter. To validate the predictive model, we combined GH13 and GH15 at a ratio of 1:6 (wt/wt) and achieved 435.8 ± 6.80 mg/liter ethanol (Table S9). This result indicated that a proper combination of multiple starch hydrolases enhances ethanol production.

DISCUSSION

Our study identified the key saccharifying enzymes associated with alcoholic fermentation from *Jiuqu* in baijiu production. The proper combination or profile of the key saccharifying enzymes could enhance ethanol production. Our study shed light on the effects of multiple saccharifying enzymes on ethanol production. Furthermore, our study also provides an approach to optimize the profile of saccharifying enzymes for enhancing ethanol production in baijiu fermentation in particular and other food fermentations in general.

***Jiuqu* was the main source of saccharifying enzymes associated with alcoholic fermentation in baijiu production.** Saccharifying activity reached a peak, and 51

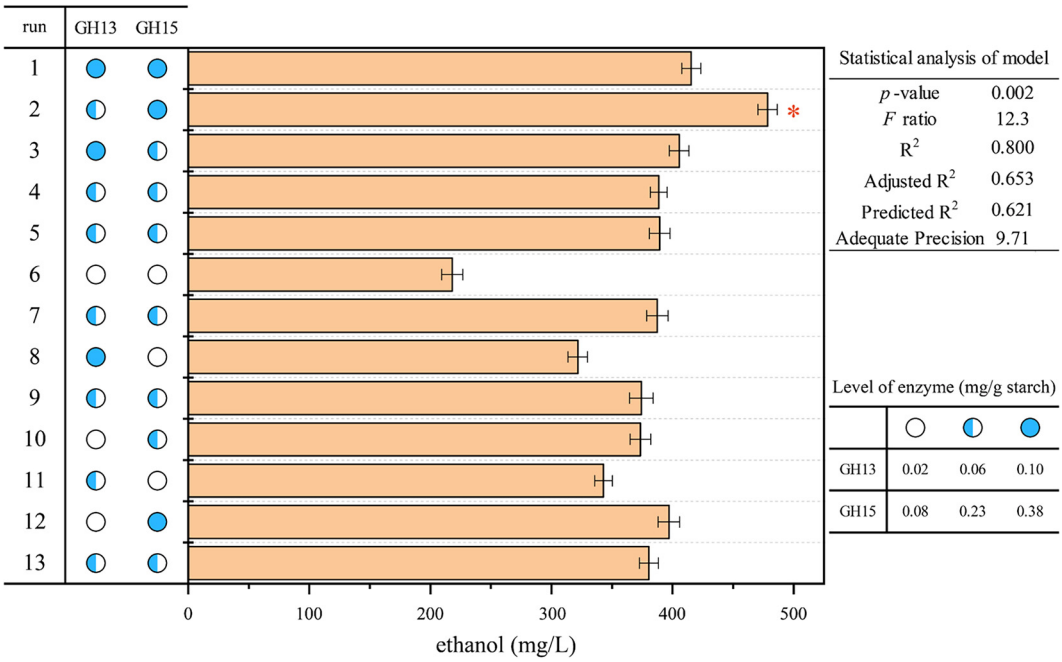

FIG 4 Statistical analysis of the predictive model for ethanol production in a similar baijiu fermentation system under laboratory conditions. Bar, ethanol concentrations in every experiment. Statistical analysis of the model indicates the evaluation of the central composite design. Circles indicate the levels of factors.

carbohydrate hydrolases were identified in the baijiu fermentation at day 3 (Fig. 1a and 2a). Approximately 80% of 51 enzymes were provided by *Jiuqu*, and they were affiliated mainly with *Aspergillus*, *Rhizomucor*, and *Rhizopus* (Fig. 2a and b). These microbes in the baijiu fermentation were also provided by *Jiuqu* (Fig. 2c).

In addition, the microbial compositions of *Jiuqu* (classified as *Xiaoqu*) in this study were different from the previous reports of *Daqu*, including bacteria such as *Bacillus*, *Enterobacter*, and lactic acid bacteria (5, 21). The universal *Jiuqu* starter can be classified as *Daqu*, *Xiaoqu*, and *Fuqu*, and their industrial characteristics were different in raw materials, inoculations, and culture conditions (1). The *Jiuqu* starter studied here is always made of stewed rice and soy beans and inoculated with strains in the initial preparation. Its preparation undergoes a back-slopped fermentation process under a lower temperature (30 to 40°C) (see Fig. S1 in the supplemental material), whereas *Daqu* is always made of raw wheat, barley, or pea and prepared in a spontaneous fermentation process under a higher temperature (40 to 70°C) (1). The industrial and host-associated stress responses in food microbes may drive the microbial community assembly in the back-slopped or spontaneous fermentation of various *Jiuqu* starters (27). According to the lifestyle of microbes in food fermentations, the microbiota of back-slopped *Jiuqu* starters may be shaped by the selection of the most competitive microorganisms, whereas the community assembly of *Daqu* may depend on microbial dispersals from plant or local environmental sources (28, 29). As a result, the selected and competitive microbes were dominated in back-slopped *Jiuqu* starters, like the vertebrate- or host-adapted lactic acid bacteria (e.g., *L. helveticus*), whereas the plant commensal microbes, like *Bacillus*, *Enterobacteriaceae*, and plant-associated lactic acid bacteria, were dominant in spontaneously fermented *Daqu* (5, 21). In addition, the microbial adaptation to environmental factors (e.g., temperature, acidity, and moisture) also may influence the community assembly in the preparation of *Jiuqu* starters (30, 31). In sum, the microbial stress responses and adaptation contribute to the community assembly in the back-slopped or spontaneous fermentation of *Jiuqu* starters.

Multiple saccharifying enzymes from multiple microbes contributed to alcoholic fermentation in baijiu production. Starch hydrolysis in cereals requires a

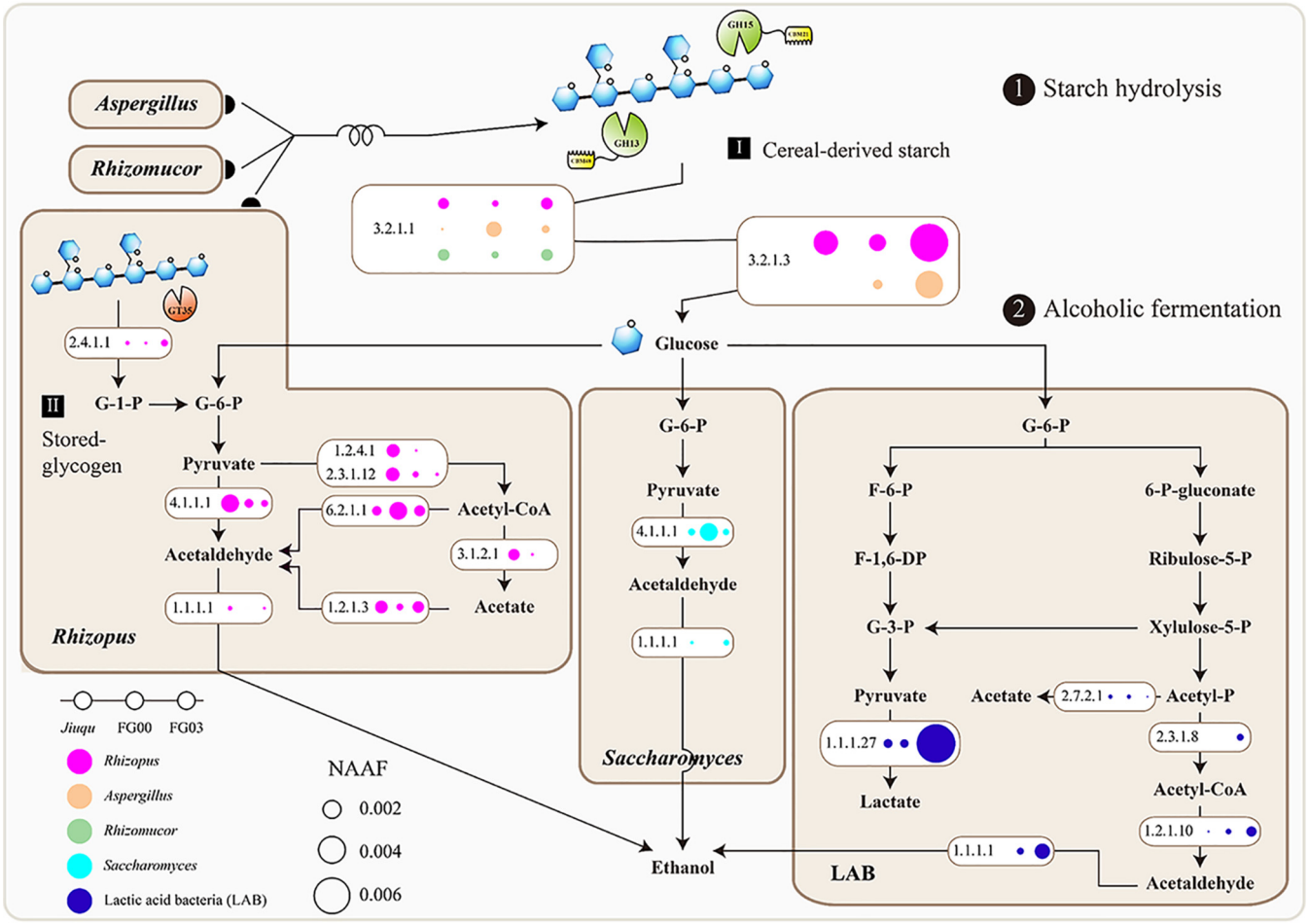

FIG 5 Functional model for polysaccharide hydrolysis and ethanol production in Chinese baijiu fermentation. G-1-P, glucose 1-phosphate; G-6-P, glucose 6-phosphate; F-6-P, fructose-6-phosphate; F-1,6-DP, fructose-1,6-diphosphate; G-3-P, 3-phospho glyceraldehyde; 6-P-gluconate, 6-phosphate-gluconate; ribulose-5-P, ribulose-5-phosphate; xylulose-5-P, xylulose-5-phosphate; acetyl-P, acetyl-phosphate. Enzymes: EC 3.2.1.1, alpha-amylase; EC 3.2.1.3, glucoamylase; EC 2.4.1.1, alpha-1,4 glucan phosphorylase; EC 4.1.1.1, pyruvate decarboxylase; EC 1.1.1.1, aldehyde-alcohol dehydrogenase; EC 1.2.4.1, pyruvate dehydrogenase; EC 2.3.1.12, acetyltransferase; EC 6.2.1.1, acetyl-coenzyme A (CoA) synthetase; EC 3.1.2.1, acetyl-CoA hydrolase; EC 1.2.1.3, aldehyde dehydrogenase; EC 1.1.1.27, L-lactate dehydrogenase; EC 2.7.2.1, acetate kinase; EC 2.3.1.8, phosphotransacetylase; EC 1.2.1.10, aldehyde-alcohol dehydrogenase.

combination of multiple endo- and exoactive enzymes, including alpha-amylase (EC 3.2.1.1), beta-amylase (EC 3.2.1.2), isoamylase (EC 3.2.1.68), glucoamylase (EC 3.2.1.3), and others (32). We combined the metaproteomics, culture-dependent, and multivariate statistical analyses to identify the key saccharifying enzymes from *Jiuqu*. Alpha-amylase (GH13) and glucoamylase (GH15) were identified as two key saccharifying enzymes and were positively related to exogenous cereal-derived starch hydrolysis and alcoholic fermentation in the baijiu fermentation (Fig. 5). Alpha-amylase (GH13) plays a central role in the endohydrolytic action of starch at alpha-1,4 bonds to release a mixture of soluble linear and branched glucans. The linear and branched glucans are hydrolyzed to release short malto-oligosaccharides or maltose by alpha-amylase, isoamylase, and beta-amylase (32). Glucoamylase (GH15) takes part in the exohydrolytic action of starch at alpha-1,4(6) bonds to release glucose (32). Furthermore, glucoamylase can act synergistically with alpha-amylase to release glucose directly from the starch granule surface (33). In this study, these alpha-amylases (GH13) and glucoamylases (GH15) were mainly secreted by *Aspergillus*, *Rhizomucor*, and *Rhizopus* in the baijiu fermentation (Fig. 3a and 5). This finding can be distinguished from a single strain being responsible for enzyme secretion and starch hydrolysis in the production of other beverages or foods (34, 35). This typical saccharification of multiple enzymes from multiple microbes contributes to the diversity of microbial communities and abundant

flavor compounds in the simultaneous saccharification fermentation process of Chinese baijiu (1).

We also applied a culture-dependent method to emphasize the metaproteomics analysis. We isolated a *Rhizopus microsporus* strain from *Jiuqu* samples, which was the most important microbe for secreting saccharifying enzymes. We found the saccharifying activity increased as *R. microsporus* grew in the simulative *Jiuqu* preparation under laboratory conditions (Fig. S4a and b). Moreover, *Aspergillus* and *Rhizomucor* species are also identified as secreted saccharifying enzymes under culture-dependent conditions (20, 36), which corresponds to the metaproteomics analysis (Fig. 5). Furthermore, *Aspergillus*, *Rhizomucor*, and *Rhizopus* species can be selected as candidates to improve the quality of *Jiuqu* by adjusting the structure of microbial communities, fortifying functional microbes, or developing pure cultures (37–39). However, a proper combination of saccharifying enzymes should be identified for enhancing ethanol production, orienting it to adjust the microbial communities of *Jiuqu*.

In addition, several intracellular phosphorylases were related to stored glycogen or oligosaccharide hydrolysis and also contributed to ethanol production in the baijiu fermentation (Fig. 5). Alpha-1,4 glucan phosphorylase (GT35) will cleave glycogen to generate glucose-1-phosphate by consuming inorganic phosphate, and glucose-1-phosphate can be converted to glucose-6-phosphate without ATP by phosphoglucomutase (40, 41). Glucose-6-phosphate then can be metabolized to generate ethanol in cells. Furthermore, the bacterial phosphorylases can select extracellular maltodextrin as a source of carbon and energy when maltodextrin is available in the environment (42). The fungal phosphorylases should get more attention in the utilization of extracellular polysaccharides. We also identified an intracellular maltose-6-phosphate hydrolase, affiliated with *Lactobacillus* species, in the metaproteomics analysis (Fig. 3a). Meanwhile, we isolated *Lactobacillus fermentum* and *Lactobacillus helveticus* strains from the baijiu fermentation. *L. fermentum* and *L. helveticus* then were cultured using the manual medium that selected maltose as the only carbon source, and we found the pH decreased as the cells grew in the fermentation (Fig. S4c and d). This finding indicated that *L. fermentum* and *L. helveticus* can utilize maltose (43, 44). These results corresponded to those from the metaproteomics analysis (Fig. 3a). Furthermore, these *Lactobacillus* species were dominant across *Jiuqu* and baijiu fermentation according to the high-throughput sequencing analysis (Fig. S5). Here, we focused on the role of glycoside hydrolases in polysaccharide hydrolysis and ethanol production in food fermentations (45, 46). Additionally, the stored glycogen or oligosaccharide hydrolysis, catalyzed by phosphorylases (starch, maltose, or other phosphorylases), also should contribute to ethanol production. These catalytic pathways are energy efficient in intracellular systems and should not be overlooked (18, 19, 47, 48). Furthermore, various phosphorylases (starch, maltose, or other phosphorylases) are widely exploited in industry because of the economic role of their glycosyltransferase reactions. Meanwhile, as starch is inexpensive and generally distributed in the local environment, multiple phosphorylases have broad applications in food, cosmetic, plastic, and pharmaceutical industries (49–51).

A proper combination of saccharifying enzymes could enhance ethanol production. The effects of two glycoside hydrolases (GH13 and GH15) were positively related to starch hydrolysis and ethanol production in baijiu fermentation (Fig. 5). Additionally, we isolated a *Saccharomyces cerevisiae* strain from samples that was generally responsible for ethanol metabolism in baijiu fermentation (52). Therefore, we combined the mixtures of two saccharifying enzymes and studied their effects on ethanol production in an *in vitro* system. As a result, we acquired a proper combination of GH13 and GH15 for enhancing ethanol production, and we obtained the responsible strains from *Jiuqu* samples. This method provides a potential approach to selecting the responsible strains to adjust microbial assembly and metabolism in the preparation of *Jiuqu* or to develop pure cultures for obtaining the optimized enzyme profile of starters to enhance ethanol production in baijiu fermentation.

In conclusion, we identified the key saccharifying enzymes associated with alcoholic fermentation from *Jiuqu* in baijiu production. Moreover, we revealed that the proper combination of the key saccharifying enzymes could enhance ethanol production. Our study shed light on the effects of multiple saccharifying enzymes on ethanol production. Furthermore, our study provides a potential approach to optimizing the profile of saccharifying enzymes for enhancing ethanol production in baijiu fermentation in particular and other food fermentations in general.

MATERIALS AND METHODS

Sample collection. Samples were collected in July 2017 from a famous baijiu distillery in Guangzhou Province, China (lat 29.43, long 115.59) (see Fig. S1 and S2 in the supplemental material). In *Jiuqu* preparations, raw materials (rice and soybean) were stewed and mixed with caky balls (inoculated with strains). Mixed materials were shaped into bricks and transferred into a brewing facility for fermentation. Matured *Jiuqu* bricks were stored for 2 weeks and smashed to powder for baijiu fermentation. The mixture of *Jiuqu* powder, raw material (rice), and water (in a ratio of 1:5:16, wt/wt/wt) was put into a fermentation silo (about 50 m^3) and then sealed for a 15-day baijiu fermentation (Fig. S1). We tracked 3 *Jiuqu* brewing facilities on 7, 8, and 9 July 2017 (Fig. S2). Each facility was an independent batch. Three *Jiuqu* powder samples (about 1,000 g) were collected before being transferred into each fermentation silo. We tracked three silos that used the corresponding *Jiuqu* samples. Samples were collected from three points (1, 2, and 3) at the upper, middle, and bottom layer (a, b, and c) in every fermentation silo (Fig. S2). One final sample was obtained by pooling samples from different sites. Samples (about 1,000 g) were collected at days 0, 3, 5, 10, and 15 from 3 fermentation silos and named FG00, FG03, FG05, FG10, and FG15. All samples were stored at −20°C until microbial and physicochemical analysis. Separate samples of *Jiuqu*, FG00, and FG03 (200 g each) were immediately frozen in liquid nitrogen after collection and then stored at −80°C for metaproteomics analysis.

Physicochemical and enzymatic activity analysis. The moisture of samples was measured by detecting its weight loss after drying 10-g samples at 105°C for 4 h (sufficient to reach constant weight). The saccharifying activity of samples was measured as previously described (53, 54). A unit of saccharifying activity was defined as the amount (in micrograms) of glucose converted from starch by 1 g of sample per minute under the assay conditions.

Protein preparation and mass spectrometry analyses. To achieve adequate predictive power for the enzyme profile, samples of *Jiuqu*, fermentation samples at day 0 and day 3, were applied to a label-free quantitative proteomics analysis. The proteomic samples were prepared as described earlier (19, 55). A ProteoExtract abundant protein extraction kit (Merck KGaA, Darmstadt, Germany) was used to remove the high-abundance proteins from the proteomic samples.

Peptides were analyzed by an EASY-nLC1200 coupled to a Q-Exactive mass spectrometer (Thermo Fisher Scientific, Waltham, MA). Five microliters (approximately 2.5 μg total peptide) was loaded into a C_{18} analytical column (75 μm [inner diameter] by 25 cm) (Thermo Fisher Scientific, Waltham, MA) for separation for 240 min. The mobile phase was controlled by solvents A (2% acetonitrile and 0.1% formic acid) and B (80% acetonitrile and 0.1% formic acid) at 300 nl/min, using the following gradients: starting with 5% B for 2 min, 23% B for min 3 to 147, 29% B for min 148 to 183, 48% B for min 184 to 208, 100% B for min 209 to 215, and 0% B until 240 min. Linear trap quadrupole mass spectrometer conditions were set as previously reported (19).

Mass spectrometric data analysis. Raw data were uploaded to the database and analyzed by Thermo Scientific Proteome Discoverer (PD) 1.4 software connected to an in-house Mascot server (V 2.4.1; Matrix Science, Boston, MA). Proteins were searched on the UniProt database (http://www.uniprot.org). The peptide and protein candidates were generated by a strict filtering based on a false discovery rate (FDR) of 0.01. Label-free quantification (LFQ) was applied by MaxQuant (56). The detailed procedure was described earlier (57). Meanwhile, we constructed the database based on the genomic and proteomic information of the dominant microbial members (identified by the high-throughput sequencing analysis) from the NCBI database (https://www.ncbi.nlm.nih.gov/), including *Lactobacillus fermentum* (CP002033.1), *Lactobacillus helveticus* (NC_010080.1), *Klebsiella pneumoniae* (FO203501.1), *Pediococcus pentosaceus* (NC_008525.1), *Weissella confusa* (NZ_CAGH00000000.1), *Aspergillus ruber* (KK088411.1), *Candida glabrata* (LMAA01000056.1), *Pichia kudriavzevii* (NC_042506.1, NC_042507.1, NC_042508.1, NC_042509.1, and NC_042510.1), *Rhizopus microsporus* (KV921258.1), *Saccharomyces cerevisiae* (NC_001136.10), *Saccharomycopsis fibuligera* (CP015978.1, CP015979.1, CP015980.1, CP015981.1, CP015982.1, CP015983.1, and CP015984.1), and others. The reconstructed database was also used for protein research. We identified parent proteins by the highest score for a given peptide mass. Identified proteins were annotated by Gene Ontology (GO), Clusters of Orthologous Groups (COG), and the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. We determined normalized area abundance factors (NAAF) to estimate protein abundances by chromatographic peak areas from the proteomics data (58).

DNA extraction, amplification, and sequence processing. Total DNA was extracted from samples by the E.Z.N.A. (easy nucleic acid isolation) soil DNA kit (Omega Bio-Tek, Norcross, GA). The 16S rRNA gene V3-V4 hypervariable region was amplified by universal primers 338F (forward) and 806R (reverse) (59). The internal transcribed spacer (ITS) was amplified by primers ITS1F and ITS2 (60). These primers contained a set of barcode sequences unique to each sample. PCR products were purified by a PCR purification kit, and their concentrations were measured by a Thermo Scientific NanoDrop 8000 UV-

visible spectrophotometer (NanoDrop Technologies, Wilmington, DE). The barcoded PCR products were sequenced by a MiSeq benchtop sequencer for 250-bp paired-end sequencing (2 by 250 bp; Illumina, San Diego, CA) at Beijing Auwigene Tech., Ltd. (Beijing, China).

The MiSeq-generated raw sequence data were processed by the QIIME pipeline (v 1.8.0) (61). We removed the sequences with quality scores of <30 for quality trimming, choosing only sequences over 200 bp to analyze. We removed sequences that did not perfectly match the PCR primer. Chimeras were removed by UCHIME software (62). The trimmed sequences then were clustered into operational taxonomic units (OTUs) with a 97% identity threshold by QIIME's uclust pipeline (63). A single representative sequence from each OTU was aligned to the Greengenes database (v13.8) and the UNITE fungal ITS database (v6.0) (64, 65). Singleton OTUs were removed before further analysis. Chao1 richness and Shannon diversity indexes were calculated by QIIME (v 1.8.0) (66).

Strain isolation and cultivation. The bacterial and fungal strains were isolated from *Jiuqu* and fermented samples by using Man-Rogosa-Sharpe medium (MRS), Wallerstein laboratory medium (WL), and potato dextrose agar (PDA). Genomic DNA extraction and identification of the single isolated strains were applied as described in previous studies (5, 19). *R. microsporus* JJ01 was inoculated into the raw materials used for *Jiuqu* preparations, and the culture conditions were set as previously described (19). *L. fermentum* JJ01 and *L. helveticus* JJ02 were cultured for 72 h at 37°C in manual medium [including 20 g/liter maltose, 10 g/liter tryptone, 10 g/liter beef extract, 5 g/liter yeast extract, 5 g/liter $CH_3COONa{\cdot}3H_2O$, 2 g/liter $K_2HPO_4{\cdot}3H_2O$, 2 g/liter $(NH_4)_2HC_6H_5O_7$, 0.58 g/liter $MgSO_4{\cdot}7H_2O$, 0.25 g/liter $MnSO_4{\cdot}H_2O$, and 1 ml/liter Tween 80]. pH was determined with a pH detector (INESA Scientific Instrument Co., Ltd., Shanghai, China). Other physicochemical parameters, real-time quantitative PCR, and enzyme activity analysis were applied as previously reported (19).

HPLC analysis. The extraction of ethanol was done as previously described (5). Ethanol was separated by a column (Aminex HPX-87H; 300 mm by 7.8 mm; Bio-Rad, Hercules, CA) and detected by high-performance liquid chromatography (HPLC; Agilent 1200 HPLC; Agilent Technologies, Santa Clara, CA) with a refractive index detector (WGE GmbH, Bergheim, Germany). The detection conditions were set as previously described (67). The quantitative standard curve was established using ethanol (99.9%, HPLC grade) purchased from J&K Chemical, Ltd. (Shanghai, China).

Optimization of enzyme profile for ethanol production. Polished rice was soaked in water at 25°C overnight (the ratio of rice to water was 2:3, wt/wt) and steamed at 121°C for 40 min. The solid fraction was suspended in the low-salt buffer (10 mmol/liter Tris-HCl, 0.1 mol/liter NaCl, pH 7.5), and a final 2% (wt/vol) suspension was used for alcoholic fermentation. Alpha-amylase (GH13) and glucoamylase (GH15) were purchased from Anhui Guangyuan Industrial Co., Ltd. (Hefei, China), and used for carbohydrate hydrolysis and alcoholic fermentation. The phylogenetic tree of commercial enzymes and the ones identified in the baijiu fermentation is shown in Fig. S3.

The experimental design and analysis were implemented by Design-Expert software (Stat-Ease, Inc., Minneapolis, MN) to assess the effect of combined GH13 and GH15 on ethanol production. A central composite design required 13 reactions based on setting the low and high levels of two enzymes. The ranges from minimum to maximum content were set as 0.02 to 0.10 mg/g starch for GH13 and 0.08 to 0.38 mg/g starch for GH15 (19). Each reaction mixture (50 ml, pH 7.5) was incubated at 30°C for a 72-h fermentation. *Saccharomyces cerevisiae* JJ01 was precultured in yeast extract-peptone-dextrose medium at 30°C and 200 rpm for 24 h. Cells were centrifuged (10,000 $\times$ *g* for 3 min), washed twice with sterile saline (0.90% wt/vol), and then inoculated into each mixture with an initial cell density of 1×10^6 cells/ml. After a 72-h fermentation, 10 ml culture broth was centrifuged at 10,000 $\times$ *g* for 5 min, and the supernatant was collected to measure ethanol. The concentration of ethanol was calculated as previously described (5, 68). All data were analyzed by ANOVA, and to develop a statistically based predictive model, *F* ratio, *P* value, R^2, adjusted R^2, predicted R^2, and adequate precision were calculated (26). All experiments were run in triplicate.

Data analysis. We calculated all possible Spearman's rank correlations to analyze the relationships among microbes, enzymes, and metabolites. A network was created by Cytoscape (ver 3.7.0) to sort through and visualize correlations.

Data availability. All generated protein data were submitted to the iProX database (http://www.iprox.org) under the project number IPX0001641000/PXD014221. All sequences generated were submitted to the DDBJ database under the accession numbers DRA008485 and DRA008486. The genomic sequences of the isolated *Rhizopus microsporus* JJ01, *Saccharomyces cerevisiae* JJ01, *Lactobacillus fermentum* JJ01, and *Lactobacillus helveticus* JJ02 strains were deposited in GenBank under the project numbers MH782032, MK994013, MN841929, and MN841930.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.7 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.4 MB.

SUPPLEMENTAL FILE 3, XLSX file, 0.02 MB.

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We have no conflicts of interest to declare.

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