Total protein extraction and activities development of major enzymes in fermentation starter of Chinese liquor

Yuxia Wang[1, #], Yongguang Huang[2, #], Bing Li[1, 3], Chao Zhang[1, 4, *]

[1]School of Life Science and Food Engineering, Yibin University, 8 Jiusheng Road, Yibin, Sichuan 644000, China. [2]School of Liquor and Food Engineering, Guizhou University, Huaxi District of Guiyang, Guizhou 550025, China. [3]School of Food and Bioengineering, Xihua University, 999 Jinzhou Road, Jinniu District, Chengdu, Sichuan 610039, China. [4]Key Laboratory of Fermentation Resources and Application of Institutes of Higher Learning in Sichuan, Yibin, Sichuan 644000, China

Received: April 2, 2019; **accepted:** May 22, 2019.

Daqu*, a naturally fermented starter, provides very abundant enzymes and diversity of microorganisms for Chinese liquor fermentation. The significant importance of enzymes and microorganisms from *Daqu* attracted much interest about their functions. However, there were few studies on enzymatic activities tracing the same batch *Daqu* from fermentation stage to maturation stage aiming at the isolation clues to special enzymes producers. Therefore, by using an orthogonal array $L_9(3^4)$, total protein of *Daqu* was extracted by a metaproteomic approach with concentration as indicator, coupled with an exploration of the variations in activities of five representative enzymes during *Daqu* fermentation and maturation. The results revealed that the optimized combination ratio of *Daqu* powder to buffer solution, pH value, ultrasound time, and ethanol volume were 1:4, 8.0, 70 min, and 4 volumes, respectively. Furthermore, the activities showed significant variations between the different enzymes, as well as between Qupi (skin layer of Daqu brick) and Quxin (core layer of *Daqu* brick) samples. Glucoamylase activity ranged from 26.62 to 3,727.80 U with two increasing and two decreasing variations in Qupi and one in Quxin. Moreover, the highest cellulase activities appeared at the final stage in both Qupi and Quxin samples. For pectinase, the highest activity (1,685.71 U) was detected in a Quxin sample (X7), which was higher than that of the Qupi samples (129.87 U). The activity levels of proteinase and esterase were similar in the Qupi and Quxin samples. These variations implied enzyme activity changes due to alterations in the microflora community, which could provide very useful clues to sample, isolate, and screen novel enzyme producers to compensate the insufficiency of Daqu's activity by adding extra enzyme preparations or enzyme producers to start the fermentation of Chinese liquor.

Keywords: Chinese liquor; *Daqu*; fermentation starter; protein extraction; orthogonal design; enzymatic activity.

[#]Equal contributors.

[*]**Corresponding author:** Chao Zhang, School of Life Science and Food Engineering, Yibin University, 8 Jiusheng Road, Yibin, Sichuan 644000, China. Email: siqi0831@163.com.

Introduction

Brandy, whiskey, gin, rum, vodka, and Chinese liquor are the six most well-known distilled spirits in the world. Chinese liquor, also called *Baijiu*, is typically obtained from cereals such as sorghum and wheat by complex fermentation processes with mixed *Daqu* in the solid state [1]. Traditional *Daqu*, a special starter culture and substrate complex used to initiate solid state

fermentation (SSF) for the production of Chinese liquor, is prepared by a natural inoculation of molds, yeasts, and bacteria as well as their growth on the grains [2, 3].

Daqu is a good source of microorganisms and abundant enzymes. It even serves to provide starting materials and flavor substances in the production of Chinese liquor; in this way, *Daqu* significantly influences the quality and style of the resulting liquor [4, 5]. The production of *Daqu* itself is also a special SSF process in an open system that aims to enrich and cultivate microorganisms from the environment named spontaneous fermentation and produces plenty of enzymes for liquor fermentation [6].

The types of *Daqu* can be distinguished into multiple types according to the fragrance of Chinese liquor (such as sauce, heavy fragrant, and light fragrant) including light-flavor *Daqu*, strong-flavor *Daqu*, and sauce-flavor *Daqu* [7, 8]. Among all the *Daqu* types, the strong-flavor type accounts for about 70% of total liquor production in China [9]. Wuliangye liquor, a typical strong-flavor Chinese liquor with a long history, is distilled from a fermented five-grain mixture (sorghum, rice, sticky rice, wheat, and corn) mixed with Wuliangye-*Daqu* powder. Wuliangye-*Daqu* is mainly prepared from wheat by six stages: (i) Moisturizing; (ii) Grinding; (iii) Watering; (iv) Shaping (pressed into a brick-shaped block); (v) Incubation (about 1-1.5 months); and (vi) Maturation (about 3-6 months) [10].

The incubation stage, also called fermentation stage, is divided into five additional steps: "Zhangmei", "Qihuo", "Dahuo", "Houhuo", and "Yangqu". The first step "Zhangmei" is a very important stage that has an irreversible effect on the final quality of the *Daqu*. Due to the natural inoculation of microorganisms derived from the raw materials, water, rush mats, bran coating, and environment, many white-gray fungi appear on the surface of the *Daqu* chunks. The temperature of the *Daqu* chunks increases gradually and reaches 30-40°C in 2-3 days. The key incubation period for microbial succession is "Qihuo", in which the temperature is increased and maintained at 50°C for the next 5-7 days. In this stage, the "Fanqu" procedure which can lower the temperature of the *Daqu* chunks is conducted every 1-2 days to ensure the uniform growth of microorganisms on the surface and between the chunks and repeated 3-5 times total. The "Dahuo" step takes about 5-7 days and the temperature gradually increases to a maximum of 60°C. The "Houhuo" step is prolonged about 9-12 days and the temperature decreases gradually to approximately 40-45°C. The "Yangqu" (pre-maturation) step takes about 15 days and aims for an equilibration of the moisture, acidity, and enzyme activity [8]. Then, the *Daqu* chunks are transferred to a large, special warehouse for a 3-6 months maturation period before use.

Due to the *Daqu*'s crucial role, up to now, more and more attention has been focused on its contents including microorganisms, aroma-active compounds, and enzymes [8, 11-14]. In particular, the microorganisms and enzymes in *Daqu* are gradually becoming an area of greater interest. Nevertheless, there are few studies concerning the effect of the range of enzymes produced by the alternative microbiota of *Daqu* and their effects on the fermentation of Chinese liquor.

Recently, metaproteomic analysis has provided novel insights into metabolic and physiological activities in mixed microbial cultures and has been used to detect microbial functional proteins [15-17] because proteins reflect the actual functionality and direct information about microbial activity [18]. Therefore, it is more direct to study the developments of enzymes during the fermentation and maturation processes of *Daqu* using a metaproteomic approach than by isolating every enzyme from each strain.

The present work is focused on the optimization of protein extraction from *Daqu* using a metaproteomics strategy under orthogonal

design conditions with protein concentration as an indicator. Furthermore, with the aim of isolating and screening the producers of special enzymes and strengthening *Daqu*-making, the variations in the activities of representative enzymes, specifically glucoamylase, cellulase, pectinase, proteinase, and esterase, were evaluated during the fermentation and maturation stages of *Daqu* chunks. This is the first systematic study of the enzymatic activities of Wuliangye strong-flavor-*Daqu* of the Yibin district throughout all of the generation, fermentation, and maturation stages before utilization.

Materials and Methods

Sampling

Wuliangye-*Daqu* samples were obtained from Zijun-Denggongye Liquor Co. Ltd. (Yibin city, Sichuan Province, China). *Daqu* was fermented and matured in stacked layers. *Daqu* bricks were collected at the end of Shaping, "Zhangmei", "Qihuo", "Dahuo", "Houhuo", and "Yangqu" steps and were sampled every ten days until they were used during the maturation stage. Each sample was obtained from 6 chunks that were randomly selected from the upper, middle, and lower stacked layers. All samples were stored at -80°C until the protein was extracted. Information on the *Daqu* samples used in this study is shown in Table 1. To explore whether there are differences between the samples' surface and core portions, each *Daqu* sample was divided into two parts: samples derived from the surface layer (0-3 cm inward) (Qupi) and those from the core layer (0-3 cm outward) (Quxin).

Method for extracting protein from *Daqu*

Ten (10) grams of each *Daqu* sample were used for total protein extraction. After grinding and sieving (< 0.25 mm), the resulting *Daqu* powder was immersed in different volumes of buffer solution from 1:1 to 1:10 (w:v, g:mL) at different pH values (pH from 3 to 12) for different lengths of time (between 1 h and 15 h). The total protein was extracted by ultrasound for different times between 10 to 80 min, and then the solution was centrifuged at 5,000 g for 15 min at 4°C to remove the insoluble matter. The different volumes of 4°C ethanol (3-5 times volume to extraction solution) were added to the supernatant to precipitate the protein. Total protein was obtained after centrifugation at 12,000 g for 30 min at 4°C and then dissolved the pellet in 0.1 M acetic acid buffer (pH 4.6) for the activity analysis of glucoamylase, cellulase, pectinase, protease, and esterase.

Table 1. Sample code and detail.

Samples from *Daqu* surface (Qupi)	Samples from *Daqu* core (Quxin)	Sample stage
Qupi (P1)	Quxin (X1)	Shaping
Qupi (P2)	Quxin (X2)	"Zhangmei"
Qupi (P3)	Quxin (X3)	"Qihuo"
Qupi (P4)	Quxin (X4)	"Dahuo"
Qupi (P5)	Quxin (X5)	"Houhuo"
Qupi (P6)	Quxin (X6)	"Yangqu"
Qupi (P7)	Quxin (X7)	One-month maturation
Qupi (P8)	Quxin (X8)	Two-month maturation
Qupi (P9)	Quxin (X9)	Three-month maturation
Qupi (P10)	Quxin (X10)	Use

Standard curves

Protein concentration was assayed by using the Coomassie blue (Bradford) protein assay reagent according to the technical instruction manual, with BSA as the standard. The concentration of glucose released from the enzymatic assay was determined via glucose standard curves using the 3, 5-dinitrosalicylic acid (DNS) method [19]. The protease activity was detected by a calibration curve for L-tyrosine. In all the calibration curves, the R^2 values were > 0.99.

Orthogonal design

In a preparatory experiment, four factors including the ratio of *Daqu* powder to buffer solution, pH of the buffer solution, ultrasound extraction time, and volumes of ethanol showed stronger influences on protein extraction than

the soak time and sedimentation time. Therefore, these four factors were used in an orthogonal array L_9 (3^4) to optimize the extraction conditions. The key parameters that influenced the yield of extracts were analyzed, which included (A) the ratio of *Daqu* powder to buffer solution; (B) pH of buffer solution; (C) ultrasound extraction time; and (D) volumes of ethanol with three levels for each factor. Nine extractions were carried out while the ratios of *Daqu* powder to buffer solution were 1:1, 1:2, and 1:3; pH values of the buffer solution were 7.0, 8.0, and 9.0; ultrasound extraction times were 50, 60, and 70 min; and volumes of ethanol were 3, 4, and 5.

Analysis of glucoamylase activity

Two milliliters of crude enzyme solution were diluted with distilled water and mixed with 2% soluble starch (Sigma-Aldrich, St. Louis, MO, USA) in 0.1 M NaAc buffer at pH 4.6 (preheat at 40°C for 5 min). After incubating the solution at 40°C for 30 min, the reaction was stopped by immediate cooling in an ice bath. Liberated glucose was determined by the DNS assay [19] and the standard curve was prepared with glucose. One unit (U) of glucoamylase activity was defined as the amount of enzyme derived from one gram of *Daqu* that liberated one milligram of glucose per minute from soluble starch. All tests and analyses were carried out in triplicate.

Analysis of cellulase activity

The cellulase activity was analyzed in an enzyme-filter paper reaction mixture containing 0.5 mL of diluted enzyme solution and a 50-mg filter paper strip (1 × 6 cm^2) (Whatman No. 1) in 0.1 M NaAc buffer (pH 4.6) and incubated at 40°C for 60 min. The cellulase activity was calculated by estimating the reducing sugars liberated from the filter paper. The reducing sugar was calculated from the standard curve of glucose by the DNS method. One unit (U) of cellulase activity was defined as the amount of enzyme from one gram of *Daqu* that liberated one milligram of reducing sugar (glucose) per min at 40°C in 0.1 M NaAc buffer (pH 4.6). All tests and analyses were carried out in triplicate.

Analysis of pectinase activity

The pectinase activity was determined by using 0.5% (w/v) pectin as the substrate. One unit (U of pectinase activity was defined as the amount of enzyme from one gram of *Daqu* that liberated one milligram of reducing sugar (glucose) equivalent to galacturonic acid per hour at 40°C in 0.1 M NaAc buffer (pH 4.6). The galacturonic acid content was measured by the DNS method with a standard curve prepared with glucose. All tests and analyses were carried out in triplicate.

Analysis of proteinase activity

Proteinase activity was investigated using 2% (w/v) casein as the substrate in 0.1 M NaAc buffer (pH 4.6) [20]. The hydrolyzed product from the mixture was then measured from a linear standard curve constructed with tyrosine. One unit (U) of proteinase activity was defined as the amount of enzyme from one gram of *Daqu* that liberated 1 µmol of tyrosine per hour under the assay condition. All tests and analyses were carried out in triplicate.

Analysis of esterase activity

Esterase activity (esterification activity) was detected by assaying the concentration of ethyl hexanoate in esterification reactions between hexanoic acid and ethanol at a molar ratio of 1:1 using 0.02 times the total volume enzyme solution. At the beginning of the reaction, samples containing the mixture of hexanoic acid and ethanol were collected and the hexanoic acid content was determined by titration with 0.05 M NaOH. After adding the enzyme, the mixture was incubated at 36°C and 100 rpm for 24 h. Then, the concentration of ethyl hexanoate was determined by the consumption of hexanoic acid using acid-base titration. One unit (U) of esterification activity was defined as the amount of enzyme in one gram of *Daqu* that was necessary to form one milligram of ethyl hexanoate per hour under the experimental conditions. All enzymatic activity determinations were carried out in triplicate.

Table 2. Result of orthogonal test.

Experiment No.	Factors				Content of protein (mg/mL)
	Ratio of *Daqu* powder to buffer solution (A)	pH values of buffer (B)	Ultrasound time (min) (C)	Ethanol volume (Times) (D)	
1	1:2	7.0	50	3	1.881 ± 0.011
2	1:2	8.0	60	4	2.124 ± 0.023
3	1:2	9.0	70	5	1.847 ± 0.014
4	1:3	7.0	70	4	1.521 ± 0.010
5	1:3	8.0	50	5	2.210 ± 0.062
6	1:3	9.0	60	3	1.843 ± 0.091
7	1:4	7.0	60	5	1.611 ± 0.026
8	1:4	8.0	70	3	3.169 ± 0.019
9	1:4	9.0	50	4	1.808 ± 0.024
k1	1.951 ± 0.016	1.671 ± 0.009	1.966 ± 0.027	2.298 ± 0.039	
k2	1.858 ± 0.032	2.501 ± 0.029	1.859 ± 0.031	1.818 ± 0.013	
k3	2.196 ± 0.007	1.833 ± 0.042	2.179 ± 0.008	1.889 ± 0.017	
R	0.338 ± 0.026	0.830 ± 0.037	0.320 ± 0.023	0.480 ± 0.027	
Best level	3	2	3	1	

Note: Ki is obtained by adding any number of columns corresponding to i factor. R is the difference between the maximum value and the minimum value of Ki of any columns.

Statistical Analysis

Experimental results were reported as the means ± SD of three parallel measurements. Analysis of variance was performed by ANOVA procedures (SPSS 19.0 for Windows). Significant differences between means were determined by Duncan's Multiple Range tests. A significance threshold of $P < 0.05$ was used to correct for multiple testing.

Results and Discussion

Analysis of orthogonal design experiment

The orthogonal design was successfully applied to improve the protein concentration extracted from *Daqu*. In the present study, the L_9 (3^4) orthogonal design was used to optimize the processing conditions. The ratio of *Daqu* powder to buffer solution (A), pH values of the buffer (B), ultrasound time (C), and volume of ethanol (D) were used as the extraction factors and the protein content was employed as the evaluation indicator.

The results from the optimization of protein extraction are shown in Table 2, which revealed that the protein concentration ranged from 1.521 to 3.169 mg/mL. These values were taken as the original data for the analysis of range and variance (ANOVA). For each factor, a higher mean value (Kji) indicated that the 'factor' exerted a positive influence on the extraction of protein. Therefore, the best level of each factor can be determined according to the highest mean value of the experimental condition (Kji). Based on the data shown in Table 2, the highest protein content was achieved with (A) 1:4 ratio of *Daqu* powder to buffer solution, (B) pH value 8.0, (C) ultrasound time 70 min, and (D) 3 volumes of ethanol. The best combination was $A_3B_2C_3D_1$ (number 8 in Table 2).

The range value (Rj) indicates the significance of the effect and a larger Rj means the factor increased the protein concentration. Therefore, the comparison of Rj for the different factors suggests that the significance of the factors on protein content, from the most significant to the least, were pH values of buffer (0.830) > ethanol

volume (0.480) > ratio of *Daqu* powder to buffer solution (0.338) > ultrasound time (0.320). The pH value was the best indicator of significance for the amount of protein extracted from *Daqu*. Due to the small range, the protein content extracted from *Daqu* changed only slightly with changes in ultrasound time. According to the result of the orthogonal design ($A_3B_2C_3D_1$), the mean protein content extracted under optimum extraction conditions was 3.131 ± 0.008 mg/mL. This proves that the constructed response model adequately predicted optimal conditions that were achievable.

The investigation of activities of representative enzymes

Barley or wheat is the main raw material used to make Wuliangye-*Daqu*. There are different kinds of substances in *Daqu* chunks such as starch, cellulose, pectin, and protein. The fermentation and maturation of *Daqu* are the processes that inoculate, enrich, and culture microorganisms derived from the materials and the environment and also the processes that lead to the accumulation of a large number of microorganism-produced enzymes that degrade substances. The activities of different enzymes can reflect not only the growth of microorganisms, but also the quality of the *Daqu*. Therefore, in order to study the relationship among the microorganisms, enzymes, and *Daqu* quality, the activities of representative enzymes including glucoamylase, cellulase, pectinase, proteinase, and esterase were investigated in this study.

Glucoamylase activity

Glucoamylase, an important enzyme in the hydrolysis of starch, can hydrolyze α-1,4-glucosidic and β-1,6-glucosidic linkages in polysaccharides to yield glucose [21]. The materials used to make *Daqu* are full of starch. Converting the raw starch to smaller oligosaccharides and glucose mainly involves α-amylase and glucoamylase hydrolysis under the conditions in the SSF of Chinese liquor. Raw starch-digesting glucoamylases are capable of directly hydrolyzing raw starch to glucose at low temperature under natural condition [22], which is significantly helpful for utilizing grain in Chinese liquor fermentation.

The activities of glucoamylases from *Daqu* were determined by investigating the concentration of glucose derived from the hydrolysis of soluble starch. The results showed that the glucoamylase activities among all the samples ranged from 26.62 to 3,727.80 U, which were consistent with the range of 1,216.62 to 3,727.80 U for the Qupi samples and 26.62 to 635.56 U for the Quxin samples (Figure 1).

The data in Figure 1A revealed that the glucoamylase activities of the Qupi samples increased very rapidly to the higher value from Shaping (P1) to "Zhangmei" (P2), and then gradually increased again to the highest, P8 (3,727.80 U), after a short-term decrease from 2,269.33 U to 1,773.62 U from "Zhangmei" (P2) to "Dahuo" (P4). Afterward, the glucoamylase activities exhibited another small decrease in variation (Figure 1A) before usage.

In comparison with the enzymatic activity in Qupi samples, the glucoamylase activities of the Quxin samples showed a marked tendency to increase and decrease during the whole stage (Figure 1B). Furthermore, the glucoamylase activities of Quxin were relatively lower than those of the Qupi (Figure 1). The highest glucoamylase activity was 635.56 U (X4), which was 23.88 times higher than the lowest value (X1) among the Quxin samples, but 5.87 times lower than the highest activity of P8. These results implied the higher possibilities to isolate strains with higher glucoamylase production or better glucoamylase property using the P8 and X4 samples. Moreover, the differences in the activities and micro-environment between the surface and core portions of *Daqu* also implied that glucoamylase producers may be aerobes, not anaerobes.

Cellulase activity

Cellulose, the highly abundant and natural polymers of 1,4 - β - linked sugar units of D-

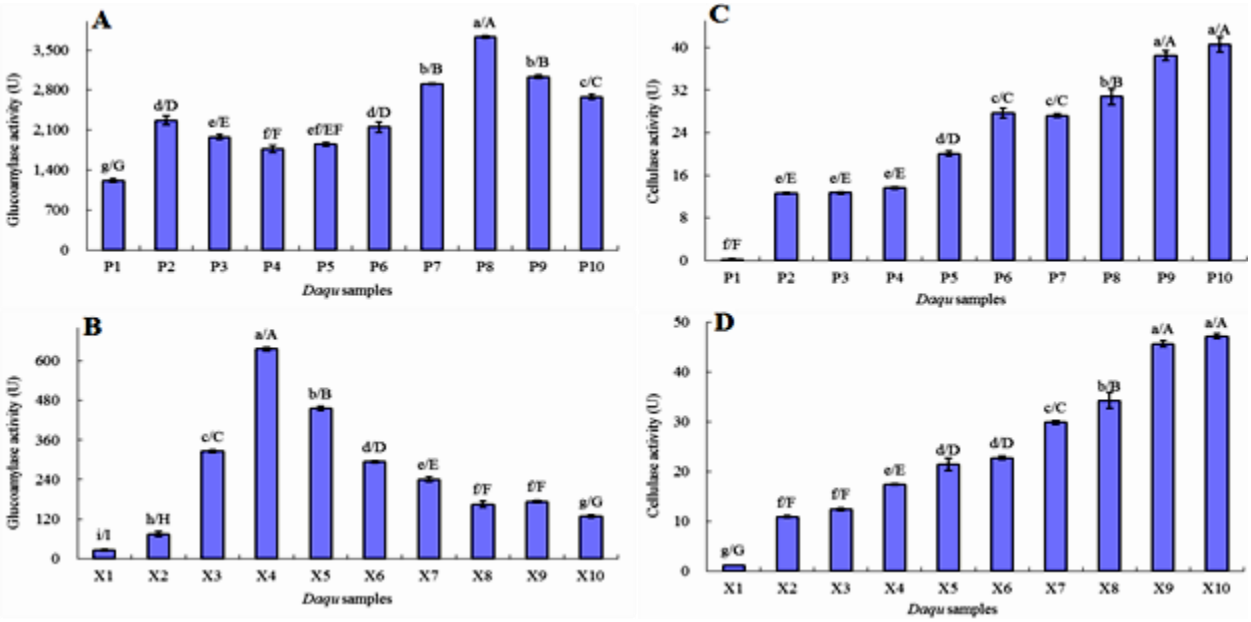

Figure 1. Activities of glucoamylase (A and B) and cellulase (C and D) of *Daqu* samples. Different letters in a column indicate significant difference at $P < 0.05$ (lowercase letters) or $P < 0.01$ (uppercase letters).

glucose, can be broken down by cellulase produced by a wide variety of microorganisms. There are abundant plant celluloses in the raw material used to make *Daqu*. The assaying the cellulase activity during the *Daqu* fermentation and maturation stages can enable a deeper insight into the microorganism diversity and provide a useful clue about the screening of microorganisms that produce cellulase.

As shown in Figure 1, the cellulase activities gradually increased to the highest values from 0.21 to 40.61 U in the Qupi samples (Figure 1C) and from 1.21 U to 47.21 U in the Quxin samples (Figure 1D). Moreover, X10 had the highest cellulase activity of 47.21 U among all the samples, which is 6.6 U higher than the highest activity of P10 with 40.61 U in Qupi sample. This result suggested that the samples from the final stage might contain some species or strains that could produce cellulases with better properties or higher production, or that some cellulases were released from intracellular locations to extracellular ones when cells died, or cell's permeability increased. Additional work is needed to elucidate these hypotheses.

Pectinase activity

Pectin, a major constituent of cereals, vegetables, fruits, and fibers, is a complex, high molecular weight, heterogeneous, and acidic-structured polysaccharide [23]. Pectinases are polysaccharidases that degrade pectins and have been widely applied in different industries such as the clarification and filtration of fruit juices, increasing the quality of wine, coffee and tea fermentation, textiles, papermaking, protoplasm isolation and purification of plant viruses, and the pre-treatment of pectin-containing wastewaters [24]. The pectin in the raw material of *Daqu* is degraded by the pectinases secreted by microorganisms in the *Daqu* chunks. Therefore, the enzymatic activities could reveal some clues about *Daqu*'s pectinase-producing microorganisms.

By assaying the concentration of galacturonic acid by the DNS method, the changes in pectinase activities of *Daqu* at different stages were evaluated (Figure 2A, 2B). Among all samples, the X7 sample exhibited the highest pectinase activity (1,685.71 U), followed by P10 (1,555.84 U). Both activities were higher than

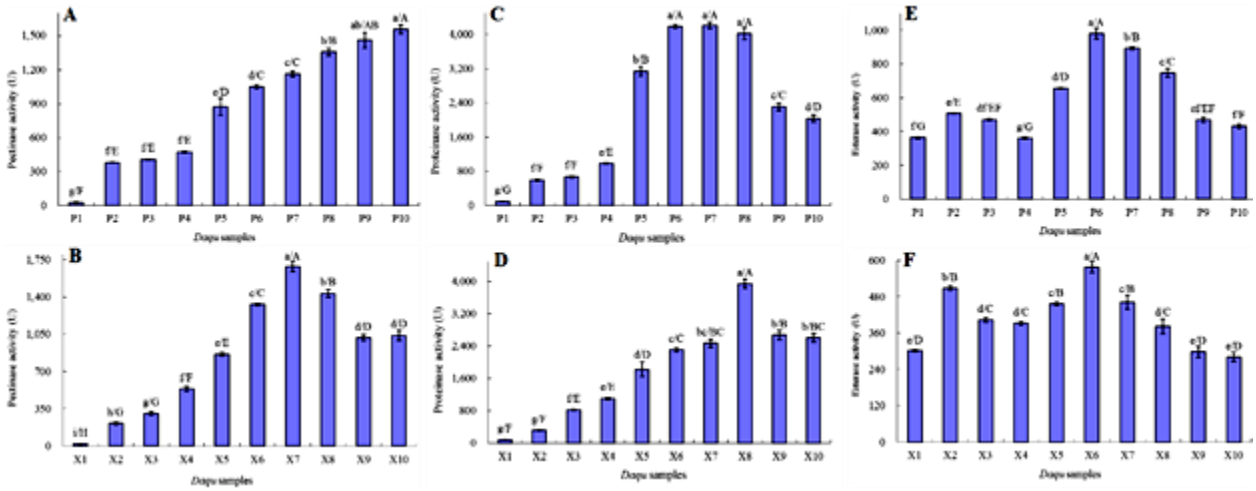

Figure 2. Activities of pectinase (A and B), proteinase (C and D), esterase (E and F) of *Daqu* samples. Different letters in a column indicate significant difference at $P < 0.05$ (lowercase letters) or $P < 0.01$ (uppercase letters).

the initial activities by 86.46 times in Quxin and 62.33 times in Qupi, respectively. As shown in Figure 2A and 2B, the pectinase activities in the Qupi samples gradually increased across the stages in contrast to those of the Quxin samples which first increased and then decreased. This suggested that the pectinase-producing microorganisms may be very abundant and have strong cell viability or have better enzymatic characteristics. Therefore, it may be a better option to screen for pectinase-producing microorganisms using the X7 or P10 samples.

Proteinase activity

Protein is an important carbon and nitrogen source for microorganisms in *Daqu* or the SSF of Chinese liquor. A diverse range of microorganisms produce proteinase during the *Daqu* fermentation and storage stages. The typical raw material of *Daqu* is barley, wheat, or peas with big fractions of starch and protein, which can be degraded by a range of enzymes derived from microorganisms and provide nutrients and substrates for their growth and even serve to form or transform the flavour compounds in Chinese liquor [25].

As shown in Figure 2C and 2D, the proteinase activities in the Qupi and Quxin samples exhibited similar trends as increasing first and then decreasing as time went on. For the two series of samples, the samples with the highest activities were all at the maturation stage. Among them, P7 had the highest proteinase activity (4,209.24 U) followed by P8 and P6. The highest proteinase activity in the Qupi was 264.56 U higher than that of the Quxin (X8, 3,944.69 U). Moreover, the lowest proteinase activity was observed in the first Quxin sample (merely 77.13 U), which was lower than the highest Qupi and Quxin samples by 54.58 and 51.14 times, respectively.

This result suggested that the microorganisms that secreted proteinases could grow better in the microenvironment of both the surface and core layers of *Daqu* although these microorganisms might not be the same species. Therefore, it may be easier to screen for proteinase microbes with high production or higher activity at the early maturation stage by using the Qupi or Quxin samples.

Esterase activity

Lipolytic enzymes, esterase (E.C. 3.1.1.1) and lipase (E.C. 3.1.1.3), belong to a group of enzymes whose biological function is to hydrolyse triacylglycerols [26]. Lipolytic enzymes have highly diversified industrial applications. They have emerged as key enzymes that have

been used in the food, dairy, paper, textile, leather, and detergent industries; and wastewater treatment [27-29].

Besides, esterase is also an important enzyme synthesizing esters in liquor production [30]. Many microorganisms can produce esterase including bacteria, filamentous fungi, and yeast. Therefore, *Daqu*, as the most important provider of microbial flora and many kinds of enzymes for Chinese liquor production, will be a good source of microorganisms that produce useful enzymes for different purposes. The esterase activity was investigated in *Daqu* samples during the *Daqu* fermentation and storage stages. As shown in Figure 2E and 2F, the changes in the esterase activities in the Qupi and Quxin samples exhibited similar tendencies. From the beginning of *Daqu* shaping, the esterase activity first increased and declined gradually with time, and then after storing the *Daqu* chunks in a special warehouse, the esterase activity increased and declined again during the maturation stages. The esterase activities ranged from 361.35 to 979.55 U in the Qupi and from 280.83 to 577.47 U in the Quxin samples, respectively. The P6 sample had the highest activity of 979.55 U (Figure 2E), which was 402.02 U higher than that of the highest Quxin sample, X7 (577.47 U, Figure 2F). Most of the esterase activities of the Qupi samples were higher than that of the Quxin samples. This implied that the better esterase producers might be more adapted to the microenvironment of the *Daqu* skin layer than that of the *Daqu* core or that the majority of esterase producers might be aerobes.

Conclusion

In this research, an orthogonal design was used to optimize the extraction of total protein coupled with the investigation of the activity development of representative enzymes from *Daqu*. The best combination of four factors was $A_3B_2C_3D_1$. The activities of the representative enzymes tended to change significantly during the fermentation and storage stages. Furthermore, the activities in both the Qupi and Quxin samples differed considerably from each other. All the activity varieties reflected the possible alterations in the microflora community. These variations also can provide very useful clues to sample, isolate, and screen novel enzyme producers which can be used to compensate the insufficiency of *Daqu*'s activity by adding extra enzyme preparations or enzyme producers to start the fermentation of Chinese liquor.

Acknowledgement

We gratefully acknowledge the financial support from the Project of Fundamental Research from Technological Office, Sichuan Province (2015JY0185 and 2016JY0159); Major project from Key Laboratory of Strong aroma liquor Solid-state Fermentation of Light Industry, China (2017JJ006); Major project from Key Laboratory of Solid-state Fermentation Resource Utilization of Sichuan Province (2015GTY001). Team Project Supported by Scientific Research Fund of Sichuan Provincial Education Department (18TD0041), and Major Science and Technology Project of Guizhou Province (2015-6012); The Advantageous Disciplines Food Science and Engineering of Yibin University.

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