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Metaproteomic Characterization of *Daqu*, a Fermentation Starter Culture of Chinese Liquor

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Abstract

Daqu is an essential fermentation starter for the prodcution of Chinese liquor, and it is closely related to the quality and yield of liquor. The aim of this study was to investigate the microbial community of *Daqu* by using the metaproteomic approach. A total of 45 protein spots in the two-dimensional electophoresis gel were excised and identified. Seventeen protein spots represent 16 proteins that originate from the secretion of bacteria, yeast, and filamentous fungi. Moreover, *Nitrobacter winogradskyi*, *Agrobacterium tumefaciens* and *Neurospora crassa* were first identified in *Daqu*. To the best of our knowledge, this is the first report on the community structure of Chinese liquor *Daqu* through metaproteomic analysis. Results presented in this study may further elucidate the microbial community structure in *Daqu* and may facilitate the development of *Daqu* for the manufacture of Chinese liquor.

Keywords: Chinese liquor *Daqu*; Metaproteomics; Microbial community

Introduction

Chinese liquor is one of the well-known distilled spirits in the world, and it has a long history of thousands of years. *Daqu* is a fermentation starter and substrate complex used to initiate fermentation for the production of Chinese liquor. It is an important saccharifying and fermenting agent and has significant impact on the flavor of the product [1]. According to the aromas of distillate, *Daqu* is mainly categorized into three different types: *Luzhou*-flavor (strong-flavor), soy sauce-flavor, and light-flavor, which depend on different manufacture techniques [2]. Generally, *Daqu* was produced through solid fermentation of grain via a natural inoculation of microbial communities originated from production environment, and the process often involves three stages: shaping, ripening and drying [3].

A diverse microbial community is associated with the *Daqu*, and consists of various types of bacteria, yeasts and filamentous fungi. Therefore, *Daqu* can be considered as a complex system containing materials (wheat, barley and/or pea), microbial community, and abundant enzymes originating from the microbes [4,5]. Already, some research has revealed the microbial community in *Daqu* based on culture-dependent and culture-independent methods including polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) and phospholipid fatty acid (PLFA) [3,6-8]. Zheng et al. [6] isolated and identified 190 microbial strains from *Fen-Daqu* by culture-dependent approach, which comprised 109 bacteria and 81 yeasts and moulds. Recently, the development of lipid- and nucleic acid-based profiling techniques has enabled the elucidation of microbial community structures in complex ecosystem, and has led to the discovery of new genes [9-11]. Unfortunately, knowledge of community structure does not necessarily provide useful information on functions such as metabolic capacity, population dynamics, and physiological responses to variable environmental conditions. Metaproteomics, also termed community proteomics, is the study of all proteins expressed at a given time in an ecosystem, and it provides an insight into cellular and community activity-information unavailable from any other approaches [12]. Microbial metaproteomics has been applied in diverse environments such as soil, marine, and human intestinal tract [13-16]. In this study, the proteins isolated from the *Daqu* extract were investigated based on metaproteomic method. To the best of our knowledge, this is the first report of metaproteome in *Daqu*. These results may aid the advancement of research on the function of *Daqu* and promote the technological development of *Daqu* manufacture.

Materials and Methods

Daqu sample

Daqu samples were collected from *Xufu* liquor brewing enterprise in Yibin city, Sichuan Province, China. Three matured *Daqu* blocks (having been stored for 3 months of maturation) were randomly selected from upper, middle and lower layers, respectively, in order to obtain an adequate representation. The samples collected from each layer were fully mixed and stored in sterile polyethylene bags at -20°C for further analysis.

Preparation of metaproteome from *Daqu* extract

The extract of *Daqu* was prepared according to Zhang et al. [17] with some modifications. Briefly, 5 g of *Daqu* sample was soaked in 10 ml of 50 mM acetate buffer (pH 4.2, containing 90 mM NaCl) for 12 h. The suspension was centrifuged twice (10000 g, 10 min) to remove the large particles. The supernatant was then filtered through a 0.45 μm filter (Sangon Biotech, Shanghai, China) and prepared for metaproteome extraction. The extracted proteins were treated with Clean-up Kit (BioRad, Hercules, CA, USA) according to the manufacturer's protocols, and dissolved in lysis buffer (8 M urea, 2%

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CHAPS, 0.5% IPG buffer). The protein concentration was determined by using the BioRad Protein Assay Kit (BioRad) with BAS as a standard.

2D gel electrophoresis and protein identification

The protein sample was applied to immobilize pH gradient strips (18 cm, pH 3-10, BioRad) with a final concentration of 700 μg protein in 350 μl rehydration buffers. Isoelectric focusing (IEF) and SDS-PAGE were performed according to the method described by Zhang et al. [17]. After SDS-PAGE, the 2D gels were stained with 0.1% Coomassie blue R-250. Three parallel gels were consistent replicates, and the stained gels were scanned using Imagescanner (GE Healthcare).

The proteins spots were excised using gel plugs, transferred to Eppendorf tubes, then digested with 20 μl of 10 ng/μl proteomics sequencing grade trypsin at 37°C for 16 h and rehydrated in 500 μl of 50 mM NH_4HCO_3 (pH 8.0). Supernatants of 0.5 μl were spotted directly onto the MALDI plate, and samples were analyzed on the Applied Biosystem 4700 Proteomics Analyzer MALDI-TOF/TOF (Applied Biosystems, Framingham, MA) in positive ion reflector mode [18]. Mass spectra were obtained for each sample by accumulating of 700-4000 Da mass range. For the MS/MS spectra, 5 most abundant precursor ions per sample were selected for subsequent fragmentation, and 1000-1200 Da laser shots were accumulated per precursor ion. The criterion for precursor selection was a minimum S/N of 50. GPS Explorer (v2.0, Applied Biosystems) was used as an interface between the raw data from the mass spectrometer. Both MS/MS and MS data were used for the identification of proteins candidates in the NCBI database by using Mascot (Matrix Science) with the following selection criteria: NCBInr database, taxonomy of all entries, trypsin of the digestion enzyme, one missed cleavage site, parent ion mass tolerance at 100 ppm, MS/MS mass tolerance of 0.5 Da, carbamidomethylation of cysteine (global modification), and methionine oxidation (variable modification). The probability score (95% confidence interval) calculated by the software was used as criteria for correct identification [17,19]. In addition, given the complexity of *Daqu* sample, the mass spectra were firstly searched against the "all entries" database in NCBInr, then the Bacteria, Fungi, and "others" databases were separately selected to avoid the failed matching. After the comparison against NCBI's "nr" database through BLAST, sequences could be classified by using the lowest common ancestor analysis based on Unipept database, which is a web application available at unipept.ugent.be.

Results and Discussion

Metaproteomic profile of *Daqu* extract was obtained by 2-DE and the representative map was shown in Figure 1. A total of 45 proteins spots were excised, and only 21 spots representing 20 proteins were successfully identified. This might be ascribed to the incomplete genome information available on environmental microbes [17]. A detailed analysis of this result showed that 17 spots representing 16 proteins originated from the secretion of bacteria, and yeasts (Table 1). Three proteins (spots 5, 6 and 21) originated from *B. subtilis*, and 3 proteins (spots 8, 9 and 17) originated from *B. licheniformis* were identified in *Daqu* sample. Previous reports demonstrated that

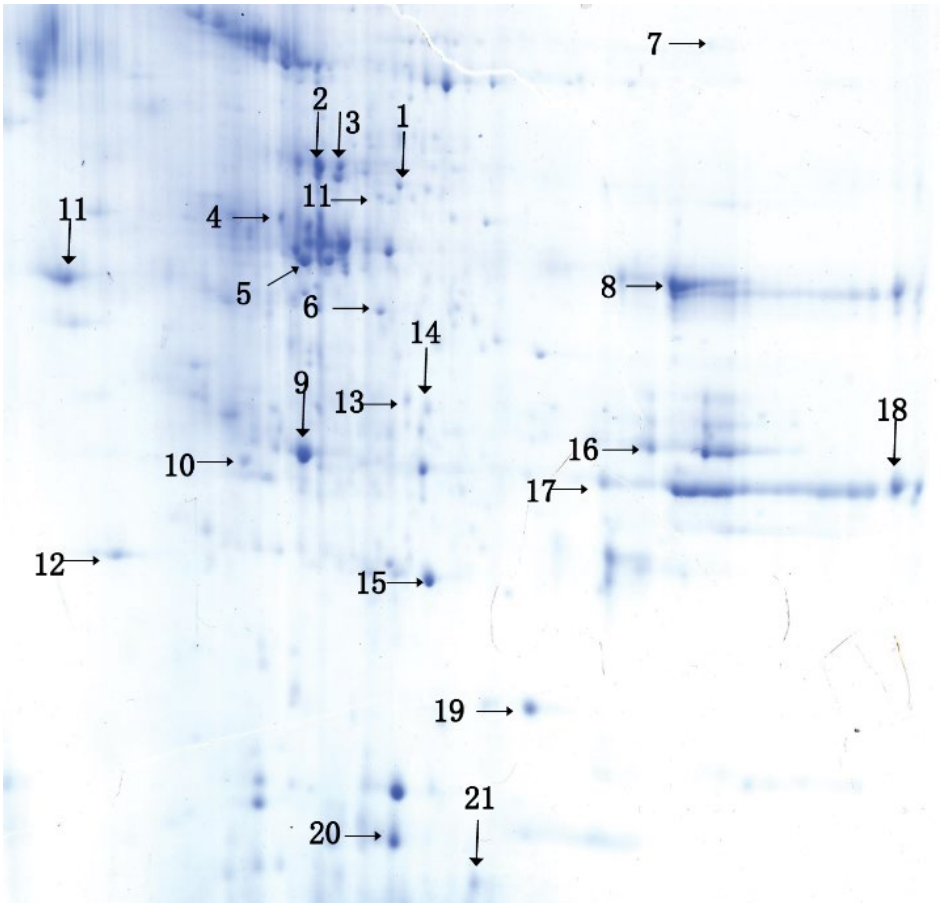

Figure 1: Metaproteomic profile of *Daqu* extract. The identified proteins are indicated by arrows and numbers and listed in Table 1.

Spot no.[a]	NCBI accession no.	Protein name	Mass (Da)	pI	MASCOT score[b] (MS/MS)	Coverage (%)	Best matched organism
1	gi|115312258	ATP synthase subunit alpha	55262.1	6.95	62.5	13	*Nitrobacter winogradskyi*
2	gi|38257741	Chromosomal replication initiator protein DnaA	50894.57813	6.15	74.8	35	*Clostridium acetobutylicum*
3	gi|38257741	Chromosomal replication initiator protein DnaA	50894.57813	6.15	68.9	29	*Clostridium acetobutylicum*
4	gi|75279910	Serpin-Z1B	43119.9	5.44	107	31	*Triticum aestivum*
5	gi|38605545	Uncharacterized protein YceH	41646.46875	5.9	74.4	78	*Bacillus subtilis*
6	gi|239938752	Uncharacterized HTH-type transcriptional regulator YcgK	36598.32813	6.73	87.9	68	*Bacillus subtilis*
7	gi|1709755	U4/U6 snRNA-associated-splicing factor PRP24	51272.51953	9.35	116.5	12	*Saccharomyces cerevisiae*
8	gi|73921052	UPF0348 protein BLi01723/BL03002	47303.39844	9.20	121.3	46	*Bacillus licheniformis*
9	gi|5200416666	Two-component response regulator	29500.4	6.20	152.2	39	*Bacillus licheniformis*
10	gi|74676389	Pyridoxamine 5'-phosphate oxidase YLR456W homolog	23449.85938	5.85	135.4	27	*Saccharomyces cerevisiae*
11	gi|109827650	Elongation factor Ts	32139.78906	5.26	72.5	15	*Nitrobacter winogradskyi*
12	gi|254767251	Nucleoside diphosphate kinase	16708.53906	5.29	96.8	31	*Staphylococcus carnosus*
13	gi|75258740	Auxin-responsive protein IAA7	32414.19922	6.34	68.1	41	*Oryza sativa* subsp. *japonica*
14	gi|73622088	Xylanase inhibitor protein 1	33538.71094	8.66	127	19	*Triticum aestivum*
15	gi|123975	Endogenous alpha-amylase/subtilisin inhibitor	19848.85938	6.77	169	26	*Triticum aestivum*
16	gi1709755	U4/U6 snRNA-associated-splicing factor PRP24	51272.51953	9.35	121	46	*Saccharomyces cerevisiae*
17	gi|52003909	Putative glutathione peroxidase	18204.5	9.23	152	39	*Bacillus licheniformis*
18	gi|42558872	Cytochrome c-556	15260.5498	8.26	68.8	16	*Agrobacterium tumefaciens*
19	gi|74631055	Chromosome transmission fidelity protein 8 OS	12471.7	9.14	61.5	27	*Schizosaccharomyces pombe*
20	gi| 189045117	Elongation factor 2	93545.40625	6.24	65.3	18	*Neurospora crassa*
21	gi|264664558	Uncharacterized protein YdzU	10791.75	9	128	45	*Bacillus subtilis*

a: Numbers corresponding to the 2-DE map in Figure 1.

b: Only results for proteins that had scores of greater than 60 and were reproducibly identified are shown.

Table 1: Proteins identification in *Daqu* extract.

Bacillus species including *B. subtilis*, *B. licheniformis*, and *B. cereus* were dominant in strong-flavor *Daqu*, and were frequently detected in *Daqu* [2,6]. The majority of Bacillus species secretes various hydrolytic enzymes and form heat-resistant spores, and the existence of such microbes may facilitate the conversion of starch into fermentable carbohydrates. In addition, *Bacillus* spp. produces nitrogenous flavor compounds such as diverse pyrazines, which may contribute to the *Daqu* flavor [2,5]. Nucleoside diphosphate kinase (spot 12), originating from *Staphylococcus carnosus* was identified in *Daqu* sample, and Staphylococcus was also identified in *Daqu* revealed by nested PCR-DGGE [2]. Generally, *S. carnosus* produces 3-methyl-1-butanol, 2-butanone, acetoin, and methyl-branched ketones, which may play an important role in liquor manufacture [20]. Two Chromosomal replication initiator proteins DnaA (spots 2 and 3) originating from *Clostridium acetobutylicum* was identified in *Daqu* extract. The Clostridium strains favorably ferment starch materials and typically produce a solvent mixture of acetone, butanol and ethanol [21]. Moreover, Clostridium was reported as important microorganism in the pit mud of Chinese liquor [22]. In addition, it should be noted that 2 proteins (ATP synthase subunit alpha and Elongation factor Ts), 1 protein (Cytochrome c-556), and 1 protein (Elongation factor 2) were identified from *Nitrobacter winogradskyi*, *Agrobacterium tumefaciens* and *Neurospora crassa*, respectively. Such strains were first identified in *Daqu*, and the functions of these microbes should be further elucidated.

Yeasts are the most important group of microorganisms contributing to liquor quality in the solid-state fermentation process of Chinese liquor. In this study, three proteins (spots 7, 10 and 16) were determined to originate from *S. cerevisiae*, which was also detected in strong-flavor, soy sauce-flavor and light-flavor *Daqu* samples [6,23]. However, previous studies showed that the band density of *S. cerevisiae* was relatively weak in DGGE profiles, and only one strain of *S. cerevisiae* was obtained by culture-dependent method, suggesting that *S. cerevisiae* was not a dominant microbe in *Daqu* [6,24]. Generally, *S. cerevisiae* usually dominates in alcoholic fermentations due to its higher ethanol tolerance and ability to grow under strictly anaerobic conditions [6]. Chromosome transmission fidelity protein (spot 19), secreted by *Schizosaccharomyces pombe* was also detected in *Daqu* extract, implying potential function of *S. pombe* in liquor fermentation. Previous research demonstrated that the population of yeasts in *Daqu* was less than 100 CFU/g, and the number in mature *Daqu* was less than that in ripening period of *Daqu*. This may be that most yeasts had died after ripening process of *Daqu* [24].

Compared to the PCR-based data, the metaproteomic approach just identified a few fungal genera and some new genera in *Daqu* sample. This may be the limitations in metaproteocmic method, which requires enough proteomic and genomic information being available on the environmental microbes. Thus, alternate research approaches could help gain a comprehensive understanding of the microbial community and its function in *Daqu*.

In this study, metaproteomic approach was employed to investigate the ecosystem of *Daqu*, the starter culture of Chinese liquor, and this has not been reported prior to this study. A total of 17 proteins originated from the secretion of microbes were identified, and strains *Nitrobacter winogradskyi*, *Agrobacterium tumefaciens*, and *Neurospora crassa* were firstly identified in *Daqu*. Results presented in this study further enhance our understanding of the microbial community in *Daqu*, and the functions of the proteins from microbes in *Daqu* remains to be elucidated.

Author's Contribution

Chongde Wu and Rongqing Zhou designed the study and prepared the manuscript, Jingcheng Deng and Guiqiang He performed the experiment. All authors review and approved the final manuscript.

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