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Transcriptome Profiling of Heat-Resistant Strain *Bacillus licheniformis* CGMCC3962 Producing *Maotai* Flavor

Qun Wu and Yan Xu*

State Key Laboratory of Food Science and Technology, Key Laboratory of Industrial Biotechnology of Ministry of Education, School of Biotechnology, Jiangnan University, Wuxi, Jiangsu, China 214122

ABSTRACT: Although *Maotai* flavor liquor is exclusive due to its soy sauce flavor, knowledge of its key compound and production mechanism is still scarce until now. To gain insight into the production mechanism of soy sauce flavor, a soy sauce flavor producing strain with high efficiency and heat-resistant capability was obtained, and the metabolic mechanism of the strain was investigated with the technique of microarray profiling. Because high temperature was a key factor for soy sauce flavor production, the global gene expression of this heat-resistant strain fermented at 55 °C was analyzed. Except for the responsive increase of heat shock proteins, which maintained cell survival during heat stress, biosynthesis of cysteine was also up-regulated. In addition, some metabolites were significantly increased when cysteine was added to the fermentation medium, such as 2,3-butanediol, 3-hydroxy-2-butanone, and tetramethylpyrazine, which were important flavor compounds in soy sauce flavor liquor and might be related with soy sauce flavor. The results indicated that cysteine might play an important role in the formation of soy sauce flavor compound, and it might act as an indirect precursor or stimulator of soy sauce flavor formation. This was the first use of the microarray profiling tool to investigate the fermentative strains for Chinese traditional liquor, which would allow a deeper insight into the mechanism of the formation of soy sauce flavor compound.

KEYWORDS: *Bacillus licheniformis, cysteine, heat stress, Maotai flavor liquor, soy sauce flavor, transcriptome profiling*

INTRODUCTION

Maotai flavor liquor, distilled from fermented sorghum, is called the national drink of China. It is as symbolic a drink of China as whiskey is of Scotland and brandy of France.[1] It is exclusive due to its soy sauce-like and roasted aroma style, which is the result of a complex microbiological and biochemical process in *Maotai* flavor liquor fermentation.

The aroma style of Chinese liquor is mainly contributed by trace aroma compounds, such as alcohols, esters, acids, acetals, ketones, aldehydes, sulfur-containing compounds, lactones, and heterocyclic compounds.[2] Many studies have reported that ethyl caproate and ethyl acetate were the key aroma compounds in the strong aroma style liquor and light aroma style liquor, respectively, which are the other two important styles of Chinese liquor.[3] However, although great efforts have been made for more than 50 years, the key aroma compound related to the soy sauce aroma style was still not clear due to the complex ingredients and low content of flavor compounds in *Maotai* flavor liquor.[4,5] Recently, Fan et al.[6] identified 76 volatile compounds in Chinese soy sauce aroma style liquor, including 25 esters, 10 alcohols, 9 aldehydes and ketones, 8 aromatic compounds, 5 furans, 3 nitrogen-containing compounds, 6 acids, 4 phenols, 3 terpenes, 1 sulfide-containing compound, 1 lactone, and 1 acetal. However, none of them exhibited soy sauce flavor. In addition, the key flavor of Chinese soy sauce aroma style liquor was also different from that of Japanese soy sauce; the main compositions and key flavor compounds between the two traditional foods were also different.[7] The uncertainty about the key soy sauce aroma compounds led to confusion about the origin and production mechanism of the flavor compound, which then made it difficult to understand the fermentation process of *Maotai* flavor liquor and, consequently, hinder the development of *Maotai* flavor liquor.

Fortunately, a strain of *Bacillus licheniformis* was isolated from the high-temperature *Daqu* during *Maotai* flavor liquor making process in this study. The strain produced a strong soy sauce flavor in wheat bran extract medium by submerged fermentation, and the flavor was the same as that of *Maotai* flavor liquor. Therefore, it was deduced that the *Maotai* flavor originated from the bacteria, which would produce the key soy sauce flavor compound. This brought hope to us, and the understanding of the metabolic mechanism of this producer would help us to shed light on the key flavor compound production process and then to improve the manufacturing process of *Maotai* flavor liquor.

Functional genomic approaches, such as microarray profiling, are powerful tools for the analysis of gene expression at the scale of the genome and provide a comprehensive view of cellular physiology.[8] The rapidly developing DNA microarray technology provided us with a highly efficient method to study cellular response to extracellular stimuli, allowing an insight into the mechanism of environmental stress on cells. Until now, global gene expression analysis has been frequently carried out for yeast. For example, the analysis of gene expression in *Saccharomyces cerevisiae* with salt stress and heat shock stress led to information about the regulatory mechanism involved in the salt and heat stress response.[9,10] On the other hand, the overview of the transcriptome profile of cotton fiber

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cell revealed a major role of ethylene in cotton fiber cell elongation.[11] However, as far as we know, the microarray profiling tool has been mainly utilized in eukaryotic cells. There were only a few reports of the utilization of this tool to deal with the expression profile of protokaryotic cells, such as *B. licheniformis*.

In this paper, we found that *B. licheniformis* producing soy sauce flavor was heat-resistant, and high temperature (55 °C) was significant for the production of the key soy sauce flavor compound by it. To gain the mechanism by which this functional strain regulated gene expression to cope with fermentative conditions and to further produce favorable flavor compound, the genome-scale DNA microarray was employed to monitor the change of global expression profile when this strain was cultured from 37 to 55 °C. The gene ontology (GO) annotations and metabolic pathways for the responsive expressed genes were combined to investigate the response logic of the cells to high temperature. This was the first time to explore the metabolic mechanism of the soy sauce flavor compound in the functional strain with the technique of microarray profiling analysis. It would help to explore the information of the ingredient of key soy sauce flavor compound and its precursors and also be beneficial for the development of *Maotai* flavor liquor fermentative industry.

MATERIALS AND METHODS

Strain Isolation and Cultural Conditions. *B. licheniformis* ATCC 14580 was obtained from Shanghai Institute of Industrial Microbiology, which would not produce soy sauce flavor. The soy sauce flavor producing strain was isolated from the high-temperature *Maotai* flavor *Daqu* from a *Maotai-flavor* liquor production factory in Guizhou, China. The sample of *Daqu* (5 g) was mixed with 25 mL of sterile deionized water and shaken for 1 h. The cell suspension was then heat treated in a water bath at 80 °C for 20 min. For the enrichment of the microorganisms, a 1% inoculum of cell suspension was added into 20 mL of basal medium [(g/L) glucose, 10; peptone, 10; beef extract, 5; NaCl, 5; initial pH, 7.2] in a 100 mL flask at 37 °C for 1 day with agitation; the culture was then diluted and spread on nutrient agar [(g/L) glucose, 10; peptone, 10; beef extract, 5; NaCl, 5; agar, 15; initial pH, 7.2] and incubated at 37 °C for 1 day. Pure cultures with different morphologies were obtained. Strains were inoculated in 50 mL of wheat bran extract medium and incubated at 55 °C for 5 days with an agitation speed of 150 rpm. One strain producing strong soy sauce flavor was picked up and identified as *B. licheniformis*. It was deposited in China General Microbiological Culture Collection Center with the accession no. of CGMCC 3962. Flask experiments in this work were the same with the above culture methods.

RNA Extraction and cDNA Preparation. Total RNA was extracted from *B. licheniformis* CGMCC 3962 cells cultured at 37 and 55 °C using RNeasy Micro Kits (Qiagen) according to the manufacturer's instruction, and DNase I was used to remove DNA contamination. The quality of the obtained RNA was evaluated using a nanobiosizing assay (Agilent). The RNA sample was then amplified and labeled using a NimbleGen One-Color DNA Labeling Kit according to the manufacturer's instruction.

Gene Expression Arrays. The obtained cDNA sample was used for gene expression array processing. The expression array chip used contained 385 000 probes representing 4196 genes (NimbleGen). Target cDNA was hybridized to microarrays using a NimbleGen Hybridization System (NimbleGen). After hybridization, the processed slides were scanned with an MS200 scanner (NimbleGen). The raw data of fluorescent intensity were extracted by NimbleScan software (version 2.5), normalized and background-corrected using the Robust Multichip Average algorithm.[12] The differentially expressed genes were further analyzed by the statistical methods previously reported.[12] Genes with P values of <0.05 and an absolute fold change of >2 were identified as significantly differentially expressed genes.

Quantitative Real Time RT-PCR (qRT-PCR). For samples to be used for q RT-PCR, Superscript III (Invitrogen) reverse transcriptase was used to prepare cDNA from amplified RNA. Random primers (Invitrogen) were used to prime the reaction. Relative transcript abundance was detected by SYBRGreen (Invitrogen) on the ABI 7900HT thermal cycler using gene-specific primers. The specificity of the primer pairs was as follows: sat, 5′-TTTCCTCAATCCGCTCGT-3′ (forward) and 5′-CGTAGTTCTTTCTCACAAGGG-3′ (reverse); yrhA, 5′-CAATACGGGAATCGGTCTG-3′ (forward) and 5′-TCAATCGCACCCTTCATCC-3′ (reverse).

Gene Ontology (GO) and Pathway Analysis. The Database for Annotation Visualization and Integrated Discovery (DAVID) (http://david.abcc.ncifcrf.gov), the Gene Ontology Project (http://www.geneontology.org), and the KEGG pathway database (http://www.genome.jp/kegg/pathway.html) were used for annotating regulated transcripts with a gene ontology and pathway designation.

Extraction and Analysis of Volatile Flavor Compounds. A total of 250 mL of fermentation broth was concentrated at 1000 rpm for 10 min to remove the cells, and then 60 g of NaCl was added to the broth to get the saturated solution. Then the solution was transferred to a separatory funnel and extracted three times with 20 mL of dichloromethane. The organic layer was recovered in a separating funnel and was concentrated in a Kuderna-Danish concentrator to 1 mL and then under a stream of nitrogen (1.5 L/min).

Identification was carried out using an Agilent 6890N GC coupled to an Agilent 5975 mass selective detector (MSD). The concentrate (1 μL) was analyzed on a DB-Wax column (30 m × 0.25 mm i.d., 0.25 μm film thickness, J&W Scientific). GC-MS analysis was performed according to the method reported.[13] Identification of all compounds was confirmed by mass spectra and retention indices with those standards. Single standard sample stock solutions of these volatile compounds were prepared by dissolving each compound in absolute ethanol. Working solutions with a range of concentrations were made from the stock solutions by dilution with wheat bran extract. Ten more calibration solutions were prepared by serially diluting the working solution by 2-fold. All calibration curves had R^2 values of at least 0.99. Triplicate analysis was performed for each sample.

RESULTS AND DISCUSSION

Effect of Culture Temperature on Flavor Production. *B. licheniformis* CGMCC 3962 was selected as the target strain, which would efficiently produce soy sauce flavor. We simulated the fermentation condition in the manufacturing process of *Maotai*–*Daqu*, using wheat bran, the raw material of *Fuqu*, as fermentation medium. The strain was cultured at different temperatures for 5 days, and the produced flavor was evaluated. As shown in Table 1, fermentation without strains inoculated in wheat bran extract exhibited only material or sweet flavor. In addition, another *B. licheniformis*, ATCC 14580, which was used as the control, would not produce soy sauce flavor. This indicated that only strain *B. licheniformis* CGMCC 3962 would produce the target flavor, which would not be produced by the medium itself at high temperature. Moreover, it was found that

Table 1. Effect of Culture Temperature on Flavor Production

	sensory evaluation		
strain	37 °C	45 °C	55 °C
none	wheat bran flavor	wheat bran flavor	sweet
B. licheniformis ATCC 14580	smelly	smelly	smelly
B. licheniformis CGMCC 3962	smelly	rancid	soy sauce-flavor

the temperature was important for soy sauce flavor formation, because only the strain cultured at 55 °C would produce soy sauce flavor. The result was in agreement with the fact that in *Maotai* flavor liquor making, as the high temperature (the fermentation temperature was kept around 55−62 °C) was also significant for *Maotai* flavor formation.

Comparative Transcriptome Analysis of *B. licheniformis* CGMCC 3962 Response to High-Temperature Stress. To gain further insight into the soy sauce flavor formation mechanism, a genome-scale microarray of *B. licheniformis* 3962 was constructed in our laboratory to investigate the global response to high temperature (55 °C) at the gene expression level. The advanced functional research on this functional strain has facilitated the elucidation of action mechanisms of temperature stress. *B. licheniformis* CGMCC 3962 was cultured at 55 and 37 °C, and the genome expression level was profiled, respectively. Of the 4196 genes on the microarray, a total of 675 genes (16%) with a change of at least 2-fold were identified as differentially expressed genes with high-temperature stress (55 °C), compared with the cell cultivation at 37 °C. Three hundred and sixty-six genes were up-regulated and 309 were down-regulated, of which 267 genes were increased between 2- and 3-fold and 99 genes were increased >3-fold.

GO annotations for the differentially expressed genes reveal several significant trends in the gene expression program. GO has been used widely to predict gene function and classification.[14] To further investigate transcriptional regulation, the 99 genes that increased >3-fold were successfully annotated and mapped to three organizing principles of GO: biological process, molecular function, and cellular component. The matches obtained from this analysis are summarized in Figure 1. Within the biological process class, the metabolic process (GO: 0008152), cellular process (GO: 0009987), and cellular metabolic process (GO: 0044237) categories were the most represented followed by "primary metabolic process (GO: 0044238)" (Figure 1A). Within the molecular function class, the "RNA binding (GO: 0003723)" term is the most represented followed by "structural constituent of ribosome (GO: 0003735)" and "structural molecule activity (GO: 0005198)" (Figure 1B). Within the cellular component class, "intracellular (GO: 0005622)" is the most highly represented, followed by "intracellular part (GO: 0044424)" (Figure 1C).

The strain was isolated from the high-temperature *Maotai* flavor *Daqu*. It could survive at 55 °C or even higher temperature. It was necessary for us to investigate the heat shock mechanism of this strain, which was the prerequisite for the cells to produce soy sauce flavor compound. Heat shock proteins protected proteins from the deleterious effects of stresses by acting as chaperones or facilitating the degradation of proteins denatured by stress.[15] The expression patterns of some heat shock proteins when the strain was fermented at 55 °C are shown in Table 2, and their expression levels all increased >2-fold. The functions of genes were mainly involved in degradation of misfolded proteins, such as *clpX*, *clpQ*, *yvtA*, and *hslU*. In addition, it was important to find *groES* and *groEL*, which induced fold changes of 4.5 and 9.1, respectively. They were class I heat shock proteins (chaperonin) and promoted the refolding of misfolded polypeptides under extremely stressful conditions. It appeared that they played important roles in thermal tolerance in maintaining this strain cultured at 55 °C.

On another hand, it was noted that the three genes involved in poly-γ-DL-glutamic acid (γ-PGA) biosynthesis were also up-regulated, and they nearly represented the highest fold change of induced genes identified, with *ywtA* increased 10.0-fold, *ywsC* 12.9-fold, and *ywtB* 21.2-fold. γ-PGA was an anionic, extracellular polymer, in which the α-amino and γ-carboxy groups of D- or L-glutamic acid were linked by isopeptide bonds.[16] It was produced primarily by *Bacillus* strains and was involved in capsule biosynthesis. The capsule was exposed to the outermost cell surface and played important roles in cell attachment for colonization, biofilm formation, and protecting cells from environmental factors such as high osmotic pressure and high temperature.[17,18] This indicated that γ-PGA also played an important role in the survival of this strain when it was cultured at high temperature.

Analysis of Metabolic Pathways in *B. licheniformis* CGMCC 3962 Response to High-Temperature Stress. Except for the responsive genes and their functions in this strain confronted with heat stress, we were most interested in the different metabolic pathways and the related intermediate metabolites of soy sauce flavor in *B. licheniformis* CGMCC 3962. To investigate how the cells had the capability to produce the related metabolites during heat stress, metabolic pathways from the 667 responsive genes obtained from the KEGG databases were characterized according to the expression profiles. As shown in Table 3, 12 of these pathways were regulated and 7 of these pathways had P values of <0.05.

The most significant responsive metabolic pathway was ribosome (bld03010, $P = 3.84 \times 10^{-6}$), which accounted for a high proportion of the overall protein synthetic capacity (Figure 1). Of all 28 responsive ribosomal protein genes, 24 ribosomal protein genes were up-regulated, including the ribosomal protein L4 (GeneID 3100325); its subunit was important during the early stages of 50S assembly. These results suggested the increasing of normal protein synthesis and vigorous growth, which indicated the high heat resistance capacity of this strain. It was different from that in the thermolabile strain *S. cerevisiae*, having a ribosome protein that was down-regulated with heat stress.[9] It was also the specialty of this thermostable strain that the high amount of expressed proteins, especially some important enzymes, might be related to cell survival during heat stress and the production of soy sauce flavor.

In addition to the metabolic pathway of ribosome, the fructose and mannose metabolism, porphyrin and chlorophyll metabolism, galactose metabolism, sulfur metabolism, pyrimidine metabolism, and starch and sucrose metabolism showed P values of <0.05 as well. Except for the two metabolic pathways sulfur metabolism (bld00920) and starch and sucrose metabolism (bli00500), the other significant responsive pathways were all related to the biological process and cellular metabolic process. The genes related to the catabolism of starch and sucrose in the starch and sucrose metabolism were down-regulated, suggesting that the decreased catabolism of starch and sucrose saved energy. On the other hand, sulfur metabolism had a P value of 0.029 and ranked fifth among all pathways identified (Table 4). It was interesting to note that the biosynthesis of homocysteine decreased, as the homocysteine formation related genes *yjc*I (Gene ID 3100356) and *yjcJ* (Gene ID 3101326) decreased 2.2- and 2.3-fold, respectively, whereas *sat* (Gene ID 3100267) and *yrhA* (Gene ID 3098113) increased 3.1- and 2.4-fold, respectively. *SAT* and *YRHA* both functioned in cysteine biosynthesis; the significant induction of these two genes indicated that cysteine anabolism was up-regulated. To confirm the microarray data, we analyzed the relative mRNA levels of two genes (*sat* and *yrhA*) by qRT-PCR,

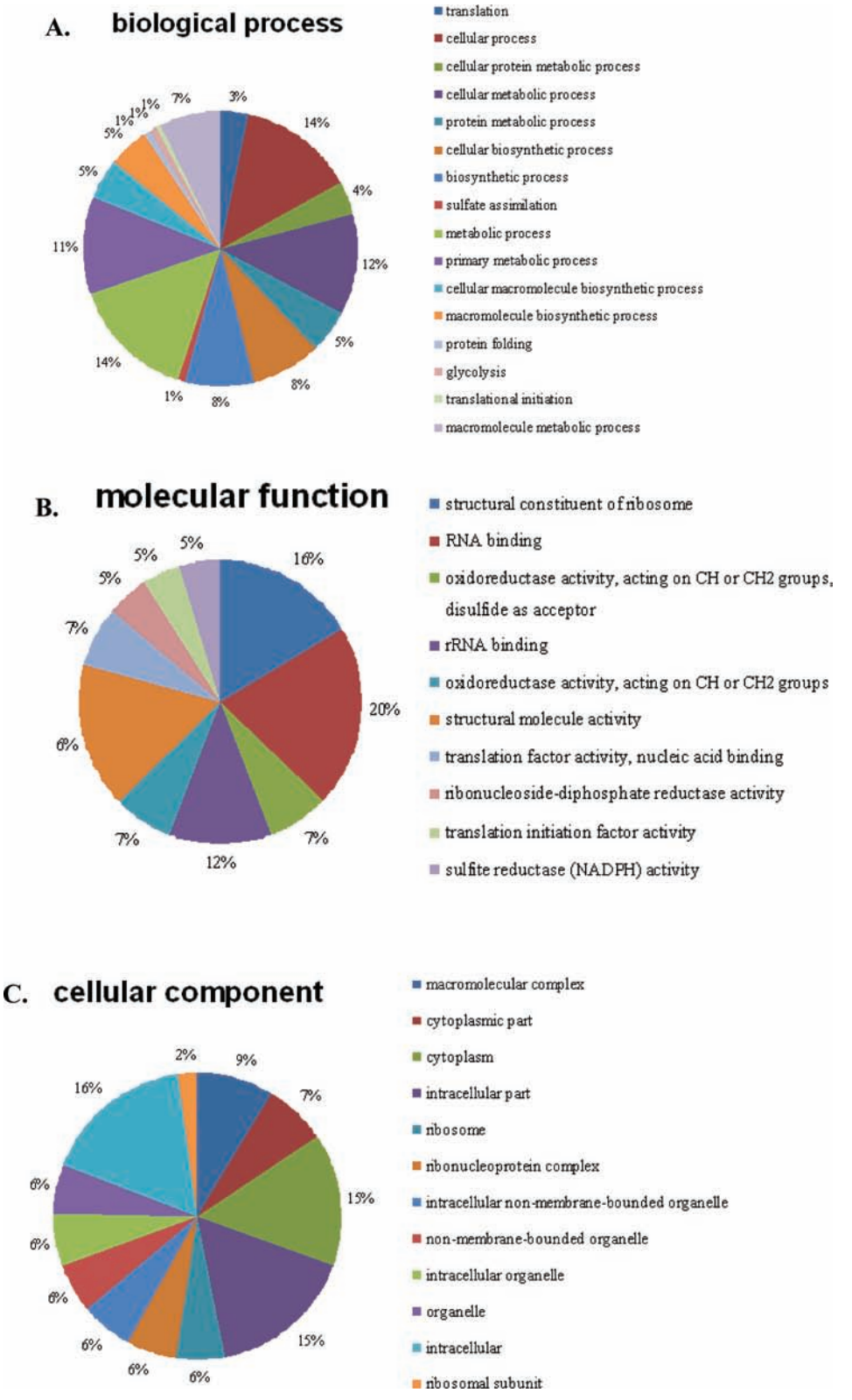

Figure 1. Summary of the gene ontology annotation of induced (>3.0-fold change) genes of *B. licheniformis* CGMCC 3962 with fermentation at 55 °C, compared with that at 37 °C, in the representatively functional classes: (A) most represented GO terms (based on number of represented sequences) of the main category "biological process"; (B) most represented GO terms of the main category "molecular function"; (C) most represented GO terms of the main category "cellular component".

and the results suggested that the expression levels of *sat* and *yrhA* were 3.8 and 2.6, respectively, which showed good agreement between the microarray and qRT-PCR analyses.

It was generally considered that the production of a specific flavor compound is a complex process, which included several biochemical reactions. The results from the global profile of

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Table 2. Heat Shock Related Genes

gene ID	gene symbol	gene function	fold induction
3098420	*clpX*	ATP-dependent *Clp* protease; binds and unfolds substrates as part of the *ClpXP* protease	2.5
3099395	*clpQ*	two-component ATP-dependent protease; heat shock protein involved in degradation of misfolded proteins	2.5
3097786	*dnaK*	class I heat shock protein (molecular chaperone)	2.6
3098618	*ftsH*	cell division protein/general stress protein (class III heat shock)	2.7
3100023	*yvtA*	similar to *HtrA*-like serine protease	2.7
3100236	*hslU*	two-component ATP-dependent protease; heat shock protein involved in degradation of misfolded proteins	2.8
3100069	*groES*	class I heat shock protein (chaperonin); binds to one or both ends of the *GroEL* double barrel in the presence of adenine nucleotides	4.5
3097938	*groEL*	class I heat shock protein (chaperonin); promotes refolding of misfolded polypeptides, especially under stressful conditions	9.1

Table 3. Responsive Metabolic Pathways

term	gene count	*P* value
bld03010:ribosome	28	3.84×10^{-6}
bld00051:fructose and mannose metabolism	14	0.003
bld00860:porphyrin and chlorophyll metabolism	9	0.010
bld00052:galactose metabolism	11	0.016
bld00920:sulfur metabolism	7	0.029
bld00240:pyrimidine metabolism	15	0.048
bli00500:starch and sucrose metabolism	13	0.049

Table 4. Responsive Genes Related with Sulfur Metabolism

gene ID	gene symbol	gene function	gene regulation	fold change
3100474	*yvgR*	similar to sulfite reductase	up	3.9
3099106	*cysC*	probable adenylylsulfate kinase	up	3.5
3100357	*yvgQ*	similar to sulfite reductase	up	3.1
3100267	*sat*	probable sulfate adenylyltransferase; converts ATP and sulfate to diphosphate and adenylsulfate; functions in cysteine biosynthesis	up	3.1
3098113	*yrhA*	similar to cysteine synthase	up	2.4
3100356	*yjcI*	similar to cystathionine γ-synthase; catalyzes the formation of L-homocysteine from cystathionine	down	2.2
3101326	*yjcJ*	similar to cystathionine β-lyase; catalyzes the formation of L-homocysteine from cystathionine	down	2.3

gene expression made us realize that the responsively increasing expression of cysteine biosynthesis genes by this functional strain might have some relationship with soy sauce flavor compound; that is, cysteine might be one of the precursors, and the increasingly produced cysteine might be quickly catabolized by this strain and then reacted to form flavor compounds, which might be related with soy sauce flavor.

Characterization of the Pattern of Cysteine Metabolism. Because cysteine might have some roles in soy sauce flavor production in this strain, the time profile of free cysteine concentration in fermentation broth was characterized during the fermentation period. As shown in Figure 2, cysteine was produced quickly in 0–2 days. It remained at around 20 mg/L at 2–4 days and decreased quickly until the end of fermentation. It was deduced that cells grew and produced cysteine at 0–2 days, and then the indirect precursor cysteine was catabolized to form some flavor metabolites at 2–4 days; the formation rate of the product might be in accordance with the consumption rate of the precursor, and after 4 days, although the consumption of cysteine was maintained, the production of cysteine decreased due to the decreasing cell activity, which led to the decrease of free cysteine concentration.

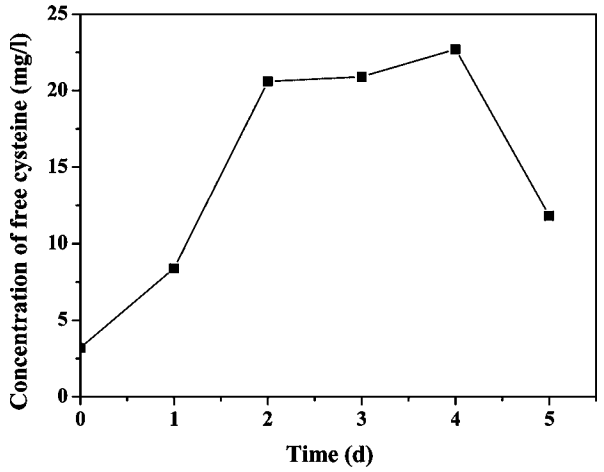

Figure 2. Time profile of free cysteine concentration during the fermentation period.

Characterization of Roles of Cysteine in Soy Sauce Flavor Formation Process. The addition of cysteine would bring some information about the effect of cysteine on soy sauce flavor formation. Then cysteine with two different concentrations was added in fermentation medium, respectively. To find some information about the flavor compounds, the profile of volatile metabolites in fermentation broth, which also exhibited soy sauce flavor, was determined. As shown in Table 5, when cysteine was added in the medium, the concentrations of 2,3-butanediol, 3-hydroxy-2-butanone, and tetramethylpyrazine all significantly increased in the fermentation broth. These three compounds were all important flavor compounds in soy sauce flavor liquor[4] and have the odor of

Table 5. Comparison of Metabolic Products with the Addition of Cysteine in Fermentation Medium

	metabolic product concentration (mg/L)		
metabolic product	without cysteine	0.05 g/L cysteine	0.1 g/L cysteine
2,3-butanediol	216.26	1128.16	1050.14
3-hydroxy-2-butanone	55.69	278.23	96.04
acetic acid	94.54	65.91	120.44
2-methylpropionate	61.04	41.59	87.04
3-methylbutanoic acid	42.97	34.84	52.20
Furaneol	10.94	2.97	12.96
maltol	1.40	2.29	11.40
2,5-dimethylpyrazine	0.14	0.14	0.25
3,5,6-trimethylprazine	12.14	10.55	17.30
tetramethylpyrazine	144.04	166.45	180.01

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"onion", "butter", and "roasted aroma", respectively. Their increase with the addition of cysteine indicated that cysteine might act as a precursor or stimulator of anabolism of these flavor compounds. In addition, it was important to note that these compounds were reported to be related to soy sauce flavor.[4,5,19] The results indicated that cysteine might play an important role in the formation of soy sauce flavor compound, and it might be an indirect precursor or stimulator of soy sauce flavor formation.

On the other hand, volatile sulfur-containing compounds were important flavor compounds in Chinese liquor,[2,3] as well as many different kinds of fermented food, such as yogurt, cheese, and beer,[20−22] and some sulfur-containing compounds were also produced by functional strains. In this paper, no volatile sulfur-containing compound was discovered in the fermentation broth of *B. licheniformis* CGMCC 3962. However, we still support the existence of volatile sulfur-containing compounds, which are the metabolites of cysteine. Usually, sulfur-containing compounds often have very low sensory thresholds,[23] and only traces of compound would exhibit distinct flavor. It was suggested that the concentrations of the volatile sulfur-containing compounds were so low that instruments were not sensitive enough to find them up to now, which was also why the key flavor compound of soy sauce flavor was still unclear.

To investigate the function of cysteine in the soy sauce flavor formation process, further efforts should be undertaken to study the metabolic pathway of cysteine, including the key enzymes and the detailed metabolites in the metabolic pathway. In addition, the regulation mechanism of cysteine metabolism should also be further investigated. It would help to further explore the production mechanism of soy sauce flavor in *Maotai* flavor liquor, which would consequently promote the development of the *Maotai* flavor liquor industry.

AUTHOR INFORMATION

Corresponding Author

*Phone: +86 510 85864112. Fax: +86 510 85864112. E-mail: yxu@jiangnan.edu.cn.

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Notes

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