JOURNAL OF PURE AND APPLIED MICROBIOLOGY, April 2013. Vol. **7**(Spl. Edn.), p. 95-100

The Development of Functional Black Bean Soy Sauce

Shoupeng Wan[1#], Meng Wang[1#], Chunling Wang[1] and Lihua Hou[1*]

[1]Key Laboratory of Food Nutrition and Safety, Tianjin University of Science & Technology, Ministry of Education, Tianjin - 300 457, China.

(Received: 03 March 2013; accepted: 14 April 2013)

Black bean soy sauce with a low-salt solid-state fermentation process was prepared by the black beans as raw material. Heilongjiang with green cotyledon was selected among different raw materials. Here, the best technique was determined. Firstly, the black beans were soaked for 4 hours under 121°, steamed for 15 min and mixed with the fried wheat (5:5, w/w). After inoculated with Aspergillus oryzae (0.4%, w/w) and cultured for 36 hours, the koji was obtained. When the brine (1:1, w/w) was added, soy sauce started to ferment. The content of total nitrogen, amino nitrogen, salt-free solids of the black bean soy sauce was even more than those of soy sauce derived from beans. Especially, the concentration of anthocyanins was 3.7g l^{-1}.

Key words: Black soybean, Soy sauce, Fermentation.

Soy sauce, which originated in China over 2,500 years ago, is one of the desired traditional seasoning in China, Korea, Japan, and Southeast Asia countries. The annual production of soy sauce in China is more than 5,000,000 tons, accounting for over 55% of the world production (Wanakhachornkrai, *et al*., 2003). Generally, soy sauce is produced by fermentation of steamed soybean and raw wheat with *Aspergillus oryzae* (solid state fermentation or koji fermentation process) (Yan, *et al*., 2008). As the development of society and the economy, more and more people pay attention to food nutrition and health problems. Therefore, black foods are more and more popular.

Black soybean (*Glycine max* (L.) Merrill) has been used as food and medicinal materials in China for a long time (Aparicio, *et al*., 2005). It is a nutritionally rich food with a plentiful supply of protein and calories. It also contains vitamin E, isoflavones, saponins and carotenoids (Lee, *et al*., 2006). Among a variety of soybeans, the black soybean is reported to have discriminating components, such as phenolic acids, anthocyanins and isoflavones (Xu, *et al*., 2008). Black soybean therefore display superior biological activities to yellow and green soybeans, such as free radical scavenging activities (Hung, *et al*., 2007) and inhibition of LDL (low-density lipoprotein) oxidation (Bae, *et al*., 2008). In addition, anti-inflammatory (Wang, *et al*., 2006) and anticancer (Baigent, *et al*., 2009) activities of black soybean have been reported, arousing strong interest in its biological and nutritional merits (Paul, *et al*., 1990; Minamino, *et al*., 1998). However, the seed coat of black bean is a little thick waxy covering outermost layer with black beans oligosaccharides. For a long time, in addition to a small amount of medicinal, edible, valuable resources of black bean has not been large-scale developed, resulting in the decrease of the economic value of the black beans and the enthusiasm of planting black beans.

Two chief fermentation techniques of soy sauce including high-salt liquid-state fermentation and low-salt solid-state fermentation are mostly used in the world. To make the consumption of black bean food to be more widespread, scientific

* To whom all correspondence should be addressed.
Tel.:+86-22-60601428;
E-mail: lhhou@tju.edu.cn

and also to the depth of the development of black beans resources, in this work, black bean was used as materials to produce the soy sauce. Using low-salt solid-state fermentation process, a high-quality black bean soy sauce was expected (Ling, *et al*., 1998) through the action of microorganisms such as *A. oryzae*.

EXPERIMENTAL

Preparation of raw materials

Black bean soy sauce was made by fermentation of a combination of black soybeans, wheat, water and salt (Ling, *et al*., 1996). The analysis method of the ingredients in black bean such as protein, crude fat, crude starch, moisture, ash content and fatty acid composition were described by the Chinese National Standard.

The steam pressure was determined to be 121!, soaking time of black beans respectively selected 2, 4, 6, 8 and 10 hours, cooking time 5, 10, 15, 20 and 25 min.

Preparation of seed koji

To prepare seed koji, wheat bran water was first blend with 100% hot water (w/w) and cooked at 121°C for 30 min. The cooked wheat bran was inoculated with *A. oryzae* spores and incubated at 30°C for 3 days, then dried in a drying cabinet at 60°C and was grounded into powders. The germination rate and the spore count were determined according to the Chinese National Standard.

Preparation of koji

Cooked Black beans were mixed with fried wheat which had been soaked in water (1:1, w/w) for 30min previously, quickly blend and cooled to a temperature of about 35!, then inoculated with a certain amount of *A. oryzae* seed koji. The initial culture temperature was 30 ± 2!. After a period of time, the culture temperature was adjusted to 25!.

Fermentation process and preparation of the samples

The resulting koji was fermented in 12% to13% brine solution at a ratio of 1:1.1 (brine raw material, w/w) to yield moromi (brine fermentation or moromi fermentation process) (Whitaker, 1987). During the first 10 days, the temperature was controlled at 45°C, and then kept at 30°C to the end of fermentation. Finally, the ripened moromi was pressed to yield soy sauce. At various time intervals of the fermentation process, samples of 100 g soy sauce mash were taken from each of the mash tanks containing the same type of koji. They were stirred individually and centrifuged at 10000 rpm for 10 min. The supernatants were filtrated through Whitman no.3 paper. The filtrate, regarded as raw soy sauce, was placed in brown bottles and kept at 4°C (Fukushima, 1987). For each analysis, tests were performed three times and all results presented are the average. As the control, the soy sauce derived from soybean was produced under the same conditions.

Parameters analysis

Determination of the quality parameters of soy sauces total nitrogen and total solids were determined by AOAC methods 991.20 and 990.2 (AOAC, 2000), respectively. Salt content (SC, expressed as sodium chloride) was analyzed by volumetric titration with $AgNO_3$ using Mohrs method. Non-salt soluble solids were calculated as TS minus SC (GB18186-2000, 2001). Amino nitrogen content was measured as described by the Chinese National Standard. Determination of the anthocyanin content was by High Performance Liquid Chromatography.

RESULTS AND DISCUSSION

The production processes of fermented soy sauce consisted of four major steps, including raw material selection, koji production, brine fermentation and refining (Zhang, 2010).

Selection of raw materials

In this study, four species of black bean were selected: Heilongjiang Province green cotyledon (HG) and yellow cotyledon (HY), Liaoning Province green cotyledon (LG) and yellow cotyledon (LY). Through comparing protein, crude fat, crude starch, moisture, ash content and fatty acid composition, the best varieties of black beans to brew soy sauce was selected. Fig. 1 showed that the highest protein content of black beans varieties was HG, followed by LG, HY, LY; the highest ash content of black beans varieties was HG, followed by LG, LY, HY; the highest crude starch content of black beans varieties was HY, followed by LY, HG, LG. As seen from Fig.2, the content of unsaturated fatty acid of HG was more than the others. In fermentation process of the soy sauce, the level of the protein content was very important.

In addition, Linoleic acid and á- Linolenic acid were the essential fatty acids of the human body. Thereby, HG was selected as the raw materials to be fermented soy sauce.

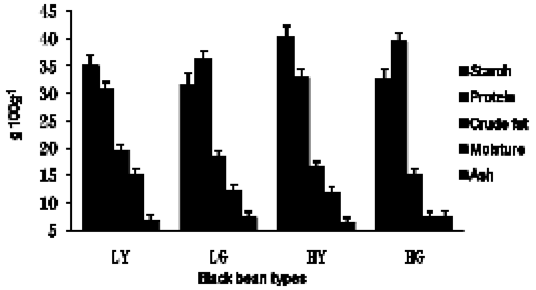

Fig. 1. Comparison of the various components of the four kinds of black beans. Data presented are the average of duplicate experiments

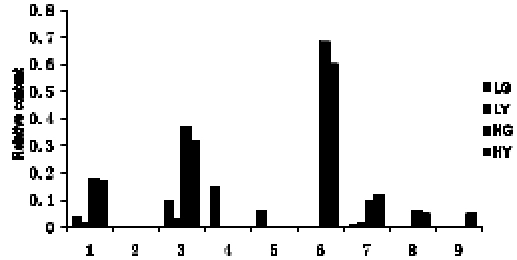

Fig. 2. Comparison of the fatty acids of the four kinds of black beans, Peak No.1 respects Palmitic acid; Peak No.3 respects Stearic acid; Peak No.6 respects Linoleic acid; Peak No.7 respects y- Linolenic acid; Peak No.8 respects α- Linolenic acid

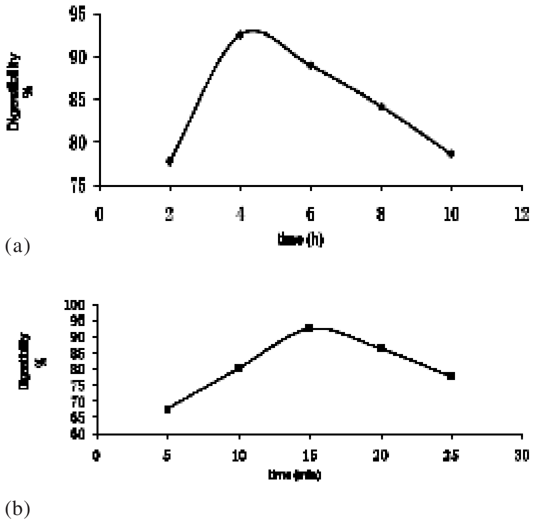

Fig. 3. The different soaking beans time (a) and cooking beans time (b) corresponding digestibility values, 121 cooking for 15min. Data presented are the average of duplicate experiments.

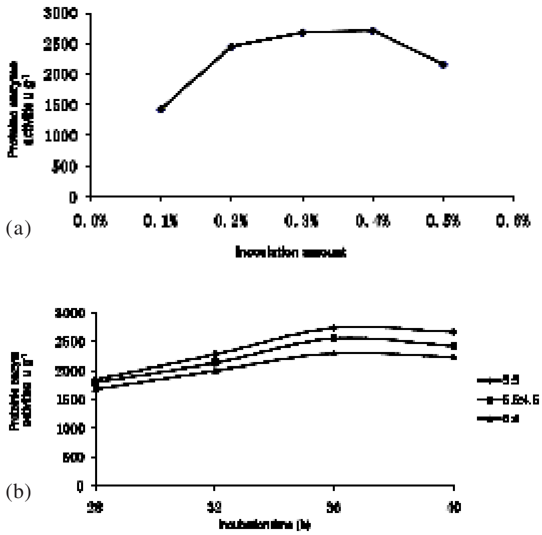

Fig. 4. The relationship of the different inoculation amount (a), the different ratio of raw materials (b) and protease activity. Black bean:wheat=5:5(w w^{-1}), Cultured for 36 hours. Data presented are the average of duplicate experiment

Determination of the cooking process

The purpose of cooking was to make moderately denaturing of the raw materials, for the reason that easy to be used by *A. oryzae*, and provide a basis for the later enzymatic decomposition (Liu, *et al*., 2008). The moderate cooking were needed due to effect obviously on the utilization of raw material and quality of soy sauce. Whether the cooking was appropriate depend on the soaking time and cooking time.

As seen from Fig. 3-a, with the extension of immersion time, the digestibility showed a rising trend, but over 6 hours, digestibility declined. The reason may be with the time longer, the water-soluble protein dissolution. Therefore, 4 hours was selected as soaking time (a little different with the seasons change). Fig. 3-b indicated that during the initial stage, digestibility was significantly increased with the extension of the cooking time. More than 20 minutes, however, digestibility began to decrease, which may be due to excessive degeneration of the protein. Finally, 15 minutes was selected as cooking time.

Determination of the koji process

The koji production process was the most important in the fermentation of the soy sauce. At this stage, the *A. oryzae* grew to a large number, and produced a complex enzyme system. The proportion of raw materials, koji duration, the amount of seed koji, moisture, humidity and temperature were the main factors that affected the production of enzymes. This study investigated the relationship between the enzyme activity and the amount of seed koji, proportion of raw materials and the duration time of koji, respectively, in order to determine the best process (Su, 1980).

Standard of high-quality of seed koji was germination rate>90%, spore count>10^9 (A g^{-1}) (Su, *et al*., 2005). The results of the amount of the seed koji was observed from Fig. 4-a. With the increase of the amount of the seed koji, the protease activity was significantly increased but once more than a certain numerical, protease activity declined. This may be because the seed koji was excessively added to the raw material, resulted in inadequate nutrition and lack of space. The growth and reproduction of the *A. oryzae* was affected, leading to the decline of protease activity. Therefore, the added amount of the koji was 0.4% (w w^{-1}). Fig. 4-b demonstrated that when the ratio of raw material was 5:5, the protease activity reached a maximum. Meanwhile, the protease activity attained the highest at 36 hours. Once more than 36 hours, *A.oryzae* began to pick spores and affect the generation of the protease.

Fermentation process and chemical indicators

Anthocyanins were the unique ingredients in black beans soy sauce. Anthocyanin remaining with polyphenolic substance can effectively scavenge oxygenic free radical. Therefore, dietary intake of soybeans can decrease the risk of the occurrence of osteoporosis, cardiovascular disease and a number of cancers, including those of breast, colon and prostate. Fig 5-a showed anthocyanins remained 3.7 g l^{-1} at the end of the fermentation.

During the fermentation of soy sauce, proteins in the raw materials were hydrolyzed into small molecular weight peptides and amino acids by the proteases produced from *A.oryzae.* The total nitrogen content was an important indicator to measure soy sauce grade (Fukushima, 1989). As can be seen from Fig. 5-b, the total nitrogen content

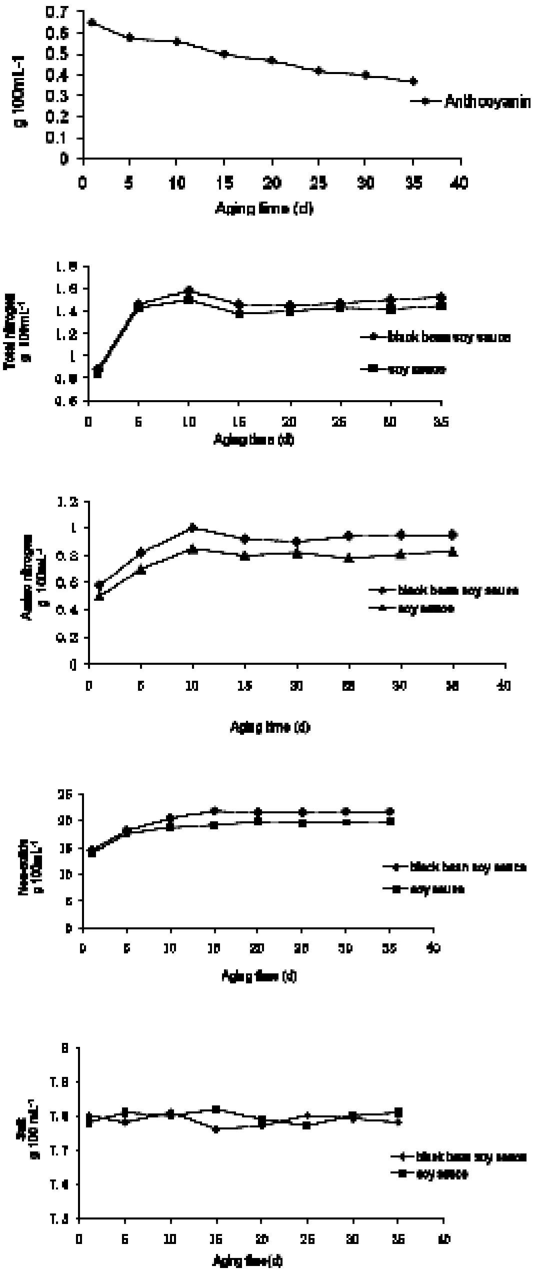

Fig. 5. Content of anthocyanin (a), total nitrogen (b), amino nitrogen (c), no-salt solids (d) and salt content (e) in raw soy sauce with various periods of aging. Data presented are the average of duplicate experiments

showed some fluctuations in the soy sauce fermentation process. Its content had been slow to improve as time progresses. The total nitrogen content in the black bean sauce was higher than that in the soy sauce. In addition, amino acid nitrogen can increase the flavor of soy sauce. Fig. 5-c indicated that the amino acid nitrogen content increased dramatically at first, then increased a little. This may be because Maillard reaction need amino and carboxyl. Amino nitrogen content was higher in the black beans soy sauce than that in the soy sauce.

Non-salt solids refer to various decomposition productions of soluble protein, such as amino acids, peptone, peptides, dextrins, low molecular weight sugars, organic acids, alcohols, esters, pigments and other substances. It was the major nutrition substance in soy sauce. There was a direct relationship between salt-free solids content and quality of soy sauce. Fig. 5-d revealed that black beans soy sauce had a larger content.

Fig. 5-e showed that throughout the sauce mash fermentation process, the salt content was maintained at a relatively stable level, showing a slight fluctuation. The stability of the salt content implied the sauce mash was fermented properly and managed under a good environment.

ACKNOWLEDGEMENTS

This work was supported by these projects (2012BAD33B04, 2012AA022108, 10ZCZDSY07000, 210-bk130006, IRT1166 and 31171731).

REFERENCES

1. Aparicio-Fernandez X, Manzo-Bonilla L, Loarca-Pina G., Comparison of antimutagenic activity of phenolic compounds in newly harvested and stored common beans Phaseolus vulgaris against aflatoxin B1. *J. Food Science.,* 2005; **70**: 73-78.
2. Bae ON, Kim YD, Lim KM, Noh JY, Chung SM, Kim K., Salsolinol, anendogenous neurotoxin, enhances platelet aggregation and thrombus formation. *Thromb Haemost.,* 2008; **100**: 52-9.
3. Baigent C, Blackwell L, Collins R, Emberson J, Godwin J, Peto R., Aspirin in the primary and secondary prevention of vascular disease: collaborative meta analysis of individual participant data from randomised trials. *Lancet.*, 2009; **373**: 1849-1860.
4. Fukushima D., Soy sauce materials and their treatment. Brewing Technology of Soy Sauceed., 1987; 1-79.
5. Fukushima D., Industrialization of fermented soy sauce production centering around Japanese shoyu. *Industrialization of Indigenous Fermented Foods*., 1989; 1-88.
6. Hung YH, Huang HY, Chou CC., Mutagenic and anti-mutagenic effects of methanol extracts of unfermented and fermented black soybeans. *J.International Journal of Food Microbiology.,* 2007; **118**: 62-68.
7. Lee IH, Chou CC., Distribution profiles of isoflavone isomers in black bean kojis prepared with various filamentous fungi. *J. Agr. Food Chem* 2006; **54**:1309-1314
8. Ling MY, Chou CC., Effect of extrusion treatment on the volatile aroma components in the raw material and product of soy sauce. *J. Chinese Agric. Chem*., 1998; **36**: 503-511.
9. Ling MY, Chou CC., Biochemical changes during the preparation of soy sauce koji with extruded and traditional raw materials. *Int. J. Food Sci. Tech*., 1996; **31**:511-517.
10. Liu LJ, Yin TF, Xue J., Effects of osmoregulation on superoxidase activity and proline content in soybean seedlings. *Soybean Sci.,* 1987; **7**: 56–60.
11. Minamino T, Kitakaze M, Asanuma H, Tomiyama Y, Shiraga M, Sato H., Endogenous adenosine inhibits P-selectin-dependent formation of coronary thromboemboli during hypoperfusion in dogs. *J. Clin Invest*., 1998; **101**: 1643-1653.
12. Paul S, Feoktistov I, Hollister AS, Robertson D, Biaggioni I., Adenosine inhibits themrise in intracellular calcium and platelet aggregation produced by thrombin:evidence that both effects are coupled to adenylate cyclase. Mol. Pharmacol., 1990; **37**: 865-870.
13. Su YC., Traditional fermented foods in Taiwan. In Proceedings of the Oriental Fermented Foods, Food Industry Research and Development Institute, Hsinchu, Taiwan, Republic of China. 1980; 15-30.
14. Su NW, Wang ML, Kwok KF, Lee MH., Effects of temperature and sodium chloride concentration on the activities of proteases and amylases in soy sauce koji. *J. Agric. Food Chem.,* 2005; **53**: 1521-1525
15. Wanakhachornkrai P, Lertsiri, S., Comparison

of determination method for volatile compounds in Thai soy sauce. *J. Food Chem.*, 2003; **83**: 619-629.

16. Wanakhachornkrai P, Lertsiri, S., Comparison of determination method for volatile compounds in Thai soy sauce. *J. Food Chem.,* 2003; **83**: 619-629.
17. Wang X, Smith PL, Hsu MY, Ogletree ML, Schumacher WA., Murine model of ferric chloride-induced vena cava thrombosis: evidence for effect of potato carboxypeptidase inhibitor. *J.Thromb Haemost,* 2006; **4**: 410-403
18. Whitaker JR., Biochemical changes occurring during the fermentation of high-protein foods. *Food Technol.,* 1978; **32**: 175-180.
19. Xu B, Chang SK., Total phenolics, phenolic acids, isoflavones, and anthocyanins and antioxidant properties of yellow and black soybeans as affected by thermal processing. *J. Agric. Food Chem.* 2008; **56**: 7175-7165.
20. Yan LJ, Zhang YF, Tao WY Wang LP, Wu SF., Rapid determination of volatile flavor components in soy sauce using head space solid phase micro extraction and gas chromatography-mass spectrometry.*J.Food SCI. Chin. Chromatogr*., 2008; **26**: 285-291.
21. Zhang YF., Biochemical changes in low-salt solid-state fermented soy sauce. *Journal of Biotechnology.,* 2010; **9**(48): 15-21.