INFLUENCES OF PRE-TREATMENT ON BIO-ACTIVE COMPOUNDS IN BLACK BEAN FERMENTED BY Aspergillus oryzae

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TÓM TẮT

ẢNH HƯỞNG CỦA QUÁ TRÌNH XỬ LÝ NGUYÊN LIỆU ĐẾN CÁC HOẠT CHẤT SINH HỌC CÓ TRONG ĐẬU ĐEN LÊN MEN NẤM MỐC *Aspergillus oryzae*

Anthocyanin trong vỏ đậu đen được sử dụng như chất màu tự nhiên tạo ra nhiều màu sắc hấp dẫn, an toàn cho thực phẩm, có thể thay thế cho chất màu tổng hợp với khả năng chống oxy hóa cao. Anthocyanin được sử dụng để bảo quản thực phẩm, kháng khuẩn, kháng nấm, chống oxy hóa cho thực phẩm. Nhóm sắc tố này kém bền nhiệt và ánh sáng nên sẽ bị phân hủy trong quá trình chế biến. Trong nghiên cứu này, hai phương pháp xử lý nguyên liệu đậu đen đã được khảo sát. Phương pháp thứ nhất (PP1) ngâm đậu trong 4 giờ sau đó hấp ở 90℃ trong thời gian 1 giờ ; phương pháp thứ hai (PP2) ngâm đậu trong 2 giờ sau đó hấp ở 121℃ với áp suất 1,5 atm trong 1 giờ. Đậu đen là một trong những nguồn giàu protein và các chất dinh dưỡng khác, sau khi lên men 30 ngày khi sử dụng các phương pháp xử lý nguyên liệu khác nhau trước khi lên men cũng cho thấy hàm lượng protein khác nhau, cụ thể ở PP1 và PP2 là 26,842 và 24,210 g/100g. Phương pháp ngâm đậu trong thời gian ngắn 2 giờ (PP2) cho protein cao hơn protein đậu đen nguyên liệu ban đầu (4%). Qua quá trình lên men, hàm lượng anthocyanin giảm từ 74,72% còn 60% và hàm lượng glucose delphinidin -3-0 giảm từ 38,66% xuống 29,38%. Bên cạnh đó, nghiên cứu còn ghi nhận được khả năng chống oxy hóa của đậu lên men cũng giảm so với đậu đen nguyên liệu ban đầu.

Từ khóa: *Anthocyanins, Aspergillus oryzae, delphinidin -3-O, DPPH, đậu đen lên men.*

1. INTRODUCTION

Solid state fermentation (SSF) involves a fermentation process using substrates that are solid materials such as grains for the growth of microorganisms. The solid-state environments provide nutrients and sufficient moisture for microorganisms to grow (Singhania *et al.* 2009). SSF commonly occurs on trays with air holes underneath, on plastic bags or cylindrical tubes. According to M. Raimbault, et al., substrates for the SSF process are usually cheap materials and agricultural waste products (Mitchell, Berovič, and Krieger 2006). SSF demonstrates several advantages that have been applied in biotechnology such as high fermentation yield, high end product performance, high product stability, cultivation of specialized microorganisms on solid substrates, especially culture media requiring no high degree of sterility because of low water activity in the solid state culture media (Hölker and Lenz 2005). There is a little amount of water used in solid-state fermentation. It has a high practical significance in the industry thanks to its less amount of wastewater thanbottom fermentation (Mitchell, Berovič, and Krieger 2006). In solid-state fermentation, the growth and

development of microorganisms on solid substrates can be seen without the presence of free water. Water exists in the form linking with the substrate or in the form of adsorption or capillary bonds in the substrate. Moisture in SSF greatly affects the growth of microorganisms. There is no optimal moisture level for all substrates and microorganisms. The moisture content usually varies from 30 to 85% (Viniegra-Gonzàlez 2013). Viniegra-González et al. (Viniegra-González *et al.* 2003) argued that higher yields in SSF result from more biomass and enzymes, but lower protein decay than those in bottom fermentation. The heterogeneity of solid substances constitutes a disadvantage of solid-state fermentation. Mold and yeast are ideal microorganisms for SSF since they demand low water activity for the growth of microorganisms. However, some bacterial species are also solid-state fermented in enzyme collection such as *Bacillus thuringenesis* (Devi, Ravinder, and Jaidev 2005).

Mold has been widely used in the food industry with global achievements. In Japan, SSF has been applied in the production of enzymes and soy sauce (alsocalled Koji). The growth and development of *Asp. oryzae*produce amylase and protease enzymes that hydrolyze substrates during solid-state fermentation (Suryanarayan 2003). The use of *Asp. oryzae* in fermentation wasstudied and published by Tominaga et al., in 2006 (Payne *et al.* 2006). In addition, over several centuries of application in food processing, *Asp. oryzae* has proven its safety and there is no strain of *Asp. Oryzae* known to produce aflatoxin. Kee-Jong Hong et al. (2004) conducted fermentation to improve the nutritional quality of soy and soy foods with *Asp. oryzae GB-107* and came to the conclusion that the effects of fermentation could make soy foods more useful in the human diet as a supplement and beneficial for livestock as a new feed ingredient (Hong, Lee, and Kim 2004).

Anthocyanins are natural pigments used in many foods, they can be red, orange, purple, blue depending on the pH of the solution. Foods that contain anthocyanins in addition to coloring also have antioxidant effects (R. Acquaviva, 2003), anti-cancer (A. Faria, 2010), and diabetes prevention (N. M. Wedick, 2012). In the previous study, when studying the pigment content in the skin of black beans, the team found a large amount of anthocyanin (Tri Nhut Pham, 2019) and also in some other surveys about anthocyanin content. In the bean peel, the main anthocyanin content of some beans is malvidin 3'-glucoside, petunidin 3'-glucoside, delphinidin 3'-glucoside, and delphinidin 3.5'-diglucoside (Choung, 2003). However, much of the research up to now has been done focus on pigment in the in black bean peels. The present research explores, for the first time, the effects of pre-treatment on bio-active compounds in black bean fermented by *Aspergillus oryzae*.

In this study, we investigated the total protein index, total anthocyanins and delphinidin 3.5'-diglucoside contents from black bean and fermented bean by *Aspergillus oryzae* using different steaming process. Based on the research results, it is possible to orient the production of fermented bean products with high nutritional contents and natural colors, without using chemicals.

2. MATERIALS AND METHODS

2.1. Materials and chemicals

Black beans was provided by Xuan Hong Company in a 20 Kg bag, then were packed in vacuum 500g PE packaging and stored at room temperature. *Aspergillus oryzae* was provided by Nosafood Co. was cultured in Potatoes-Glucose-Agar media. Broken rice corn used in prolific breeding had a moisture content of (13 ± 1 %) and (14 ± 1 %), respectively. Salt was supplied by Tan Thanh Salt Company.

Chemicals used in the study such as peptone (China), yeast extract powder (HiMedia Laboratories Pvt. Ltd - India); absolute ethanol, hydrochloric acid, methanol, ethyl acetate, formic acid, sodium acetate (Merck - Germany), phosphoric acid 85% (China), and glucose (China), delphinidin -3-0 glucose (Merck - Germany). DPPH (Merck -

Germany), DMSO (Merck - Germany).

All other ingredients (Monosodium Glutamat-MSG, sugar, salt, Modified starch E1422, acetic acid,…) were of standard of food grade.

2.2. Starter culture production processing

Aspergillus oryzae was transplanted and kept in the PGA (potato glucose agar) medium for preparation the starter cultures. Microorganism was first transferred to the high concentration of the nutrients with the ratio of mixed medium. The ratio of the medium in the first level cultures (called a beaker cultures) and the second level cultures (called the production starter cultures) were included broken rice: broken corn: water = 5:12:6 (by wt.) and 10:18:9 (by wt.), respectively. The first level cultures was incubated at 32- 35ºC in 24 hours to transplanted into the trays. 600g of the first level cultures was mixed well with 1400g the second level cultures and then was incubated in trays 40 cm x 30 cm at the laboratory temperature for 30 hours. The thickness of mixed medium layer was 1 - 2cm and there was a sterile cloth covered on the tray. The colony-forming units per gram the production starter culture was required an average $3x10^8$ CFU/g.

2.3. Steps for solid state fermentation

Each batch of fermented beans was done with 500g of dry black beans in the following sequence: Firstly, Black beans were treated by 2 methods. Method 1: beans were soaked for 4 hours and steamed at 90°C. Method 2: beans were soaked for 2 hours and steamed at 121°C with pressure of 1.5 atm for 1 hour.

Secondly, steamed black beans were mixed with enrichment culture. *Aspergillus oryzae*, then put into sterilized 2L glass jars, added with 600 g of salt solution concentration C% of 180% (w/w).

Finally, the jars were covered tightly and placed at ambient temperature during fermentation. The ratio of culture and salt solution used in this work were calculated by % weight of dry black beans. Fermented black beans were used as raw materials for making hoisine sauce according to the procedures in Figure 1.

2.4. Sample preparation for analysis

Black bean and two fermented black beans (20 g for each sample) were extracted in solvents of ethanol (85%) and HCl 1.5M (5%) (v/v) (Liqin Yin et al, 2016) at the rate of solvent: sample (10:1 ml/g) with ultrasonic support (Elmasonic S60H) under conditions of darkness at room temperature for 15 hours separately. The extracts were collected and filtered on filter paper, the solid residue were continued to be soaked twice in 50 ml previous solvent and the extracts were combined. The entire extracts was concentrated by vacuum evaporation at 30°C and extracted residues maintain anthocyanins were dissolved in 0.1% hydrochloric acid before analysis of the total anthocyanins and the delphinidin -3-0 glucose contents.

2.5. Methods for determining the total anthocyanin contents and delphinidin-3-O-glucose contents

2.5.1. Total anthocyanin contents

The total anthocyanin contents were analysed by pH-differential method (AOAC method 2005-02), which bases on the structural changes in chemical forms of anthocyanin and absorbance measurements at pH 1.0 and 4.5. The extracts (5 ml) and solution of sodium acetate buffer were added separately in a 25-mL volumetric flask (pH level 1.0) and diluted to pH level 4.5. Used instrument of UV-Vis spectrophotometer (Lambomed, USA) with ultraviolet region λ = 520nm and λ = 700nm. The total anthocyanin contents were expressed as delphinidin-3-O-glucose equivalent.

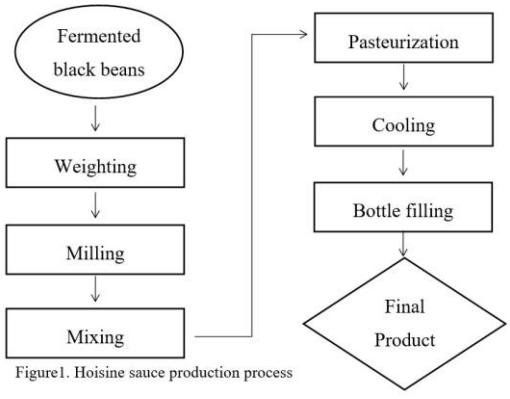

Figure1. Hoisine sauce production process

2.5.2. Delphinidin-3-O-glucose contents

The delphinidin-3-O-glucose content was analysed on an HPLC of analytical samples containing anthocyanins, the anthocyanine extracts were treated by passing 5 ml of each extract through the C-18 Sep-Pak (SPE) column to remove polar substances such as sugar, salt and less polar polar phenolic substances. First of all, the SPE column was activated with methanol and then 0.01% HCl (v/v) in water, the impurities were removed by 2 volumes of 0.01% HCl in water (v/v) and next ethyl acetate (10 ml), finally the anthocyanin extracts werec ollected by two volumes of 0.01% HCl (v/v) in methanol. The anthocyanin extracts were used for HPLC analyses.

Delphinidin-3-O-glucose were dissected by HPLC (Shimadzu LC-2030C 3D, Kyoto, Japan) following Susana Gonza´lez-Manzano et al., (2008) with Shin-pack GIST C18 column (250 mm × 4.6 nm, 5 µm particle size,) at the temperature 40^{o}C using injection volumes of 20 µL , detector PDA. Mobile phases consisted of formic acid (5%) and water (95%, v/v) (solvent A); formic acid (5%) and methanol (95%, v/v) (solvent B) with gradient elution (Chuan-guang Qin et al., 2011) following in Table 1.

The absorbance spectra were recorded every 2 s between 200 nm and 600 nm, with a bandwidth of 4 nm, and chromatography absorbent at 520 nm, 440 nm, 310 nm, and 280 nm. The wavelength used for anthocyanin was 520 nm.

Table 1. Method of delphinidin-3-O-glucose analysis by HPLC

Run time (min)	Solvent A (%)	Solvent B (%)	Flow rate (mL/min)
0 - 2	85	15	1
2.1 - 30	55	45	1
30.1 – 40	85	15	1
40 - 60	0	100	1

2.6. Methods for determining antioxidant activity

Antioxidant activity was computed according to W.Brand-Williams *et al.* (1995). 0.1 mL of extract was added to 2.9 mL of DPPH (2,2-diphenyl- 1-picrylhydrazyl) reagent, which was prepared with 0.1mL DPPH 6mM and 2,8mL methanol. The mixture was shaken manually and then incubated for 30 min at room temperature (25°C). The absorbance was then computed at 520 nm. To quantify the antioxidant activity, a standard ascorbic acid (0.20 to 1µmol/mL) was prepared and the sample extract was dissolved in DMSO (0.2 to 1 mg/L); the blank (100 µl of DPPH 6mM in 2800 µl methanol), each reference substance concentration repeated three times.

2.7. Methods for statistical analysis

The experiments were repeated 3 times and the results were presented by Mean ± SD values. Microsoft Excel 2010 and Statgraphics applications were used to analyze statistical data of experiments and evaluate differences between treatments

3. RESULTS AND DISCUSSIONS

In the chromatographic conditions under investigation, the mobile phase system: formic acid 5% and water 95% (v/v) (phase A), formic acid 5% and methanol 95% (v/v) (phase B) with the program runs the gradient, the delphinidin -3-0 glucose signal showed at RT (Retention time) 5.368 min and the calibration curve had been constructed with 5 concentrations of 2.5 ppm, 5 ppm, 10 ppm, 25 ppm, 100 ppm to give the equation f (x) = 8320.31x-10447.9 with the correlation coefficient R^2 = 0.9856 (Figure 1).

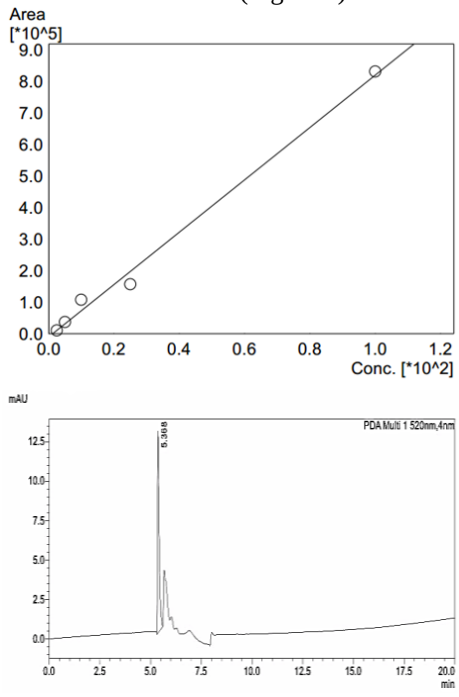

Figure 1. Chromatogram and calibration curve of delphinidin-3-O-glucose in HPL

According to the linear regression the LOD value is calculated as follows: y_B is accepted by the cut-off on the vertical axis (a) of the linear

regression line, while S_B is accepted by the standard error of the measured signal on the vertical axis (Sy); from there, the LOD is calculated (Miller, 2010):

$$LOD = 3 \times S_y/b$$

Where, b is the slope of the linear regression line.

Limit of Quantitation is a parameter that shows the quantitative ability of the analytical method. LOQ is defined as the minimum concentration on a reliable calibration curve and it is calculated from LOD (Miller, 2010):

$$LOQ = (3 - 4) \times LOD$$

Table 2. LOD and LOQ values of delphinidin-3-O-glucose

Compound	Parameter				
	a	b	S_y	LOD, ppm	LOQ, ppm
Delphinidin-3-O-glucose	10448.10325	8320.305377	47419.88765	17	51

Appling the equation to calculate the delphinidin -3-0 glucose contents of the black bean and the two fermented bean extracts, the results showed on the Table 1.Delphinidin -3-0 glucose is the main ingredient in anthocyanin extracted from black beans (Xu *et al.*, 2007). In the whole black beans, delphinidin -3-0 glucose (0.194 mg/g) accounted for 11% of the total anthocyanin, fermented beans of method one (M2) decreased by 38.66% (0.111 mg / g), accounted for 11.13% of the total anthocyanin, the one of method two (M3) had a higher contents, decreased by 29.38% compared to whole beans (0.1137 mg/g) and accounted for 8.1% of the total content. The above results show that, the longer steaming, the total anthocyanin was higher but the content of delphinidin -3-0 glucose content was lower.

Table 2. Delphinidine and anthocyanine contents in black bean and fermented bean

Samples	**Delphinidine (mg/g DM)**	**Anthocyanine (mg/g DM)**
Black bean - M1	$0.194^{cb} \pm 0.002$	$4.229^{c} \pm 0.155$
Fermented 1– M2	$0.119^{ab} \pm 0.001$	$1.069^{a} \pm 0.019$
Fermented 2 – M3	$0.137^{bb} \pm 0.002$	$1.690^{bb} \pm 0.010$

Table showing average values ± standard deviation, different letters a, b, c denoted the difference in columns with statistical significance P-value <0.05.

According to the results obtained (Table 2), the anthocyanin contents in whole black beans (M1, 4.229 mg/g) suited the previous survey of the author (Elia Nora Aquino-Bolaños *et al.*, 2016) (1.3-5.4 mg/g) and higher than black beans after fermentating by method one (M2) and method two (M3), anthocyanin contents decreased 74.72% and 60% respectively, because of fermented conditions and long fermentation, some anthocyanin compounds were modified. Within two fermented methods, M3 showed greater anthocyanin content than M2(1.690 and 1.069 mg/g) corresponding to longer steaming times. Different anthocyanin contents gave different colors of the beans with different methods of fermentation which would help fermented bean products such as sauces have different colors.

The total protein of the whole black beans and two fermented bean showed on Table 2. After 30 days of fermentation used method one, the total protein increase 4% (26.842 g/100g), this result was consistent with the research by Kee-Jong Hong, Chan-Ho Lee and Woo Kim Sung that conducted fermentation to improve the nutritional quality of soybeans and soy foods with *Aspergillus oryzae* GB-107, but using method two, the protein reduce 6.162% (24.210 g/100g). It shows that with two methods of processing raw materials before fermenting gave different protein contents.

Table 3. Moisture and total protein contents in black bean and fermented bean

Samples	**Moisture (%)**	**Protein (g/100g DM)**
Whole black bean-M1	$10^{a} \pm 0.037$	$25.800^{b} \pm 0.748$
Fermented 1– M2	$81^{b} \pm 1.062$	$26.842^{c} \pm 0.533$
Fermented 2 – M3	$81^{b} \pm 0.700$	$24.210^{a} \pm 0.339$

Table showing average values ± standard deviation, different letters a, b, c, denoted the

difference in columns with statistical significance P-value <0.05.

According to Table 3, when the extract concentrations of M1, M2, M3 were increased (0.1 to 1.0 mg/l), the percentages of DPPH free radical scavenging activity increased, making the antioxidant capacities of the fluids potentiometer increased. The free radical scavenging activityof M1>M3>M2, such as the concentration of 1.0 mg/l, the percentage of free radicals captured in M1 (74.32%) higher than M2 (31.67%) and M3 (37.95%). The difference data of free radical scavenging activity of M1, M2, M3 samples were consistent with total anthocyanin and delphinidin -3-0 glucose data. Among the concentrations in the same extract, the percentage of DPPH free radical scavenging was statistically different with P-value ≤ 0.05.

Table 4. The percentage of DPPH free radical scavenging activity of the extracts

Samples	Black bean (M1)	Fermented 1 -M2	Fermented 2 - M3	Ascorbic acid
	% DPPH	% DPPH	% DPPH	% DPPH
NH 0.1	$18.47^{a} \pm 0.68$	$1.48^{a} \pm 0.71$	$5.45^{a} \pm 0.59$	$37.65^{a} \pm 0.57$
NH 0.2	$25.66^{b} \pm 0.70$	$4.85^{b} \pm 0.94$	$8.62^{b} \pm 0.74$	$47.10^{b} \pm 0.52$
NH 0.4	$43.22^{c} \pm 0.54$	$2.61^{c} \pm 0.13$	$28.95^{c} \pm 0.27$	$84.42^{d} \pm 0.43$
NH 0.6	$53.87^{d} \pm 0.80$	$28.77^{d} \pm 0.57$	$29.68^{d} \pm 0.15$	$79.88^{c} \pm 0.11$
NH 0.8	$56.35^{e} \pm 0.59$	$30.05^{e} \pm 0.44$	$34.71^{e} \pm 0.48$	$88.64^{e} \pm 0.55$
NH 1.0	$74.32^{f} \pm 0.19$	$31.67^{f} \pm 0.33$	$37.95^{f} \pm 0.39$	$90.41^{f} \pm 0.55$

Table showing average values ± standard deviation, different letters a, b, c, d, e, f denoted the difference in columns with statistical significance P-value <0.05.

The graph shown when concentrations of M1, M2 and M3 increased, the antioxidant ability also increased and antioxidant capacity of fermented black beans (M3) was higher than M2 and lower than M1 (Figure 2). The resistance of ascorbic acid was higher 2.6 times at the concentration of 0.6 (mg/l) than antioxidant ability of M3. These results were consistent with the data of free radical scavenging activity shown on Table 4 and also were consistent with the original hypothesis that the soaking and steaming process significantly reduces the antioxidant and biological activity and thus affects the quality of activity of fermented beans.

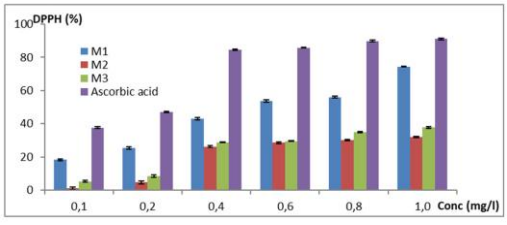

Figure 2. Graph showing antioxidant capacity of surveyed samples

M1: Black bean material sample; M2 is a fermented black bean sample according to method one; M3 is a black bean sample according to method two; the ascorbic acid was a control sample.

4. CONCLUSION

The present study was determined the effect of black bean pre-treatment method on bio-compounds in fermented black bean. Fermented black bean in this study can be considered as a high-quality for making hoisine sauce that has not been yet on the market because of its high protein content and no use of coloring agents. This work contributes to existing knowledge of fermented beans by providing the process of material processing as well as traditional fermentation of black beans.

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