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Original Article

Changes in physico-chemical properties and antioxidant activities during reduced-salt fermentation of green mussel (*Perna viridis*, Linnaeus 1758)

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Abstract

Green mussels were fermented at reduced salt condition by controlling the salt to mussel ratio at 1:6, in order to compare the physico-chemical and antioxidant properties with the control, at a ratio of 1:3, at the ambient temperature for 60 days. Salt content in traditional and reduced salt fermentation conditions were 8.74 ± 0.03 and 14.62 ± 0.03 %, respectively. Results showed that reduced-salt fermented mussels have significantly increased pH value, ammonia nitrogen and formaldehyde nitrogen, which could indicate faster protein degradation of mussel meat than the control. In turn, significant production of total soluble protein and phenolic compounds of reduced-salt fermented mussels were observed. Browning development, as an index of Maillard reaction products (MRPs) formation was also detected. The fermented mussel at reduced-salt condition showed higher total phenolic content and radical scavenging activity than that of control. The DPPH and ABTS radical scavenging activities were found at 28.02, 22, and 89.66%, respectively. High correlation in the physico-chemical properties and antioxidant activities were observed, suggesting bioactive substances could have been produced during fermentation. Results also suggest that reduced-salt fermented product can be produced from green mussels, as a potential source of antioxidants.

Keywords: *Perna viridis*, reduced-salt fermentation, physico-chemical changes, antioxidant activity, Maillard reaction products (MRPs)

1. Introduction

Green mussels are widely distributed mytilid bivalves in most Asian regions. Green mussels are an in-expensive source of protein, vitamins C, D, E, and other vitamins and essential minerals; e.g. zinc, manganese and copper (Gopalakrishnan & Vijayavel, 2009; Ismail, Marjan, & Fong, 2004). Commonly mussels are sold live, therefore, if not properly handled and processed, it may result in food wastage and spoilage. Hence, there is a need for maximum utilization of this perishable foods.

Fermentation is the process of breaking down of organic substances into simpler components generally by the action of enzymes from fermentative microorganisms (Mackie, Hardy, & Hobbs, 1971). Fermented products can have added benefits such as enhanced flavor, increased digestibility, and improved nutritional and pharmacological value. Furthermore, the technology is simple, and the operating cost is minimal. In the Philippines, the two well-known fermented products are fish sauce (*patis*) and fish paste (*bagoong*) (Montaño, Gavino, & Gavino, 2001; Peralta *et al.*, 2005). However, these contain high salt contents (14–30%), making it un-attractive to people who have health dysfunctions, e.g. cardiovascular diseases, hypertension, kidney stones, stomach cancer, cataract, asthma, Meniere's disease, osteoporosis, hypercalciuria and hyper thyroidism/hypothyroidism (Cappuccio, 2013; Cappuccio, Capewell, Lincoln, & McPherson, 2011; Cappuccio & MacGregor, 1997; Marcisz, Jonderko, & Kucharz, 2001). As discussed by Cappuccio (2013), WHO has set a global goal of

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reducing salt intake by 2025 of 5g per person per day. Hence, the emergence of researches for the production of reduced salt food products.

Previous studies on green mussels have been found to exhibit cytotoxic activities, anti-microbial, radio-protective, anti-angiogenic, and anti-inflammatory properties (Annamalai, Anburaj, Jayalakshmi, & Thavasi, 2007; Chakraborty *et al*., 2010; Sreejamole, Radhakrishnan, & Padikkala, 2011). Anent to food products from mussels, the study of Je, Park, Byun, Jung, and Kim (2005) on fermented blue mussels (*Mytilus edulis*) sauce with 25% NaCl for 12 months was found to have strong angiotensin I converting enzyme (ACE) inhibitory activities. Pharmacological studies involving antioxidant activities of mussels, however, are sparse. Proteins, peptides, and amino acids derived from fermentation are known to have regulatory function in the human body. Also, a high total phenol content in samples is usually associated with high antioxidant activities. Thus, the study on the anti-oxidant properties derived from green mussels is appropriate.

The current study aims to produce a reduced salt fermented mussel product. In this condition, there is now a need to evaluate the process behavior during fermentation. Therefore, main objective of the study is to investigate and monitor the physico-chemical changes and antioxidant properties of reduced-salt fermented mussel during 60 days of fermentation.

2. Materials and Methods

2.1. Preparation and sampling of salt-fermented mussel

Green mussels were collected from Capiz, Philippines. Upon harvest, the samples were immediately cleaned, shucked, and salted at a ratio of 1:6 (salt: mussel meat with intravalvular fluid) on site. A ratio of 1:3 salt: mussel were also prepared as control. The mixtures were immediately transported to the Institute of Fish Processing Technology laboratory. Upon arrival, mixtures were homogenized thoroughly using a mechanical blender and allowed to equilibrate overnight for even salting. The mixture (500 g) was packed in sterile glass jar and allowed to ferment anaerobically for 60 days at room temperature (28–32 °C). Samples were withdrawn at Day 1, and every 6 days thereafter for 60 days. Collected samples were kept frozen (–18 to –21 °C) prior to analyses. The experimental set-up used in the study was complete randomized blocked design (CRBD).

2.2 Preparation of 80 % ethanol sample extract

The salt-fermented mussel samples (1:6 and 1:3) collected per time interval were homogenized in ice bath. A 5-gram sample was mixed with 5 mL of water and 20 mL of 95 % ethanol. The mixture was then centrifuged (CENTURION, Scientific Limited) at 1,500 rpm for 10 min. The supernatant was recovered, and filtered into a 50 mL volumetric flask using a funnel with defatted cotton. The residue was treated twice with 10 mL of 95% ethanol as described above and all the supernatant was collected. The recovered supernatants were combined and adjusted to 50 mL by adding 95% ethanol (Peralta *et al*., 2005). The resulting concentration of the crude sample extract was calculated and diluted to come up with 5 $mg.mL^{-1}$. This concentration was used in all antioxidant assays.

2.3 Determination of the physico-chemical changes during fermentation

2.3.1 Salt analysis

Triplicate samples of the mussel sauce; experimental sample (1:6) and control (1:3), salt to mussel ratios; were reacted with standard $AgNO_3$ solution, according to Volhard method (AOAC, 1990); the resulting AgCl precipitate, dissolved and digested, and reacted with standard ammonium ferric sulfate solution, and back titrated with standard potassium thiocyanate (KSCN), until end point.

2.3.2 pH determination

pH was determined using analytical pH meter (HM Digital pH meter PH-200) at ambient temperature. The samples were allowed to thaw first before conducting the experiment in triplicates.

2.3.3 Formol nitrogen content determination

Formol nitrogen content was determined following the method of Chaveesuk, Smith, and Simpson (1993). Formol nitrogen of the samples was calculated as follows:

$$\text{Formol nitrogen}\left(\frac{mg}{mL}\right) = \frac{(mL_{NaOH})(1.4)}{W_{sample}}$$

2.3.4 Ammonia nitrogen content determination

Ammonia nitrogen content was determined following the method of Faithong and Benjakul (2014). Ammonia nitrogen content ($mg.mL^{-1}$) was calculated as follows:

$$\text{Ammonia nitrogen content}\left(mg\frac{N}{mL}\right) = \frac{(28.0 \times M_{conc.\ H2SO4} \times Vol_{\ H2SO4})}{W}$$

2.3.5 Total soluble protein (TSP) determination

The protein content of sample was estimated by Lowry's method using Bovine serum albumin (BSA) as a standard (Lowry, 1951). The samples were read at 750 nm absorbance using the 96-well BMG CLARIOSTAR Microplate Reader. The standard curve was generated and the protein content of the samples were estimated by extrapolation of calibration curve generated using the BSA standard solution as a standard protein wherein the result was expressed in mg $BSA.L^{-1}$.

2.3.6 Brown color development determination

The brown color intensities of the sample extracts were measured following the method of Benjakul *et al*. (2005) with slight modification. Appropriate dilution of sample extract (5 $mg.mL^{-1}$) was made and the absorbance was read at 420 nm using the 96-well BMG CLARIOSTAR Microplate Reader. 80% ethanol was used as blank.

2.4 Assessment of the antioxidant activities

2.4.1 DPPH radical scavenging activity assay

DPPH radical scavenging activity was measured as described by Peralta *et al*. (2005) with slight modification. Briefly, 2 mL of the 80% ethanol sample extract with a concentration of 5 mg.mL^{-1} was used. Then, 0.50 mL of 0.5 mM 2,2-diphenylyl-1-picryly hydrazyl (DPPH) in ethanol solution was added. The solution was mixed vigorously and incubated at room temperature in the dark for 20 min. The absorbance of 200 µL sample aliquot was measured at 517 nm using the 96-well BMG CLARIOSTAR Microplate Reader. Distilled water was used as blank. The activity was expressed as %DPPH scavenging activity was calculated as follows:

$$\%\ \text{DPPH scavenging activity} = \frac{\text{blank Abs} - \text{sample Abs}}{\text{blank Abs}} \times 100$$

2.4.2 ABTS radical scavenging activity assay

ABTS radical scavenging activity was determined as described by Faithong, Benjakul, Phatcharat, and Binsan (2010). The absorbance of a 200 µL sample aliquot was measured at 734 nm using the 96-well BMG CLARIOSTAR Microplate Reader. ABTS radical scavenging activity was expressed as %ABTS scavenging activity was calculated as follows:

$$\%\ \text{ABTS scavenging activity} = \frac{\text{blank Abs} - \text{sample Abs}}{\text{blank Abs}} \times 100$$

2.4.3 Total phenol content assessment

Total phenol content was determined following the method of Benzie and Strain (1996) or known as Folin-Ciocalteu's method. For the standard, gallic acid was prepared with concentrations of 0, 1, 2, 3, 5, 10, and 20 mg.L^{-1}. After incubation, absorbance of the sample was read at 765 nm using 96-well BMG CLARIOSTAR Microplate Reader. A blank was prepared by doing the same step but the addition of extract was substituted with the diluent. Phenolic content was determined by extrapolation of calibration curve generated using the gallic acid standard solution as a standard phenolic compound. The results were expressed in gallic acid equivalents (GAE in mg.L^{-1}).

2.5 Statistical analysis

The significant difference between physico-chemical and antioxidant activities of the reduced-salt fermented mussel sample (1:6) and control (1:6) were analyzed using the standard T-Test, at a significance level of 0.05 alpha, using Excel of MS Office 2013.

3. Results and Discussion

3.1 Assessment of the physico-chemical properties during fermentation

3.1.1 Salt analysis

The fermented mussel with 1:6 salt to mussel ratio were found to have 8.74 ± 0.03 % salt, whereas the fermented mussel with 1:3 salt to mussel ratio were found to have 14.62 ± 0.03 % salt.

3.1.2 pH value evaluation

The pH of the experimental samples was below pH 7.0 initially at Day 1, and then lowered abruptly at Day 6 (Figure 1). This may be due to the dissociation of amino acids and small peptides (Sanchez, 2008). It then showed an increasing trend but remained stable from Day 12 until Day 30. Thereafter, the pH significantly ($P < 0.05$) increased from Day 30 until Day 60 of fermentation period. This suggest that volatile basic substances such as ammonia, trimethylamine, and others, may have been formed by fermentative microorganisms, predominantly gram negative psychrophiles (Bell & Shelef, 1978). Increase in pH could also be attributed to the metabolites produced by bacterial action on protein and amino acids (Kandeepan, Anjaneyulu, Kondaiah, & Rajkumar, 2011).

Meanwhile, the pH of the control was observed to be more or less stable below 7.0, on the low acid side, throughout the fermentation. The control contains high salt, 15–20% salt or more, wherein proteolytic activities are somewhat inhibited during fermentation (Chaveesuk *et al*., 1993).

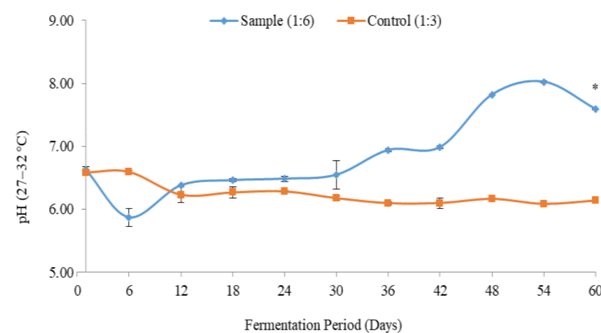

Figure 1. Changes in the pH value of the reduced salt-fermented mussel samples (1:6) and control (1:3) during 60 days of fermentation period, n=3. (*) denotes significant difference throughout fermentation period ($P < 0.05$), except for Day 0 and 12.

3.1.3 Formaldehyde nitrogen analyses

The formaldehyde nitrogen content of the experimental samples incrementally increased from Day 1 until Day 30 of fermentation, slightly lowered in Day 36 and Day 42, and achieved the highest degree of hydrolysis at Day 48, and lowered until Day 60 (Figure 2). This may indicates the formation of peptides and free amino acids through protein hydrolysis (Faithong & Benjakul, 2014). It should be noted in Figure 1 that the pH of the experimental samples lies on the basic side, thus, pH condition and low salt could have encouraged the hydrolysis of myofibliar proteins by fermentative microorganisms. As a result, more hydrolysis products were produced (Faithong *et al*., 2010). This also implies that the protein hydrolysis in the experimental sample was more or less complete. As compared to the control, the formaldehyde N were observed to be low, and remained

almost constant throughout the fermentation period. It is noted that low acid conditions, may have stabilized the conversion of amines and other fermentative products; e.g. formaldehyde N. Moreover, Amano (1962) implied that high salt concentration slows down the activities of fermentative enzymes, resulting in a slower rate of fermentation. Fermentation in high-salt content usually takes more than six months to complete its hydrolysis, and ripening.

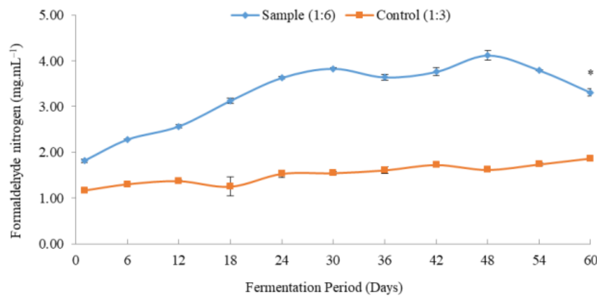

Figure 2. Changes in formaldehyde nitrogen (mg.mL^{-1}) of the reduced-salt fermented mussel samples (1:6) and control (1:3) during 60 days of fermentation period, *n*=3. (*) denotes significant difference throughout fermentation period ($P < 0.05$)

3.1.4 Ammonia nitrogen analyses

The ammonia N content of experimental samples significantly ($P < 0.05$) increased as the fermentation progresses (Figure 3). A notable increase can be observed starting from Day 12 of fermentation period, wherein, the highest was at Day 54, and slightly decrease in Day 60. Increase in ammonia N could be attributed to the decomposition or de-amination of nitrogenous compounds, i.e. peptides and amino acids during fermentation due to low concentration of salt that resulted in rapid protein hydrolysis (Beddows *et al*., 1980; Lopetcharat & Park, 2002; Uyenco *et al*., 1983). Whereas, the ammonia N content of the control was significantly lower ($P < 0.05$) and almost remained low throughout the experiment. This can be attributed to its high salt content, wherein the activities of fermentative microorganisms are slowed down (Chaveesuk, 1993). Proteins may have hydrolyzed, but not converted into ammonia N. Therefore, decomposition or deamination of peptides and amino acids occurred slowly; or did not occur at all.

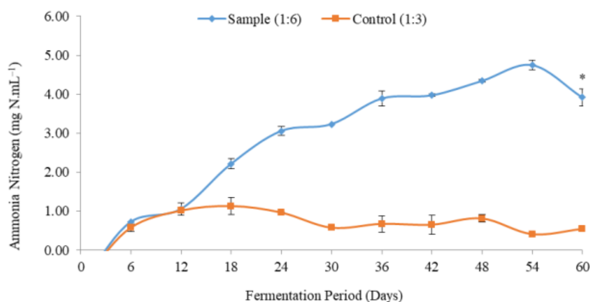

Figure 3. Changes in ammonia nitrogen content (mg N.mL^{-1}) of the reduced-salt fermented mussel samples (1:6) and control (1:3) during 60 days of fermentation period, *n*=3. (*) denotes significant difference starting with Day 18 and throughout the fermentation period ($P < 0.05$)

3.1.5 Total soluble protein assessment

The total soluble protein (TSP) of the experimental sample was found to be significantly ($P < 0.05$) higher with that of control, except for Day 48 (Figure 4). It increased rapidly until Day 30 (73.63 ± 0.27 mg BSA.L^{-1}) and started to decrease at Day 36 until Day 48 of fermentation period. The observed abrupt decrease may be attributed in the increase in the ammonia N (r = 0.675), which suggests that some of the hydrolyzed peptides were degraded and converted. Factors such as pH, degree of hydrolysis, and temperature may affect the protein hydrolysis during fermentation (Normah & Asmah, 2016). On the other hand, the TSP concentration of control gradually increased and almost remained stable throughout the fermentation period; which implies that the proteins were hydrolyzed slowly throughout the experiment.

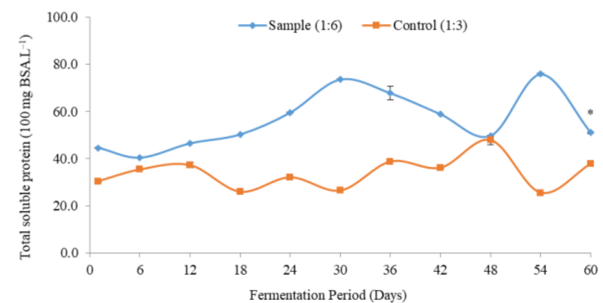

Figure 4. Total soluble protein (TSP) of the salt-fermented mussel samples (1:6) and control (1:3) during 60 days of fermentation period, *n*=3. (*) denotes significant difference throughout fermentation period ($P < 0.05$), except for Day 48

3.1.6 Brown color development

Maillard reaction products (MRPs) are formed by the non-enzymatic browning reaction between reducing sugars and amino compounds (Benjakul, 2005; Morales & Jimenez-Perez, 2001); and the degradation of ATP (Saisithi, 1994; Jing & Kitts, 2004). The brown color formation of the experimental samples during fermentation was more intense and significantly ($P < 0.05$) higher compared to that of control (Figure 5). This suggests that brown color, as index of Maillard reaction (production of MRPs) may have developed during the fermentation (Peralta *et al*., 2008). According to Ajandouz, Tchiakpe, Ore, Benajiba, and Puigserver (2001) high browning intensity (A_{420}) happens in the final stage of the Maillard reaction. Also, the browning intensity developed, alongside with the increase in formaldehyde N in the experimental samples (Faithong & Benjakul, 2014). This phenomenon, further implies that the more proteins are hydrolyzed during fermentation period, the more MRPs are formed, resulting in the increased browning intensity. Free amino acids are usually formed during fermentation, and the abundance of these free amino acids may result to increased formation of MRPs. Several studies have reported that when MRPs are formed in fermented foods, formation of melanoidins, an end product Maillard reaction, also exhibit antioxidant activities (Benjakul, Lertittikul, & Bauer, 2005; Yilmaz & Toledo, 2005). Furthermore, results in the control suggests that the use of high-salt levels in the fermentation

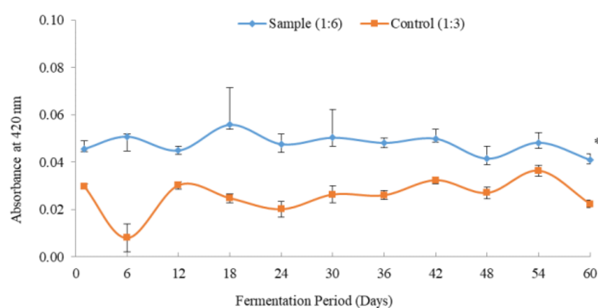

Figure 5. Browning intensity of the fermented mussel samples (1:6) and control (1:3) during fermentation period of 60 days, n=3. (*) denotes significant difference throughout fermentation period ($P < 0.05$)

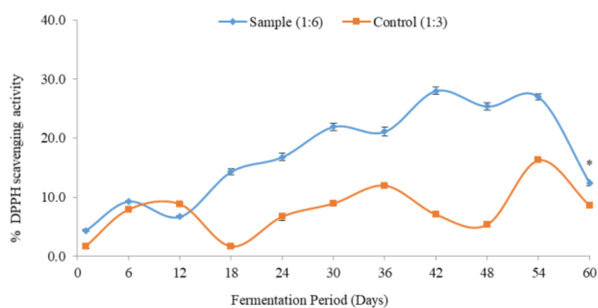

Figure 6. DPPH radical scavenging activity of fermented mussel samples (1:6) and (1:3) during fermentation period of 60 days, n=3. (*) denotes significant difference throughout fermentation period ($P < 0.05$)

significantly delayed the hydrolysis of proteins into amino acids and peptides, thus, most probably result to minimal production of MRPs.

3.2 Determination of the antioxidant activities

The 80% ethanolic extract (5mg.mL^{-1}) of the experimental samples scavenge effectively the free radical DPPH as compared to that of control; although both samples initially have almost similar activities from Day 1 until Day 12 of fermentation (Figure 6). However, from Day 12 until Day 54, % DPPH activity of the experimental samples significantly ($P < 0.05$) increased and was higher than the control. Likewise, the control was found to have significantly ($P < 0.05$) lower DPPH activities than the experimental samples throughout the fermentation.

Figure 7 shows that the experimental samples exhibited significantly ($P < 0.05$) higher ABTS radical scavenging activity than the control. The activities of the experimental samples dramatically increased from Day 1 until Day 18, and then eventually plateaued until the end of fermentation period. Likewise, the control samples showed fluctuating ABTS activities throughout the fermentation. Hence, results suggest that reduced-salt fermentation produced considerable amounts of substances with antioxidant capabilities to scavenge ABTS radicals.

High total phenol content in samples is usually associated with high antioxidant activities. Figure 8 shows a fluctuating trend in the total phenolic content for both experimental samples and control, but levels of the former were significantly higher ($P < 0.05$) than the latter. Polyphenols are usually found to be present only in plants. Since green mussels consumed algae and phytoplankton, it is believed that green mussels have accumulated these compounds (Landete, 2012; Naczk & Shadihi, 2004). It has been reported that some phenolic compounds are usually formed during fermentation, due to the endogenous and bacterial enzymes of fermentative microorganisms (Muñoz *et al*., 2017; Selma, Espin, & Tomas-Barberan, 2009). Similarly, Othman, Roblain, Chammen, Thonart, and Hamdi (2009) reported that the presence of LAB in fermentation contributes to phenolic conversions and depolymerization of phenolic compounds. Furthermore, the total phenol content of the experimental sample was positively correlated with pH, formaldehyde N, ammonia N, browning intensity, and TSP (r = 0.527, 0.572, 0.603, 0.215, and 0.524, respectively).

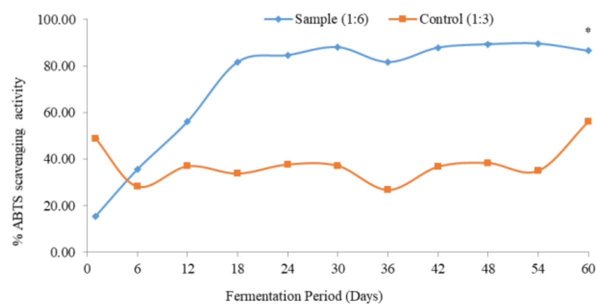

Figure 7. ABTS radical scavenging activity of fermented mussel samples (1:6) and (1:3) during of fermentation period of 60 days, n=3. (*) denotes significant difference throughout fermentation period ($P < 0.05$).

Protein hydrolysis during fermentation produces huge amounts of short chain peptides, and amino acids that are associated with antioxidant activities (Benjakul, Binsan, Visessanguan, Osako, & Tanaka, 2009). In general, the 80% ethanol-extract (5 mg.mL^{-1}) from reduced-salt fermented mussel samples have higher ability to quench DPPH and ABTS radicals, compared to the control. These results could be attributed to the phenolic contents of green mussel, and through fermentation, phenolic compounds are released, exhibiting free radical scavenging properties (Ng, Wang, Wang, Tzeng, & Shyu, 2011; and Bobo-Garcia *et al*., 2015). The increase in the antioxidant (DPPH and ABTS) activities of the experimental samples were found to be highly correlated with the increased total phenol content (r = 0.754 and 0.595) and TSP (r = 0.717, 0.639) during fermentation. In addition, during fermentation, increased browning intensity was also observed. These are believed to lead in the formation of MRPs that may have contributed to the observed antioxidative properties (Lertittikul, Benjakul, & Tanaka, 2007). Moreover, the hydrolysis of protein occurred producing low-molecular weight peptides, and free amino acids that are believed to possess strong antioxidant activities (Binsan *et al*., 2008; Faithong *et al*., 2010; Kittiphattanabawan *et al*., 2012).

4. Conclusions

The production of fermented mussel at reduced-salt condition provided a fast fermentation, and fermentation time could be possible for 30 days at room temperature. In

addition, higher natural antioxidants were produce more, on a reduction of salt during fermentation. This would be an excellent strategy to produce healthier product from fermented green mussel.

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