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DEGRADATION OF HISTAMINE IN SALTED FISH PRODUCT BY HALOTOLERANT *BACILLUS POLYMYXA*

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ABSTRACT

Bacillus polymyxa D05-1 isolated from salted fish product which possess histamine degrading activity was added to the salted fish products and held at 30C for 6 weeks in this study. Aerobic plate counts (APCs) in low spiked samples (4.0 log CFU/g) were remained constant during storage time, whereas, those of control samples gradually increased during first 3 weeks of storage and thereafter remained constant. However, the aerobic plate counts (APCs) of high spiked samples (6.0 log CFU/g) rapidly decreased to 4.0 log CFU/g at first week of storage, and thereafter increased slightly. The both spiked samples had considerably lower levels of total volatile basic nitrogen ($P < 0.05$) than control samples at each sampling time except for second week storage. In general, histamine contents in the both spiked samples markedly decreased during 6 weeks storage, whereas those of control samples increased with increased storage time. After 6 weeks storage, the histamine contents in high and low spiked samples were reduced for 45.8% and 36.0%, respectively, as compared with control samples. These results indicated that *B. polymyxa* D05-1 could be utilized in salted fish fermentation to reduce histamine levels in fermented products.

PRACTICAL APPLICATIONS

This study shows that *Bacillus polymyxa* D05-1 can be used as an additive to degrade histamine for salted fish product. In addition, *B. polymyxa* D05-1 could retard the increase of total volatile basic nitrogen in salted fish product. Therefore, this is an alternative method for the control of histamine in salted and fermented seafood.

INTRODUCTION

Biogenic amines are basic nitrogenous compounds occurring in meat, fish, cheese and wine products, mainly due to amino acid decarboxylation activities of certain microbes (Hungerford 2010). High levels of histamine in foods can have important vasoactive effects in humans (Lehane and Olley 2000). Scombroid fish, such as tuna, mackerel, bonito and saury, which contain high levels of free histidine in their muscle are often implicated in scombroid poisoning incidents when not properly processed and stored (Lehane and Olley 2000). Some of the nonscombroid fish, cheese and other foods have also been implicated in incidents of histamine poisoning (Lehane and Olley 2000). Fermented fish products may contain high contents of histamine (Mah *et al.* 2002). Although incidents of histamine poisoning following the consumption of these fermented fish products have not been reported, they may have occurred but went unnoticed because symptoms of histamine poisoning closely resemble those of food allergies (Lin *et al.* 2012).

Salted fish products are the traditional salted and fermented fish products used as a condiment or side dish in Asia countries. To prepare salted fish products, salt should be added at the level of 10–20% to raw fish, and then

allowed to ferment for 3–6 months depending on the processes until the fish tissue has decomposed (Lin *et al.* 2012). Typically, no starter culture was applied in salted fish products, as it merely relies on indigenous bacteria from raw materials. At present, application of starter cultures with high proteinase activity is a common practice to accelerate fermentation process (Yongsawatdigul *et al.* 2007). Salted fish products was reported to contain >5.0 mg/100 g of histamine, although it also contains many nutritious compounds (Yongsawatdigul *et al.* 2007).

Histamine may be physiologically degraded through the oxidative deamination process catalyzed by either histamine oxidase or histamine dehydrogenase. The presences of histamine dehydrogenase have been found in *Rhizobium* sp., *Nocardioides simplex, Natrinema gari* and *B. polymyxa* (Siddiqui *et al.* 2000; Sato *et al.* 2005; Naila *et al.* 2010; Tapingkae *et al.* 2010; Lee *et al.* 2015a), whereas some bacteria producing histamine oxidase included *Staphylococcus xylosus*, *Staphylococcus carnosus*, *Bacillus amyloliquefaciens*, *Arthrobacter crystallopoietes, Brevibacterium linens, Lactobacillus plantarum, Lactobacillus sake, Lactobacillus pentosus* and *Pediococcus acidilatici* (Leuschner *et al.* 1998; Sekiguchi *et al.* 2004; Zaman *et al.* 2010). The application of bacteria possessing histamine degrading enzyme has become an emerging method for reducing histamine concentration in foods, especially in fermented products. Mah and Hwang (2009) studied biogenic amine reduction in *Myeolchi-jeot*, a salted and fermented anchovy (*Engraulis japonicas*) by applying starter culture (*S. xylosus* No 0538) during ripening and found it degraded histamine by 38%. Recently, our study revealed that application of *B. polymyxa* D05-1 as a starter culture in salted fish products fermentation was effective on inhibiting histamine accumulation and enhanced the safety of salted and fermented fish products (Lee *et al.* 2015b).

In Taiwan, some commercial salted fish products had histamine contents greater than the 5.0 mg/100 g allowable limit suggested by the US Food and Drug Administration (Lin *et al.* 2012). Although histamine-degrading bacteria was used as a starter culture in inhibit histamine accumulation in fermented seafood product as described above, little information is available on the effect of histamine-degrading bacteria on histamine degradation for commercial salted fish products. Therefore, the objective of this study was to investigate the effects of histamine-degrading isolate, *B. polymyxa* D05-1, added in commercial salted fish product for degrading histamine as an alternative method for the control of histamine in seafood.

MATERIALS AND METHODS

Preparation of *B. polymyxa* D05-1

B. polymyxa D05-1 used in this study was isolated from salted fish products of Taiwan (Lee *et al.* 2015a). It exhibited a high activity in degrading histamine in Trypticase soy broth (TSB) (Difco) supplemented with 0.1% histamine (histamine TSB broth). One hundred microliters of the 20-h-old bacterial cultures in 5 mL of histamine TSB broth at 30C were inoculated into fresh 100 mL histamine TSB broth and incubated at 30C for another 24 h. The culture was centrifuged at 10,000 × g for 10 min at 4C and the cell pellet was washed and resuspended in sterile saline solution, serially diluted with sterile saline solution to obtain the suspension with the desired bacterial concentration.

Degradation of Histamine in Salted Fish Products by *B. polymyxa* D05-1

Salted fish product (sardine fish, *Sardinops sagax*) was purchased from the fishing village store in Taiwan on October, 2014. The salted fish product was packed in glass bottles and kept at room temperature in the stores before purchase. After purchase, it was immediately transported to the laboratory for use. On arrival, salted fish product was tested to determine the microbiological and chemical quality, and subsequently divided into three equal portions (each of 900 g samples). Following the addition of the 100 mL diluted bacterial culture suspension to reach a concentration of 1×10^4 CFU/g or 1×10^6 CFU/g for two lots, the spiked samples were well mixed by blending at low speed. The other lot was added with the 100 mL volume of sterile saline solution (control). Each lot was prepared in triplicate. All treatments were kept in an incubator at 30C for 6 weeks. Samples were drawn periodically for chemical and microbiological analysis.

Determination of pH Value, Salt Content, Water Activity and Total Volatile Basic Nitrogen

The salted fish sample (10 g) was homogenized in blenders (Omni International Waterburry, CT) for 5 min at 5,000 rpm with 40 mL of distilled water to make a thick slurry. The pH of this slurry was then measured using a Corning 145 pH meter (Corning Glass Works, Medfield, MA). Salt content in each sample was determined according to the AOAC (1995). Two grams of sample was homogenized with 18 mL of distilled water, and then was titrated with 0.1 M $AgNO_3$ using 10% w/v K_2CrO_4 solution as an indicator. Water activity (Aw) was determined at 27C using an electric hygrometer (Hygrodynamics, Inc., Silver Spring, MD). The total volatile basic nitrogen (TVBN) content of the sample was measured by the method of Conway's dish (Cobb *et al.* 1973). The TVBN extract of the sample in 6% trichloroacetic acid (TCA, Sigma, St. Louis, MO) was absorbed by boric acid and then titrated with 0.02 N HCl. The TVBN content was expressed in mg/100 g sample.

TABLE 1. VALUES OF PH VALUE, SALT CONTENT, WATER ACTIVITY, TOTAL VOLATILE BASIC NITROGEN, HISTAMINE CONTENT, AEROBIC PLATE COUNT (APC), TOTAL COLIFORM AND *ESCHERICHIA COLI* IN SALTED FISH PRODUCT

pH value	Salt content (%)	Aw	TVBN content (mg/100 g)	Histamine content (mg/100 g)	APC (log CFU/g)	TC (MPN/g)	*E. coli* (MPN/g)
6.1 ± 0.01*	18.0 ± 0.3	0.77 ± 0.01	117.9 ± 2.1	4.81 ± 0.04	2.2 ± 0.7	<3.0	<3.0

* Mean values ± standard deviation.

Microbiological Analysis

A 25-g portion of the salted fish sample was homogenized at high speed for 2 min in a sterile blender with 225 mL sterile potassium phosphate buffer (0.05 M, pH 7.0). The blender was sterilized by autoclaving for 15 min at 121C before use. The homogenates were serially diluted with a sterile phosphate buffer (1:9), and 1.0-mL aliquots of the dilutes were poured onto Petri dishes (9 cm diameter). Then, 15–20 mL of plate count agar (Difco, Detroit, MI) containing 3.0% NaCl at 45–50C was added and gently mixed. The poured plates were allowed solidify under a biological clean bench. Bacterial colonies were counted after the plates were incubated at 35C for 48 h. Bacterial numbers in the salted fish samples were expressed as $\log_{10}$ colony forming units (CFU)/g.

Analyses of total coliforms (TCs) and *Escherichia coli* in the salted fish product were conducted using the three-tube most probable number method (U.S. Food and Drug Administration 1998). Lauryl sulphate tryptose broth (LST broth) and brilliant green lactose bile (2%) broth (BGLB broth) incubated at 35C for 48 h were used for presumptive and confirmation tests for TC, respectively. *E. coli* was determined using the LST broth and *E. coli* broth (EC broth) incubated at 35C and 44.5C for 48 h, respectively. Cultures that showed positive production of gas in BGLB or EC broth were then confirmed by eosine methylene blue agar and by the indole, methyl red, Voges-Proskauer, and citrate tests.

Histamine Content Analysis of High Pressure Liquid Chromatograph

A 5-g sample was transferred into 50-mL centrifuge tubes and homogenized with 20 mL of 6% trichloroacetic acid (TCA) for 3 min. The homogenates were centrifuged (10,000 × g, 10 min, 4C) and filtered through Whatman No. 2 filter paper (Whatman, Maid-stone, England). The filtrates were then placed in volumetric flasks, and TCA was added to bring to the final volume to 50 mL. Samples of standard histamine solution and 1 mL aliquots of the sample extracts were derivatised with dansyl chloride according to the previously described method (Chen *et al.* 2010). The dansyl derivatives were filtrated through a 0.45-μm filter, and 20 μL aliquots were used for high pressure liquid chromatograph (HPLC) injection.

The histamine contents in the salted fish samples were determined with HPLC (Hitachi, Tokyo, Japan) consisting of a Model L-7100 pump, a Rheodyne Model 7125 syringe loading sample injector, a Model L-4000 UV–Vis detector (set at 254 nm) and a Model D-2500 Chromato-integrator. A LiChrospher 100 RP-18 reversed-phase column (5 μm, 125 × 4.6 mm, E. Merck, Damstadt, Germany) was used for chromatographic separation. The gradient elution programme began with 50:50 (v/v) acetonitrile: water at a flow rate of 1.0 mL/min for 19 min, followed by a linear increase to 90:10 acetonitrile:water (1.0 mL/min) during the next 1.0 min. Finally, the acetonitrile:water mix decreased to 50:50 (1.0 mL/min) for 10 min.

Statistical Analysis

The difference between different spiked concentrations of *B. polymyxa* D05-1 culture was determined using analysis of variance. Comparison of means was carried out with Duncan test. All statistical analysis was performed using the Statistical Package for Social Sciences, SPSS Version 16.0 for windows (SPSS Inc., Chicago, Illinois). Values of $P < 0.05$ were used to indicate significant deviation.

RESULTS AND DISCUSSION

Aerobic Bacterial Counts

As shown in Table 1, values of the pH, salt content, Aw, TVBN, histamine and aerobic plate count (APC) in the salted fish product used in this study was 6.1, 18.0%, 0.77, 117.9 mg/100 g, 4.81 mg/100 g and 2.2 log CFU/g, respectively. TC and *E. coli* were not detected in this sample (Table 1). The changes in aerobic plate counts (APCs) during storage of salted fish samples added without (control) or with low (4.0 log CFU/g) and high (6.0 log CFU/g) cultures of *B. polymyxa* are shown in Fig. 1. Initially, the APCs of control, and low and high spiked samples were 2.2 log CFU/g, 3.9 log CFU/g and 6.3 log CFU/g, respectively. APCs of control samples gradually increased during first 3 weeks of storage and thereafter remained constant to 3.8 log CFU/g after 6 weeks of storage. Conversely, APCs in low spiked samples were remained constant in the range of 3.6–3.9 log CFU/g during storage. The APCs of high spiked samples rapidly decreased to 4.0 log CFU/g at first week of storage, and thereafter

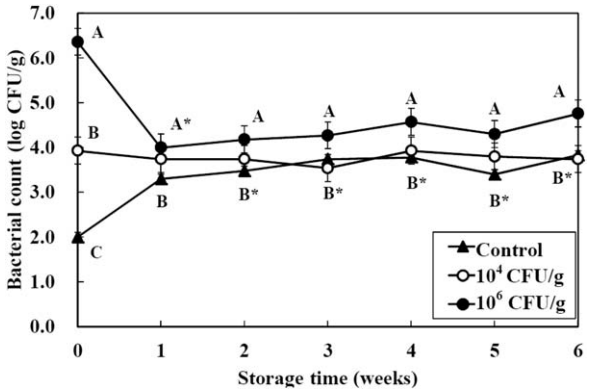

FIG. 1. CHANGES IN BACTERIAL COUNT DURING STORAGE OF SALTED FISH SAMPLES ADDED WITHOUT (CONTROL) OR WITH CULTURE OF *BACILLUS POLYMYXA* D05-1 AT 30C FOR 6 WEEKS (▲: WITHOUT ADDITION, ○: ADDED WITH *B. POLYMYXA* D05-1 FOR 1 × 10^4 CFU/G •: ADDED WITH *B. POLYMYXA* D05-1 FOR 1 × 10^6 CFU/G). EACH VALUE REPRESENTS MEAN ± SD OF THREE REPLICATIONS. POINT AT THE SAME STORAGE TIME MARKED WITH DIFFERENT LETTERS ARE SIGNIFICANTLY DIFFERENT ($P < 0.05$). *: NO SIGNIFICANTLY DIFFERENT

increased slightly to 4.7 log CFU/g at the end of storage. Moreover, the APCs were significantly lower ($P < 0.05$) in control as compared with the low or high spiked samples at first week of storage, but there was no significantly ($P > 0.05$) between control samples and low spiked samples for the next 5 weeks (Fig. 1). Meanwhile, APCs of high spiked samples were significantly ($P < 0.05$) higher than control and low spiked samples after 2 weeks of storage. The result of control samples in this study is in agreement with a previous report by Paludan-Muller *et al.* (2002) that halotolerant bacteria (including Lactic acid bacteria and yeast) began growth and propagate, and lead to increase in microbiological counts in Thai fermented fish product. Moreover, the result of high spiked samples in this study is in partial agreement with a recent report by Mah and Hwang (2009) that the total plate counts of inoculated sample (*S. xylosus* No.0538, 7 log CFU/g added to salted anchovy) rapidly decreased to 2 log CFU/g and thereafter increased slowly to 5 log CFU/g. Recently, the range of salt concentration for growth of *B. polymyxa* D05-1 was 0.5–5% NaCl, whereas levels of NaCl in excess of 15% inhibited their growth (Lee *et al.* 2015a). Therefore, as the salted fish product contained 16–18% NaCl, bacterial growth of *B. polymyxa* might be inhibited or retarded.

AW, pH Value, Salt Content and TVBN

The initial Aw of salted fish product was 0.77 (Table 1). Aw in control and spiked samples ranged from 0.76 to 0.79 remained constant and no significant different ($P > 0.05$) of Aw was found between control, and low and high spiked samples at each sampling time during 6 weeks storage (Data not shown). In agreement with our result, Mah and Hwang (2009) reported that the Aw of salted and fermented anchovy samples were constant to be in the range of 0.73–0.76, showing little change throughout ripening. As shown in Fig. 2, the pH value (6.1) in samples was detected at the initial time, and maintained to be constant in the range of 6.0–6.2 for the 6 weeks of storage in all samples. Meanwhile, there was no significant difference ($P > 0.05$) between the pH of control, and low and high spiked samples at each time of sampling. Similarity, Zaman *et al.* (2014) demonstrated that pH in fish sauce samples treated with 6.0 log CFU/g of histamine-degrading bacterium, *S. carnosus* FS19, were not changed after incubation, as compared with control. The changes in salt content during storage of salted fish product added without (control) or with low and high cultures of *B. polymyxa* are shown in Fig. 3. The initial salt content of salted fish samples was 18.0%. Salt content in control, and low and high spiked samples ranged from 16% to 18% remained constant throughout 6 weeks of storage and no significant different ($P > 0.05$) of salt content was found between control, and low and high spiked samples at each sampling time. Similarity, Zaman *et al.* (2014) demonstrated that salt contents in fish sauce samples treated with 6 log CFU/g of histamine-degrading bacterium, *S. carnosus* FS19, were not changed after incubation, as compared with control.

The changes in TVBN during storage of salted fish samples added without (control) or with low and high culture of *B. polymyxa* are shown in Fig. 4. The initial TVBN content of salted fish product was 117.9 mg/100 g (Table 1). The

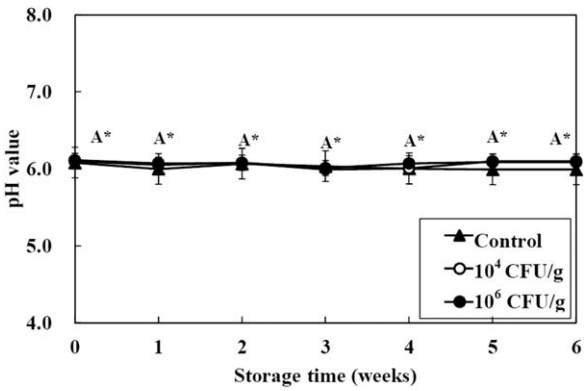

FIG. 2. CHANGES IN PH DURING STORAGE OF SALTED FISH SAMPLES ADDED WITHOUT (CONTROL) OR WITH CULTURE OF *B. POLYMYXA* D05-1 AT 30C FOR 6 WEEKS (▲: WITHOUT ADDITION, ○: ADDED WITH *B. POLYMYXA* D05-1 FOR 1 × 10^4 CFU/G •: ADDED WITH *B. POLYMYXA* D05-1 FOR 1 × 10^6 CFU/G). EACH VALUE REPRESENTS MEAN ± SD OF THREE REPLICATIONS. POINT AT THE SAME STORAGE TIME MARKED WITH DIFFERENT LETTERS ARE SIGNIFICANTLY DIFFERENT ($P < 0.05$). *: NO SIGNIFICANTLY DIFFERENT

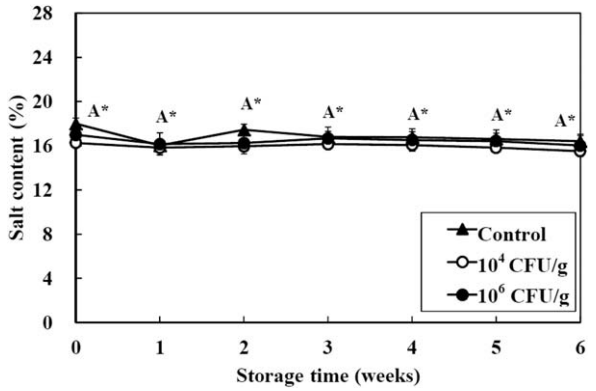

FIG. 3. CHANGES IN SALTED CONTENT DURING STORAGE OF SALTED FISH SAMPLES ADDED WITHOUT (CONTROL) OR WITH CULTURE OF *B. POLYMYXA* D05-1 AT 30C FOR 6 WEEKS (▲: WITHOUT ADDITION, ○: ADDED WITH *B. POLYMYXA* D05-1 FOR 1×10^4 CFU/G •: ADDED WITH *B. POLYMYXA* D05-1 FOR 1×10^6 CFU/G). EACH VALUE REPRESENTS MEAN ± SD OF THREE REPLICATIONS. POINT AT THE SAME STORAGE TIME MARKED WITH DIFFERENT LETTERS ARE SIGNIFICANTLY DIFFERENT ($P < 0.05$). *: NO SIGNIFICANTLY DIFFERENT

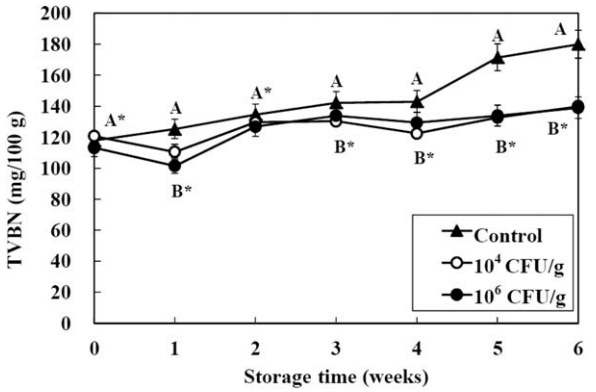

FIG. 4. CHANGES IN TVBN DURING STORAGE OF SALTED FISH SAMPLES ADDED WITHOUT (CONTROL) OR WITH CULTURE OF *B. POLYMYXA* D05-1 AT 30C FOR 6 WEEKS (▲: WITHOUT ADDITION, ○: ADDED WITH *B. POLYMYXA* D05-1 FOR 1×10^4 CFU/G •: ADDED WITH *B. POLYMYXA* D05-1 FOR 1×10^6 CFU/G). EACH VALUE REPRESENTS MEAN ± SD OF THREE REPLICATIONS. POINT AT THE SAME STORAGE TIME MARKED WITH DIFFERENT LETTERS ARE SIGNIFICANTLY DIFFERENT ($P < 0.05$). *: NO SIGNIFICANTLY DIFFERENT

levels of TVBN in control samples increased gradually during storage time, reaching 180 mg/100 g at the end of storage. In low and high spiked samples, the TVBN levels only slowly increased during storage time, reaching 139 mg/100 g and 140 mg/100 g at the end of storage, respectively. Conversely, TVBN levels of control were significantly ($P < 0.05$) higher than both spiked samples at each sampling time, except for the samples stored at second week (Fig. 4). TVBN, including trimethylamine (TMA), dimethylamine (DMA) and ammonia (NH_3), is one of the most widely used indicators for fish quality and spoilage (Gill 1990). Hernandez-Herrero *et al.* (1999) proposed that the increase of TVBN value in salted anchovies during ripening was due to proteolytic bacteria and porteolytic enzyme actions. Zaman *et al.* (2011) demonstrated that proteolytic bacterial count was higher in control than samples inoculated with starter cultures, capable of degrading histamine and biogenic amine during fish sauce fermentation. Therefore, the lower levels of TVBN in both spiked samples observed in this work might be due to inhibiting proteolytic bacterial proliferation and proteolytic enzyme activity by the culture of *B. polymyxa* during salted fish storage.

Histamine Profile

The changes in histamine during storage of salted fish samples added without (control) or with low and high culture of *B. polymyxa* are shown in Fig. 5. Initially, the histamine contents of control and both spiked samples were 4.81 mg/100 g. The contents of histamine in control samples increased gradually during storage time, reaching 5.72 mg/100 g at the end of storage, which might be due to histamine formation by histamine-forming bacteria present in salted fish product. In low and high spiked samples, the histamine contents slowly decreased during storage time, reaching 3.66 mg/100 g and 3.10 mg/100 g at the end of storage, respectively. Therefore, histamine contents of control samples were significantly ($P < 0.05$) higher than those of both spiked samples at each sampling time, except for the samples stored at first week (Fig. 5). In addition, histamine contents of high spiked

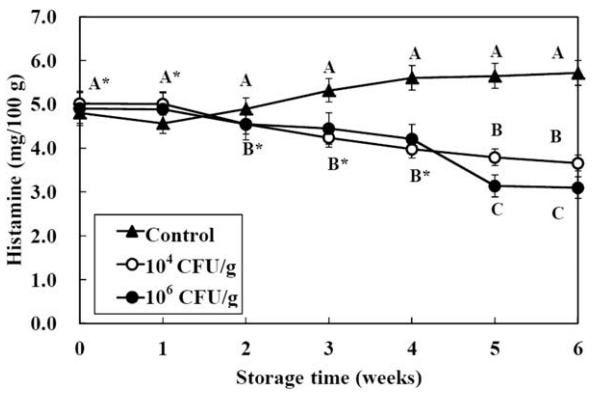

FIG. 5. CHANGES IN HISTAMINE CONTENT DURING STORAGE OF SALTED FISH SAMPLES ADDED WITHOUT (CONTROL) OR WITH CULTURE OF *B. POLYMYXA* D05-1 AT 30C FOR 6 WEEKS (▲: WITHOUT ADDITION, ○: ADDED WITH *B. POLYMYXA* D05-1 FOR 1×10^4 CFU/G •: ADDED WITH *B. POLYMYXA* D05-1 FOR 1×10^6 CFU/G). EACH VALUE REPRESENTS MEAN ± SD OF THREE REPLICATIONS. POINT AT THE SAME STORAGE TIME MARKED WITH DIFFERENT LETTERS ARE SIGNIFICANTLY DIFFERENT ($P < 0.05$). *: NO SIGNIFICANTLY DIFFERENT

sample were significantly ($P < 0.05$) lower than those of low spiked sample after 5 week of storage. After 6 weeks storage, histamine content was 36.0% and 45.8% less in low and high spiked samples, respectively, as compared with control samples. The result in this study is in agreement with a recent report by Zaman *et al.* (2014) that the histamine contents of treated sample (*S. carnosus* FS19, 6 log CFU/g added to fish sauce product) slowly decreased to 44.1 mg/100 g after 5 days incubation, as compared with control sample (51.4 mg/100 g of histamine). This proved that *B. polymyxa* D05-1 could degrade histamine and reduce histamine accumulation in salted fish products.

Kuda *et al.* (2012) demonstrated that the inhibitory activity of histamine-suppressing strain *Tetragenococcus halophilus* against histamine-forming bacteria HmF131 growth in salted and fermented sardine samples was due to competition in the environment, rapid nutrient utilization and depletion. The *S. xylosus* No.0538 as a starter culture was also found to produce bacteriocin-like inhibitory substance and have the highest antimicrobial activity against amine-producer of *Bacillus licheniformis* strains in salted and fermented anchovy (Mah and Hwang 2009). To explain the inhibitory effect of *B. polymyxa* D05-1 culture on histamine formation, two speculations are possible: (i) The culture of *B. polymyxa* D05-1 may be against histamine-forming bacterial growth by competition in the environment, rapid nutrient utilization and depletion, or production of bacteriocin-like inhibitory substance. (ii) The histamine dehydrogenase produced from *B. polymyxa* D05-1 may degrade histamine produced by histamine-forming bacteria in salted fish product.

Mah and Hwang (2009) reported in their study that *S. xylosus* which was applied as a protective culture in salted and fermented anchovy could reduce histamine by 18%, as compared with the control. Similarity, Zaman *et al.* (2014) also demonstrated that histamine content was reduced by 27.7% and 15.4% by *S. carnosus* FS19 and *B. amyloliquefaciens* FS05, respectively, as compared with control in fish sauce. In this study, the degradation percentages of 36% and 45.8% histamine were occurred in salted fish samples by spiked low and high levels of *B. polymyxa* D05-1, respectively. Consequently, these results suggest that *B. polymyxa* D05-1 can be used as additive to degrade histamine in salted fish products, enhancing food safety.

CONCLUSION

This study, to investigate the effect of *B. polymyxa* D05-1 as additive in salted fish product during storage, showed that the low and high spiked samples had lower TVBN and histamine contents than control samples. The degradation percentages of histamine in low and high spiked samples were 36.0% and 45.8% at the end of storage, as compared with control samples, respectively. This study demonstrated that application of *B. polymyxa* D05-1 as additive in salted fish fermentation could degrade histamine and reduce the levels of histamine in fermented products.

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