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## Original Article

# Reduction of histamine and biogenic amines during salted fish fermentation by *Bacillus polymyxa* as a starter culture



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## ABSTRACT

*Bacillus polymyxa* D05-1, isolated from salted fish product and possessing amine degrading activity, was used as a starter culture in salted fish fermentation in this study. Fermentation was held at 35°C for 120 days. The water activity in control samples (without starter culture) and inoculated samples (inoculated with *B. polymyxa* D05-1) remained constant throughout fermentation, whereas the pH value rose slightly during fermentation. Salt contents in both samples were constant in the range of 17.5–17.8% during the first 60 days of fermentation and thereafter increased slowly. The inoculated samples had considerably lower levels of total volatile basic nitrogen ( $p < 0.05$ ) than control samples at each sampling time during 120 days of fermentation. Aerobic bacterial counts in inoculated samples were retarded during the first 60 days of fermentation and thereafter increased slowly, whereas those of control samples increased rapidly with increased fermentation time. However, the aerobic bacterial counts of control samples were significantly higher ( $p < 0.05$ ) than those of inoculated samples after 40 days of fermentation. In general, overall biogenic amine contents (including histamine, putrescine, cadaverine, and tyramine) in the control samples were markedly higher ( $p < 0.05$ ) than those of the inoculated samples throughout fermentation. After 120 days of fermentation, the histamine and overall biogenic amine contents in the inoculated samples were reduced by 34.0% and 30.0%, respectively, compared to control samples. These results emphasize that the application of starter culture with amines degrading activity in salted fish products was effective in reducing biogenic amine accumulation.

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## 1. 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 [1]. High levels of histamine in foods can have important vasoactive effects in humans [2]. Scombroid fish, such as tuna, mackerel, bonito, and saury, which contain high levels of free histidine in their muscles are often implicated in scombroid poisoning incidents when not properly processed and stored [2]. Some nonscombroid fish, cheese, and other foods have also been implicated in incidents of histamine poisoning [2]. Fermented fish products may contain high contents of histamine [3]. 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 the symptoms of histamine poisoning closely resemble those of food allergies [4].

Salted fish products are the Taiwan traditional salted and fermented fish products used as a condiment or side dish in Taiwan. 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 processing store until the fish tissue has solubilized [4]. Typically, no starter culture was applied in salted fish products, because it merely relies on indigenous bacteria from raw materials. At present, the application of starter cultures with high proteinase activity is common practice to accelerate fermentation time [5,6]. The end of fermentation is determined by color, aroma, flavor, and clarity, which are typical qualities of salted fish products [7]. Salted fish products are reported to contain considerable amounts of biogenic amines, in addition to many nutritious compounds. Biogenic amine concentration in salted fish products was predominated by histamine, putrescine, cadaverine, and tyramine [8,9].

Histamine is physiologically degraded through the oxidative deamination process catalyzed by either histamine oxidase or histamine dehydrogenase. Histamine oxidase catalyzes the conversion of histamine, in the presence of water and oxygen, to imidazole acetaldehyde, ammonia, and hydrogen peroxide [10]. The presence of histamine oxidase was found in *Staphylococcus xylosum*, *Staphylococcus carnosus*, *Bacillus amyloliquefaciens*, *Arthrobacter crystallopoietes*, and *Brevibacterium linens* [9–12]. In addition, several bacteria also utilize histamine dehydrogenase to catalyze the oxidative deamination of histamine to imidazole acetaldehyde and ammonia [13]. Some bacteria that produce histamine dehydrogenase include *Rhizobium* sp., *Nocardioides simplex*, and *Natrinema gari* [14–18]. Recently, the application of bacteria possessing histamine-degrading enzymes has become an emerging method for reducing histamine concentration in foods, especially in fermented products [14,15,19]. Mah and Hwang [15] studied biogenic amine reduction in *Myeolchi-jeot*, a salted and fermented anchovy (*Engraulis japonicas*), by applying starter culture (*S. xylosum* No. 0538) during ripening and found that it degraded histamine and tyramine by 38% and 4%, respectively, and the total biogenic amine level was decreased by 16% [15]. After 120 days of fermentation in fish sauce, the histamine concentration was 27.7% and 15.7%

reduction in samples inoculated with *S. carnosus* FS19 and *B. amyloliquefaciens* FS05, respectively, compared to control samples [19]. In Taiwan, some salted fish products had a histamine content greater than the 5.0 mg/100 g allowable limit suggested by the US Food and Drug Administration [4]. Recently, the histamine-degrading isolate, *Bacillus polymyxa* D05-1, isolated from Taiwanese salted fish product, was found to exhibit high potential to degrade histamine [20]. Therefore, the objective of this study was to investigate the effects of the histamine-degrading isolate, *B. polymyxa* D05-1, as a starter culture in inhibiting histamine and biogenic amines accumulation during salted fish fermentation.

## 2. Materials and methods

### 2.1. Preparation of starter culture

The starter culture used in this study was *Bacillus polymyxa* D05-1, which was isolated from salted fish products of Taiwan [20]. It has high ability to degrade histamine and lesser ability to degrade putrescine and cadaverine in trypticase soy broth (TSB; Difco, Detroit, MI, USA) supplemented with 0.1% histamine (histamine TSB broth). Samples (100  $\mu$ L) of the 20-hour-old bacterial cultures in 5 mL of histamine TSB broth at 30°C were inoculated into fresh 100 mL histamine TSB broth and incubated at 30°C for another 24 hours. The culture was centrifuged at 10,000g for 10 minutes at 4°C and the cell pellet was washed and resuspended in sterile saline solution, adjusted to approximately  $1 \times 10^7$  cells/mL.

### 2.2. Preparation of salted fish sample fermentation

Sardines (*Sardinops sagax*, 8–12 cm long, 12–15 g/sardine) caught in the Taiwan strait were purchased from Tungkung (Pingtung, Taiwan). The fish samples were wrapped in bags, placed in ice, and immediately transported to the laboratory for use within 2 hours. Upon arrival, fish samples were mixed thoroughly with 17% salt (w/w) and divided into two equal portions (each of 1000 g raw sardines). One lot was treated with 100 mL (10% of sardine weight) of starter culture suspension ( $1 \times 10^7$  cells/mL). Another lot was treated with the same volume of sterile saline solution (control). Each lot was prepared in triplicate. The mixture of all treatments were transferred into glass jars and kept in an incubator at 35°C for 120 days. Samples were drawn periodically for chemical and microbiological analysis.

### 2.3. Determination of pH value, salt content, water activity, and total volatile basic nitrogen

The salted fish fermentation sample (10 g) was homogenized in blenders (Omni International Waterbury, CT, USA) for 5 minutes at 5000 rpm with 10 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, USA). Salt content in each sample was determined according to the Association of Official Agricultural Chemists (AOAC) [21]. Two grams of sample were homogenized with 18 mL of distilled water, and then titrated with 0.1 M AgNO<sub>3</sub> using 10%

w/v  $K_2CrO_4$  solution as an indicator. Water activity ( $A_w$ ) was determined by an Electric Hygrometer (HygroDynamics, Inc., Silver Spring, MD, USA) at 27°C. Examined samples were homogenized in CombiMax 600 blender (Brown GmbH, Kronberg, Germany). Approximately 5 g of homogenous and slurry sample was put in a disposable cup, completely covering the bottom of the cup and filling not more than half of it. The  $A_w$  was directly measured using a hygrometer with accuracy of  $\pm 0.003$ . The total volatile basic nitrogen (TVBN) content of the sample was measured using Conway's dish method [22]. The TVBN extract of the sample in 6% trichloroacetic acid (TCA, Sigma, St. Louis, MO, USA) was absorbed by boric acid and then titrated with 0.02 N HCl. The TVBN content was expressed in mg/100 g sample.

#### 2.4. Microbiological analysis

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

#### 2.5. Biogenic amine content analysis of HPLC

A 5-g sample was transferred into 50-mL centrifuge tubes and homogenized with 20 mL of 6% TCA for 3 minutes. The homogenates were centrifuged (10,000g, 10 minutes, 4°C) and filtered through Whatman No. 2 filter paper (Whatman, Maidstone, Kent, UK). The filtrates were then placed in volumetric flasks, and TCA was added to bring to the final volume to 50 mL. Samples of standard biogenic amine solutions and 1-mL aliquots of the sample extracts were derivatised with dansyl chloride according to the previously described method [23]. The dansyl derivatives were filtrated through a 0.45- $\mu$ m filter, and 20- $\mu$ L aliquots were used for high-pressure liquid chromatography (HPLC) injection.

The contents of biogenic amines (including histamine, putrescine, cadaverine, and tyramine) in the salted fish samples were determined using 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  $\mu$ m, 125 mm  $\times$  4.6 mm; E. Merck, Darmstadt, Germany) was used for chromatographic separation. The gradient elution program began with 50:50 (v/v) acetonitrile:water at a flow rate of 1.0 mL/min for 19 minutes, followed by a linear increase to 90:10 acetonitrile:water (1.0 mL/min) during the next minute. Finally, the acetonitrile:water mix was decreased to 50:50 (1.0 mL/min) for 10 minutes.

#### 2.6. Statistical analysis

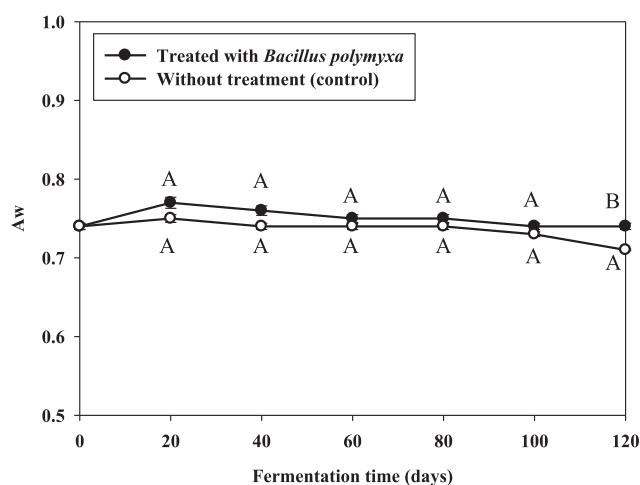
The significance of difference was determined using analysis of variance. Comparison of means was carried out using Duncan test. All statistical analysis was performed using SPSS Version 16.0 for Windows (SPSS Inc., Chicago, IL, USA). Values of  $p < 0.05$  were used to indicate significant deviation.

### 3. Results and discussion

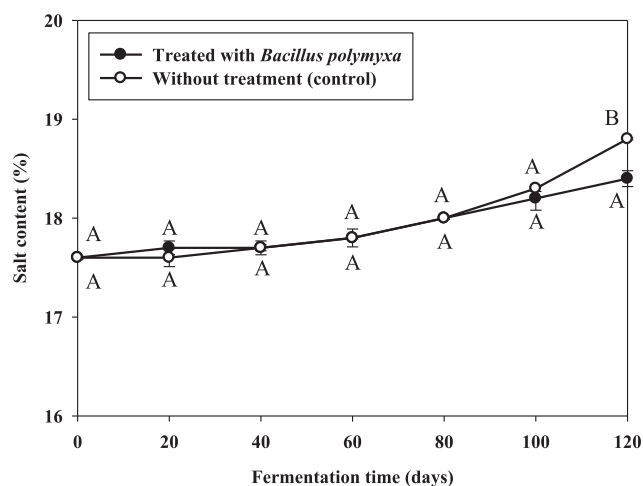
#### 3.1. $A_w$ , pH value, and salt content

The changes in water activity during fermentation of salted fish samples added without (control) or with starter culture of *B. polymyxa* are shown in Fig. 1. The initial  $A_w$  of salted fish samples was 0.74.  $A_w$  in both samples ranged from 0.73 to 0.77, remained constant, and no significant difference ( $p > 0.05$ ) of  $A_w$  was found between control and inoculated samples at each sampling time before Day 100 fermentation. In agreement with our result, Mah and Hwang [15] reported that the  $A_w$  of salted and fermented anchovy samples was constant in the range of 0.73–0.76, showing little change throughout ripening. However, there was significant difference ( $p < 0.05$ ) between the  $A_w$  of control (0.71) and inoculated sample (0.75) at the end of fermentation (Day 120). The difference between the observations could be attributed to the higher salt content in control sample than inoculated sample at Day 120 fermentation (Fig. 2).

The changes in salt content during fermentation of salted fish samples added without (control) or with starter culture of *B. polymyxa* are shown in Fig. 2. The initial salt content of salted fish samples was 17.5%. The salt content in both samples ranged from 17.5% to 17.7%, remained constant before Day 60 of fermentation, and no significant difference ( $p > 0.05$ ) of salt content was found between control and inoculated



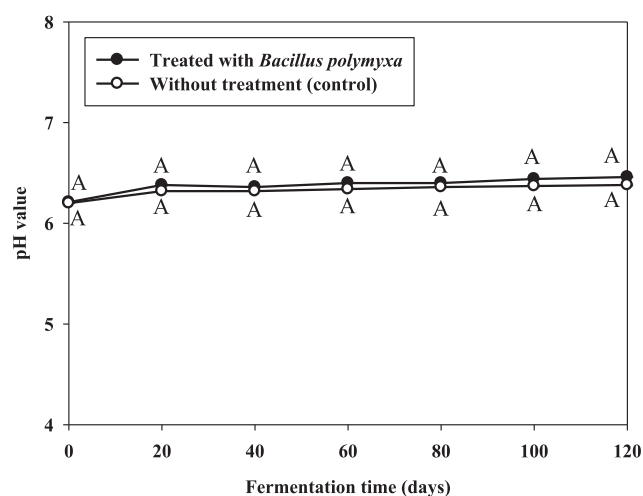
**Fig. 1** – Changes in water activity ( $A_w$ ) during fermentation of salted fish samples treated without (control) or with starter culture of *Bacillus polymyxa* at 35°C for 120 days. Each value represents mean  $\pm$  standard deviation of three replications. Points at the same fermentation time marked with different letters are significantly different ( $p < 0.05$ ).



**Fig. 2** – Changes in salt content during fermentation of salted fish samples treated without (control) or with starter culture of *Bacillus polymyxa* at 35°C for 120 days. Each value represents mean  $\pm$  standard deviation of three replications. Points at the same fermentation time marked with different letters are significantly different ( $p < 0.05$ ).

samples at each sampling time. Thereafter, the salt contents in control and inoculated samples increased to 18.8% and 18.4% at the end of fermentation (Day 120), respectively. There was significant difference ( $p < 0.05$ ) between the salt contents of control and inoculated sample at Day 120 of fermentation. Similarly, Xu et al [24] demonstrated that the salt content in fish sauce increased gradually after 30 days of fermentation.

As shown in Fig. 3, the pH value (6.2) in samples was detected at the initial time, increased slightly during 120 days

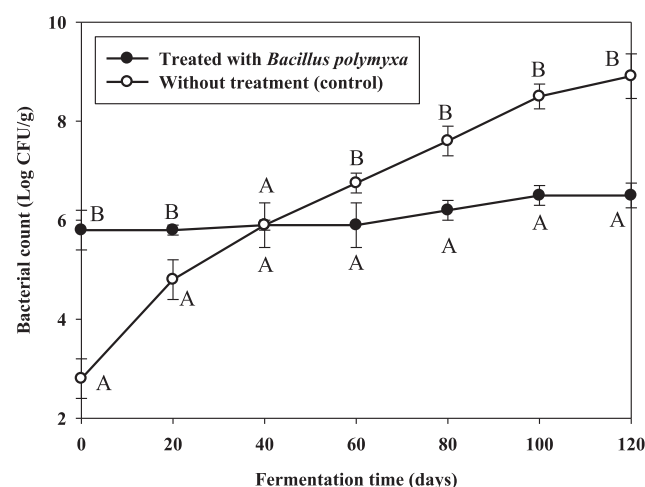


**Fig. 3** – Changes in pH during fermentation of salted fish samples treated without (control) or with starter culture of *Bacillus polymyxa* at 35°C for 120 days. Each value represents mean  $\pm$  standard deviation of three replications. Points at the same fermentation time marked with different letters are significantly different ( $p < 0.05$ ).

of fermentation, and reached pH 6.5 at the end of fermentation. However, there was no significant difference ( $p > 0.05$ ) between the pH of control and inoculated samples at each time of sampling. The pH value of salted fish samples was found to increase during fermentation in both samples. Biogenic amines were regarded as the main factor causing the increase in pH, because their concentration increased in accordance with the increase in pH [25].

### 3.2. Aerobic bacterial counts

The changes in aerobic bacterial counts during fermentation of salted fish samples added without (control) or with starter culture of *B. polymyxa* are shown in Fig. 4. Initially, the aerobic bacterial counts of control and inoculated samples were 3.0 log CFU/g and 5.8 log CFU/g, respectively. Aerobic bacterial counts of control samples rapidly increased with increased fermentation days and reached 8.9 log CFU/g after 120 days of fermentation, however, those of inoculated samples were retarded during the first 60 days of fermentation and then slightly increased with increased fermentation days, reaching 6.5 log CFU/g at the end of fermentation (Day 120). Moreover, the aerobic bacterial counts were significantly lower ( $p < 0.05$ ) in control compared to the inoculated samples before Day 40 of fermentation, whereas those of control were significantly ( $p < 0.05$ ) higher than inoculated samples after Day 40 of fermentation (Fig. 4). The result of control samples in this study is in agreement with a previous report by Paludan-Müller et al [26] that halotolerant bacteria (including lactic acid bacteria and yeast) began to grow and propagate, and lead to an increase in microbiological counts during fermentation in Thai fermented fish products. Kuda et al [27] demonstrated that the inhibitory activity of histamine-suppressing strain *Tetragenococcus halophilus* against histamine-forming bacteria HmF131 growth in salted and fermented sardine samples was



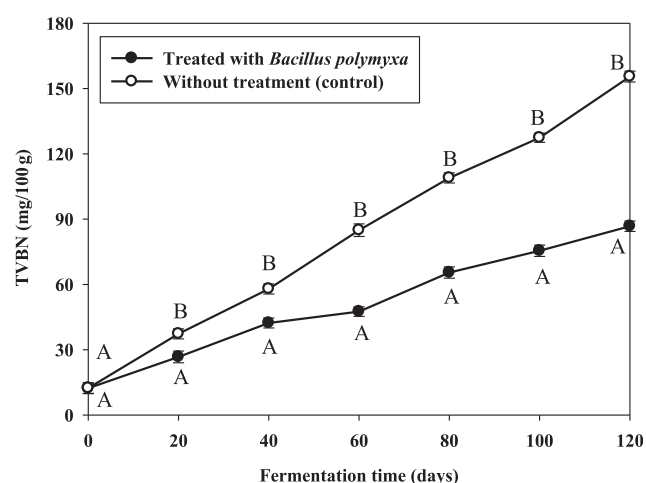
**Fig. 4** – Changes in bacterial count during fermentation of salted fish samples treated without (control) or with starter culture of *Bacillus polymyxa* at 35°C for 120 days. Each value represents mean  $\pm$  standard deviation of three replications. Points at the same fermentation time marked with different letters are significantly different ( $p < 0.05$ ). CFU = colony forming units.



due to competition in the environment, and rapid nutrient utilization and depletion. *S. xylosum* No.0538 as a starter culture was also found to produce a bacteriocin-like inhibitory substance and have the highest antimicrobial activity against amine-producer of *Bacillus licheniformis* strains in salted and fermented anchovy [15]. Therefore, the aerobic plate counts for slight increase in inoculated samples during fermentation could be due to the inhibitory activity of starter culture of *B. polymyxa* against other bacterial growth by competition in the environment, rapid nutrient utilization and depletion, or production of bacteriocin-like inhibitory substance. Moreover, the range of salt concentration for growth of *B. polymyxa* was 0.5–5% NaCl, whereas levels of NaCl in excess of 10% inhibited their growth [20]. Therefore, because the inoculated samples contained > 17% NaCl, bacterial growth of *B. polymyxa* might be inhibited or retarded.

### 3.3. TVBN

The changes in TVBN during fermentation of salted fish samples added without (control) or with starter culture of *B. polymyxa* are shown in Fig. 5. The initial TVBN content of salted fish samples was 12.35 mg/100 g. The levels of TVBN in control samples increased rapidly during fermentation time, reaching 155.5 mg/100 g at the end of fermentation (Day 120). In inoculated samples, the TVBN levels only gradually increased during fermentation time, reaching 86.7 mg/100 g at the end of fermentation (Day 120). In other words, TVBN levels of control were significantly ( $p < 0.05$ ) higher than inoculated samples at each sampling time (Fig. 5). TVBN, including trimethylamine, dimethylamine, and ammonia, is one of the most widely used indicators for fish quality and spoilage [28]. Hernandez-Herrero et al [29] proposed that the increase of TVBN value in salted anchovies during ripening was due to proteolytic bacteria and proteolytic enzyme actions. Zaman



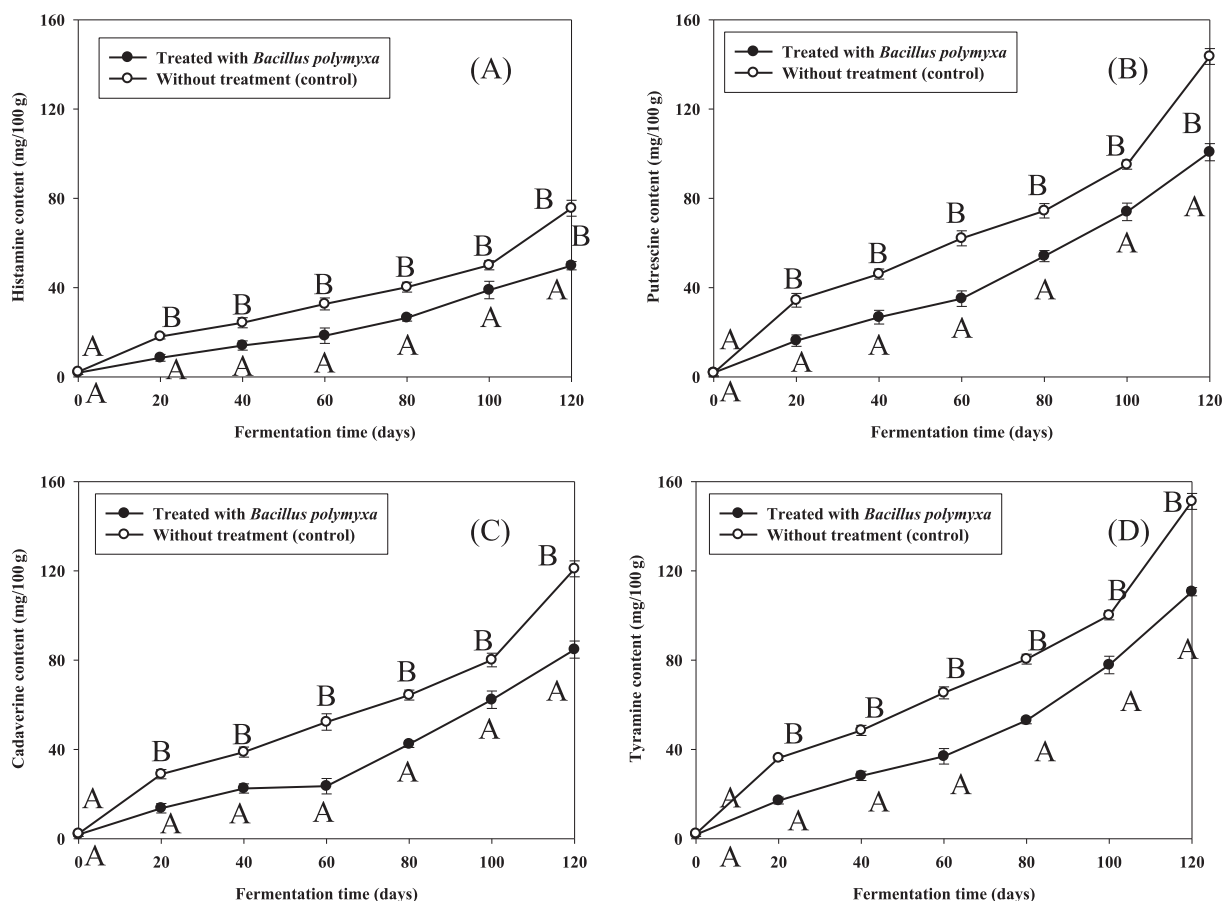
**Fig. 5 – Changes in total volatile basic nitrogen (TVBN) during fermentation of salted fish samples treated without (control) or with starter culture of *Bacillus polymyxa* at 35°C for 120 days. Each value represents mean  $\pm$  standard deviation of three replications. Points at the same fermentation time marked with different letters are significantly different ( $p < 0.05$ ).**

et al [19] demonstrated that proteolytic bacterial count was higher in control than samples inoculated with starter cultures, capable of degrading histamine and biogenic amines during fish sauce fermentation. Therefore, the lower levels of TVBN in inoculated samples observed in this work might be due to inhibiting proteolytic bacterial proliferation and proteolytic enzyme activity by the starter culture of *B. polymyxa* during salted fish fermentation.

### 3.4. Biogenic amine profile

The changes in biogenic amines during fermentation of salted fish samples added without (control) or with starter culture of *B. polymyxa* are shown in Fig. 6. Initially, the histamine contents of control and inoculated samples were 2.30 mg/100 g. The contents of histamine in control samples increased rapidly during fermentation time, reaching 75.56 mg/100 g at the end of fermentation (Day 120). In inoculated samples, the histamine contents only gradually increased during fermentation time, reaching 49.84 mg/100 g at the end of fermentation (Day 120). In other words, histamine contents of control samples were significantly ( $p < 0.05$ ) higher than those of inoculated samples at each sampling time (Fig. 6A). As shown in Fig. 6B, putrescine contents in control and inoculated samples (1.88 mg/100 g in the beginning) progressively increased with increased fermentation days and reached 143.56 mg/100 g and 100.70 mg/100 g at the end of fermentation (Day 120), respectively (Fig. 6B). Putrescine contents in control samples were significantly higher ( $p < 0.05$ ) than those of inoculated samples during fermentation. Similarly, cadaverine contents in control and inoculated samples (2.35 mg/100 g at the beginning) gradually increased with increased fermentation days and reached 120.90 mg/100 g and 84.74 mg/100 g at the end of fermentation (Day 120), respectively (Fig. 6C). We observed that cadaverine contents in control were significantly higher ( $p < 0.05$ ) than those of inoculated samples during fermentation. Fig. 6D shows that initial tyramine content was approximately 2.50 mg/100 g in both samples. In general, tyramine contents markedly increased to 151.12 mg/100 g in control samples and 110.68 mg/100 g in inoculated samples at the end of fermentation (Day 120). Tyramine contents in control were significantly higher ( $p < 0.05$ ) than those of inoculated samples during fermentation (Fig. 6D).

After 120 days of fermentation, histamine content was 34.0% less in inoculated samples compared to control samples. This shows that starter culture *B. polymyxa* D05-1 could reduce histamine accumulation. Moreover, the use of starter culture reduced the overall amine content (histamine + putrescine + cadaverine + tyramine) in salted fish samples (Fig. 6). After 120 days of fermentation, overall biogenic amines contents were approximately 30.0% less in inoculated samples compared to control samples. To explain the inhibitory effect of starter culture on biogenic amine formation, two speculations are possible: (1) starter culture presumably serves as a competing organism possessing less efficient amino acid decarboxylases than strong biogenic amine producers; and/or (2) it may degrade biogenic amines produced by strong amine producers. It is not discussed in depth herein, however, it is evident that



**Fig. 6** – Changes in biogenic amines (A) histamine, (B) putrescine, (C) cadaverine, and (D) tyramine during fermentation of salted fish samples treated without (control) or with starter culture of *Bacillus polymyxa* at 35°C or 120 days. Each value represents the mean  $\pm$  standard deviation of three replications. Points at the same fermentation time marked with different letters are significantly different ( $p < 0.05$ ).

the use of starter culture is an effective way to (at least partially) inhibit biogenic amine formation and that starter cultures having an inhibitory effect on biogenic amine formation may be applicable to fermentation and/or ripening processes for the production of other salted and/or fermented fish products [15].

Mah and Hwang [15] reported in their study that *S. xylosum*, which was applied as a protective culture in salted and fermented anchovy, could reduce histamine and overall amines by 18% and 16%, respectively, compared to the control. Similarly, Zaman et al [19] also demonstrated that histamine content was reduced by 27.7% and 15.4% by *S. carnosus* FS19 and *B. amyloliquefaciens* FS05, respectively, and the overall biogenic amines content was 15.9% and 12.5% less in inoculated *S. carnosus* FS19 and *B. amyloliquefaciens* FS05, respectively, compared to control in fish sauce. In this study, the reduction percentages of 34% histamine and 30% overall amines in inoculating samples by *B. polymyxa* D05-1 were higher than those previously reported by Mah and Hwang [15] for histamine and overall amines by 18% and 16%, respectively, and Zaman et al [19] for histamine and overall

amines by 27.7% and 15.9%, respectively. Consequently, these results suggest that *B. polymyxa* D05 can be used as a starter culture for salted and fermented fish products, enhancing food safety.

#### 4. Conclusion

This study, to investigate the effect of *B. polymyxa* D05-1 as starter culture during salted fish fermentation, showed that the inoculated samples had lower TVBN and biogenic amines contents than control samples. The reduction percentage of histamine in inoculated samples was 34% at the end of fermentation, compared to control samples. Inoculation of *B. polymyxa* D05-1 could inhibit other bacterial growth to retard the increase of aerobic bacterial counts in salted fish fermentation. Our results emphasize that application of *B. polymyxa* D05-1 as a starter culture in salted fish products fermentation is effective to inhibit biogenic amines accumulation and to enhance the safety of salted and fermented fish products.

## Conflicts of interest

All authors declare no conflicts of interest.

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