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Short communication

Histamine accumulation and histamine-forming bacteria in Indian anchovy (*Stolephorus indicus*)

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Abstract

Accumulation of histamine, trimethylamine (TMA), and total volatile base nitrogen (TVB-N), as well as microbial population incidence in Indian anchovy (*Stolephorus indicus*) during storage in ice and at 15 and 35 °C were investigated. Histamine was as low as 1.9 mg/100 g in 15 days at ice storage, but it increased to 19.0 mg/100 g after 32 h at 15 °C. Histamine rapidly increased to 25.4 mg/100 g when stored at 35 °C for 8 h. TVB-N and TMA began to sharply increase after 11 days in ice storage, but abruptly increased after 16 and 8 h of storage at 15 and 35 °C, respectively. A high number of *Enterobacteriaceae* (10^{10}–10^{11} cfu/g) was detected and shown to be the dominant group of microbial flora during spoilage of Indian anchovy at both 15 and 35 °C. A total of 153 bacterial strains were selected from the prescreening step using various selective media. Only 75.8% of these selected isolates showed a positive reaction in Niven's differential medium, and 27.6% of the positive isolates were true histamine formers when confirmed by the enzymatic method. Prolific histamine formers were identified as *Morganella morganii*, *Proteus vulgaris*, and *Enterobacter aerogenes*, and produced high histamine content of 104.1–203.0 mg/100 ml. Optimum growth and histamine production of selected strains of these three species was at 35 °C in histamine evaluation broth (HEB) containing 0.5% NaCl, pH 5. *E. aerogenes* produced the highest histamine of 500 mg/100 ml at the optimum condition. All studied strains did not produce histamine at ⩾10% NaCl.

Keywords: Histamine-forming bacteria; Anchovy; Histamine; *Stolephorus indicus*

1. Introduction

Indian anchovy (*Stolephorus indicus*) is abundant fishery resource in Southeast Asia. It is a major raw material for commercial fish sauce production. Fish sauce is clear amber liquid with a unique aroma and flavor, and is rich in amino acids (Saisithi, 1994). It is widely used as a condiment and seasoning in most countries of Southeast Asia and gradually gained acceptance worldwide. Fish sauce made from Indian anchovy was found to contain histamine content ranged from 20.97 to 78.30 mg/100 ml (Sanceda et al., 1999; Brillantes et al., 2002; Yongsawatdigul et al., 2004). Salted and fermented anchovy also contained high histamine of 15.5–57.9 mg/100 g (Mah et al., 2002). The maximum allowable histamine in fish sauce imposed by the Canadian Food Inspection Agency (CFIA) is 20 mg/100 g (CFIA, 2003). Therefore, some commercial fish sauce products contain histamine exceeding the standard. The presence of histamine in fish sauce does not pose any health threat compared to other fishery products because average uptake of fish sauce is relatively small, about 20 ml/person/day (Anonymous, 2000). However, it implies poor hygienic qualities of raw material and/or manufacturing processes. Yongsawatdigul et al. (2004) reported the subtle changes of histamine during fish sauce fermentation, indicating that high histamine level in Indian anchovy fish sauce could be associated with histamine content of raw material. However, histamine formation in Indian anchovy (*S. indicus*) has not yet been studied.

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Indian anchovy is typically caught and stored on board without ice or salt, resulting in deterioration of freshness quality. In addition, fish is normally transported to a processing plant in an opened container with exposure to sunlight. Therefore, fish is subjected to severe temperature abuse before being processed. However, changes of chemical and microbiological qualities of Indian anchovy with respect to storage temperature and time have not been established.

Several species of the *Enterobateriaceae* family have been primarily associated with high histamine level of decomposed scombroid fish. The species included *Morganella morganii*, *Klebsiella pneumoniae*, *Hafnia alvei*, *Proteus vulgaris*, *Enterobacter aerogenes*, *Enterobacter cloacae*, *Serratia fonticola*, *Providencia stuartii*, and *Citrobacter freundii* (Ababouch et al., 1991; Kim et al., 2001; López-Sabater et al., 1994b; Okuzumi et al., 1984). In addition to these species, *Clostridium* spp., *Vibrio alginolyticus*, *Acinetobacter lwoffii*, *Plesiomonas shigelloides*, *Pseudomonas putida*, *Pseudomonas fluorescens*, *Photobacterium phosphoreum*, and *Aeromonas* spp. have been isolated and found to produce histamine in various fish species (López-Sabater et al., 1996a; Middlebrooks et al., 1988; Morii et al., 1988; Ryser et al., 1984). However, bacteria responsible for histamine formation in Indian anchovy have not been isolated, identified, and thoroughly investigated.

The objectives of our study were to investigate histamine accumulation, as well as changes of trimethylamine (TMA) and total volatile base nitrogen (TVB-N), and microbial population incidence in Indian anchovy (*S. indicus*) stored at various temperatures. Prolific histamine-forming bacteria were then isolated from spoiled anchovy using the two-step procedure, and identified to species. Some factors affecting growth and histamine formation of strong histamine-forming isolates were also elucidated.

2. Materials and methods

2.1. Fish samples and sample storage

Fresh Indian anchovies (*S. indicus*) caught off the Gulf of Thailand were obtained from Chonburi province. Samples were kept in ice on board and transported to Suranaree University laboratory within 4 h after catch. Upon arrival, samples were placed in sterile plastic bags and stored in ice and at 15 and 35 °C. In the ice storage, samples were kept in polystyrene foam boxes filled with ice and stored in a cold room (7 °C). Water was drained off containers, and fresh ice was added every day throughout the study. All samples were taken at each time intervals for chemical and microbiological analyses. Chemical changes including histamine, TMA, and TVB-N were monitored. To isolate histamine-forming bacteria, fish samples were stored at 35 °C up to 16 h.

2.2. Chemical analyses of anchovy during storage

Histamine content in anchovy during storage in ice and at 15 and 35 °C was analysed in duplicate using spectrofluorometric method (Association of Official Analytical Chemists (AOAC), 1995). Extraction was performed in methanol. The methanol filtrate was loaded onto the Dowex 1-X8 ion-exchange column. The eluant was derivatized with *o*-phthaldialdehyde, and fluorescence intensity was determined using a spectrophotometer (RF-1501, Shimadzu Co., Kyoto, Japan) at excitation wavelength of 350 nm and emission wavelength of 444 nm.

TMA was determined by the Modified Dyer Picrate method (AOAC, 1995). Whole fish (20 g) was homogenized in 80 ml cold 7.5% (w/v) trichloroacetic acid. The homogenate was centrifuged at 8000 rpm (PK 121R, ALC International Srl, Italy) at 4 °C for 10 min. The supernatant was further extracted in toluene and reacted with 1% picric acid. Absorbance was measured at 410 nm using TMA as a standard.

TVB-N was determined by stream distillation as described by Botta et al. (1984). Ten grams of homogenized anchovy were added 2 g MgO and 40 ml distilled water. Steam distillation was performed using Kjeldahl distillation unit (Vapordest 30, Gerhardt, Germany) for 5 min. The distillate was titrated with 0.1 N HCl, and TVB-N was calculated and expressed as mgN/100 g sample.

2.3. Enumeration of microbial flora in anchovy during storage

The enumeration of microbial flora in Indian anchovy during storage in ice and at 15 and 35 °C was performed by homogenizing 25-g fish samples with 225 ml of sterile peptone water (0.1%) in a stomacher 400 (Seward, London, England). The homogenates were serially diluted and spread onto plate count agar (PCA, Difco Laboratories, Detroit, Michigan, USA), violet red bile glucose (VRBG) agar (Difco Laboratories, Detroit, Michigan, USA), and thiosulfate citrate bile salt (TCBS) agar (Difco Laboratories, Detroit, Michigan, USA), for the enumeration of total viable cells, *Enterobacteriaceae*, and vibrios, respectively. The plates were incubated at 15 °C for 7 days for the enumeration of psychrotrophic microorganisms in all fish samples stored in ice and at 15 °C, and at 35 °C for 24 h for aerobic mesophiles in fish samples stored at 35 °C. The enumeration of these microorganisms was carried out in duplicate.

2.4. Two-step isolation of histamine-forming bacteria

The isolation of histamine-forming bacteria was performed by prescreening for a target group of bacteria using selective agar media and screening for histidine decarboxylase activity on Niven's differential medium as described by Kim et al. (2001). The target bacteria were *Enterobacteriaceae*, pseudomonads, vibrios, and moderately halophilic and halotolerant bacteria, which have been previously reported as histamine formers (Ababouch et al., 1991; Hernández-Herrero et al., 1999; Kim et al., 2000; López-Sabater et al., 1994a, 1996b; Middlebrooks et al., 1988; Ryser et al., 1984; Yatsunami and Echigi, 1993).

Fish stored at 35 °C for 16 h were homogenized and diluted as previously described, then spread onto selective media: VRBG for *Enterobacteriaceae*, pseudomonad isolation (PI) agar (Difco Laboratories, Detroit, Mich.) for pseudomonads, TCBS agar for vibrios, halobacterium medium (Atlas and Parks, 1997) containing 10% NaCl for moderately halophilic and halotolerant bacteria, as well as the Niven's medium (Niven et al., 1981) for the direct detection of histamine-forming bacteria. All plates were incubated at 35 °C for 24 h. Colonies obtained from each plate were randomly selected. The morphological characteristics of colonies were also considered as a selection criterion. Selected colonies were then purified, and kept on tryptic soy agar (TSA, Difco Laboratories, Detroit, Mich.) at 4 °C throughout the study. To screen for histamine-forming bacteria, the overnight cultures on TSA were streaked on the Niven's medium, and incubated at 35 °C for 24 h. Purple colonies with purple halos on the yellow background were considered positive for histamine production. All positive colonies were collected for the confirmation of histamine production in broth medium.

2.5. Confirmation of histamine formation of selected bacterial isolates

Histamine formation by the presumptive histamine producers was confirmed by analyzing the culture broth for histamine. A loopful of each presumptive isolate on the Niven's medium was inoculated into 9 ml of histamine evaluation broth (HEB) containing 0.5% tryptone, 0.5% NaCl, 0.25% K_2HPO_4, 1% histidine-HCl, pH 5.7 (Rodríguez-Jerez et al., 1994) in a glass tube (160 mm × 15 mm). All isolates were incubated at 35 °C for 20 h to reach an approximate cell concentration of 10^8 cfu/ml. One-milliliter aliquot of the each culture was transferred to a new tube containing 9 ml of the same medium, then incubated for 18 h at 35 °C. The bacterial cultures were then centrifuged at 13,000 rpm (Centrifuge 5415D, Eppendorf, Hamburg, Germany) for 15 min. The supernatant was analysed for histamine using the enzymatic method (Rodríguez-Jerez et al., 1994). Potential histamine formers capable of producing histamine >1 mg/100 ml were selected to be identified, and strong histamine-forming isolates producing histamine >100 mg/100 ml were selected for their growth and histamine production studies.

2.6. Identification of histamine-forming bacteria

The bacterial isolates producing histamine in HEB were initially classified on the basis of Gram staining, cell morphology and motility, catalase and cytochrome-oxidase production, and oxidation/fermentation tests (Holt et al., 1994). Further identification to the species was accomplished using API identification systems (Bio-Mérieux, Marcy-l'Etoile, France).

2.7. Effects of temperature, NaCl, and pH on growth and histamine production of selected isolates

Prolific histamine-producing bacteria isolated from spoiled anchovy were selected and tested for their growth and histamine-producing ability in HEB at various conditions. An overnight culture on TSA was inoculated into TSB and incubated at 35 °C for 18 h. One milliliter of inoculum containing approximate 10^8 cfu/ml was added into 9 ml HEB containing 0.5%, 5%, 10%, 20% and 25% NaCl (pH 5.7) and incubated at 35 °C. Bacterial cultures in the HEB containing 0.5–10% NaCl were incubated for 18 h, while those containing 20% and 25% NaCl were incubated for 7 days. Subsequently, total viable counts of all samples were determined. Bacterial cells were removed by centrifugation at 13,000 rpm (Centrifuge 5415D, Eppendorf, Hamburg, Germany.) for 15 min. Supernatant was collected and analysed for histamine content using spectrofluorometric method (AOAC, 1995).

The effect of pH (5.0, 5.7, 6.5, and 7.0) was carried out using HEB containing 0.5% NaCl. Samples were incubated at 35 °C for 18 h. The effect of temperature (0, 15, 25, 35, 45, and 55 °C) was also conducted using HEB containing 0.5% NaCl at pH 5.7. All cultures were incubated for 18 h.

2.8. Statistical analysis

Experiments were replicated twice with two different lots of fish. All analyses were run in duplicate for each replication. Mean values were presented. Analysis of variance (ANOVA) was performed and Duncan's multiple range test (DMRT) was used to determine differences between mean at $p<0.05$ (SAS Institute, Inc., NC, USA).

3. Results and discussion

3.1. Chemical and microbiological changes of anychovy during storage

Histamine content of anchovy stored in ice gradually increased within the first 15 days (Fig. 1a). TVB-N and TMA also gradually increased during the first 11 days, and began to sharply increase afterwards (Fig. 1b). Based on odor and appearance, anchovy became spoiled after 11 days of storage in ice. Although spoilage was observed, histamine level of spoiled anchovy remained relatively low during 15 days in ice. This could be because histamine-forming bacteria did not grow well at low temperature. The total viable counts slowly increased and reached 10^7 cfu/g after 15 days of ice storage (Fig. 1c). Counts of bacteria belonging to the *Enterobacteriaceae* family also gradually increased and exceeded 10^6 cfu/g at day 13. None of vibrios was detected but non-vibrio colonies were found on TCBS agar (Fig. 1c). These results indicated that ice storage could minimize the formation of histamine in anchovy. Psychrotrophic bacteria contributing to spoilage of anchovy were unlikely to form histamine.

Histamine content slowly increased within 16 h at 15 °C, and rapidly increased to reach the maximum value of 129.9 mg/100 g after 104 h (approximately 4 days) (Fig. 2a). TMA and TVB-N also gradually increased during the first 16 h, and abruptly increased thereafter (Figs. 2b,c). An increase in histamine, TMA, and TVB-N corresponded with the outgrowth of microbial flora, especially total and *Enterobacteriaceae*

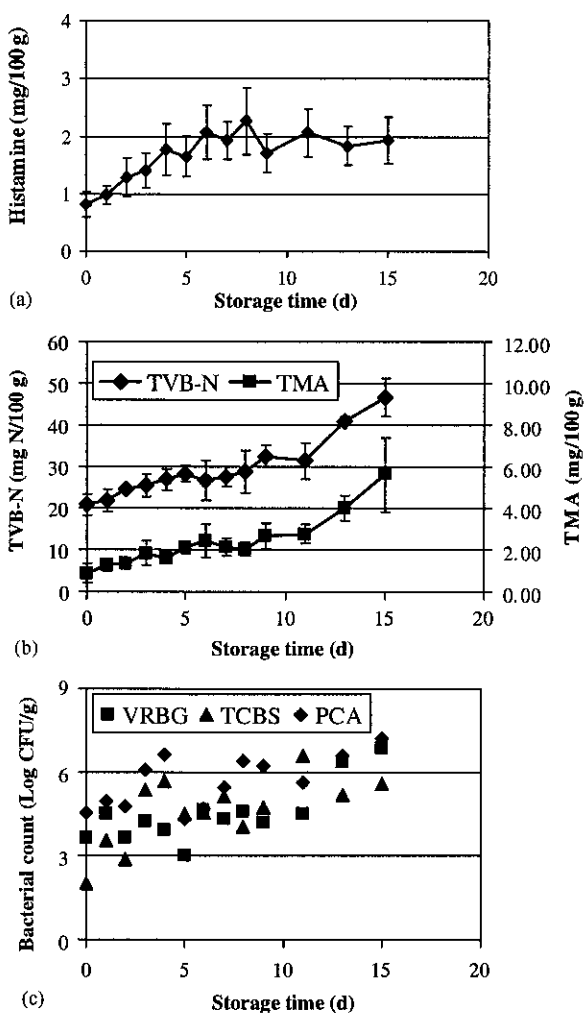

Fig. 1. Changes of histamine (a), TMA and TVB-N (b), and microbial counts (c) in Indian anchovy during storage in ice.

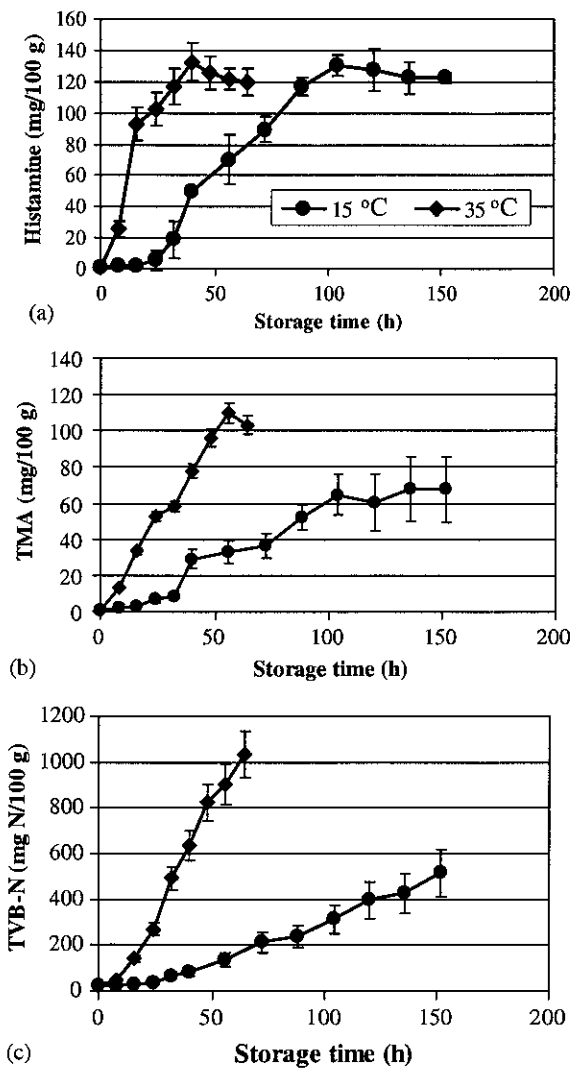

Fig. 2. Changes of histamine (a), TMA (b), and TVB-N (c) in Indian anchovy during storage at 15 and 35 °C.

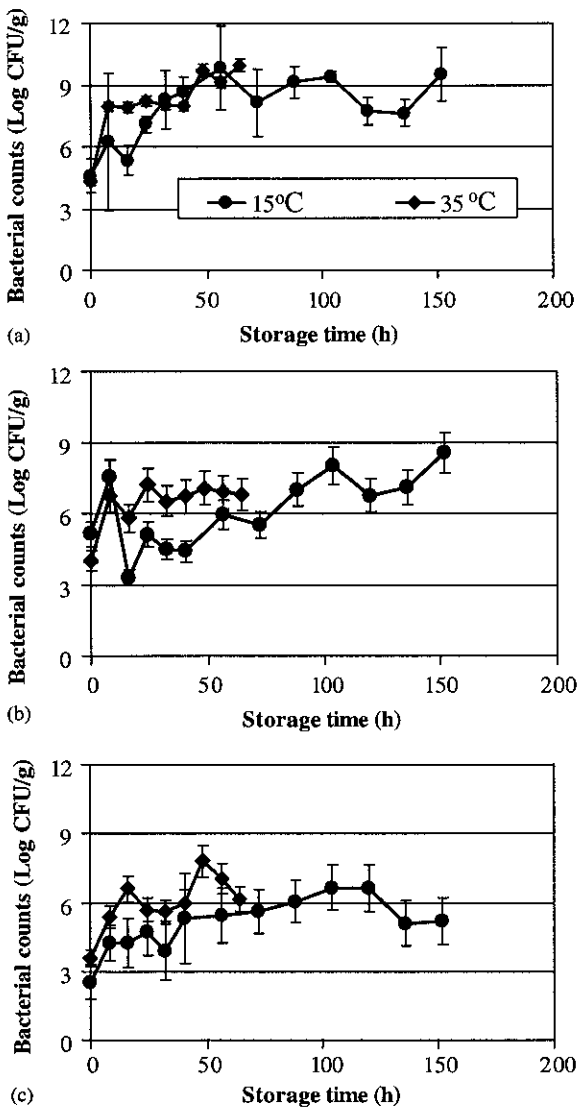

Fig. 3. Occurrence of total viable counts (a), *Enterobacteriaceae* (b), and non-vibrios from TCBS agar (c) in Indian anchovy during storage at 15 and 35 °C.

counts (Figs. 3a,b). Low temperature delayed the growth of bacteria, resulting in minimal changes of histamine, TMA, and TVB-N, during the first 16 h of storage. An increase in a number of both psychrotrophic and mesophilic bacteria took place as storage time was prolonged, leading to an abrupt increase in histamine, TMA, and TVB-N after 16 h.

Histamine, TMA, and TVB-N contents in anchovy stored at 35 °C continually increased at a faster rate than those at 15 °C (Figs. 2a–c). The highest level of histamine content, 132.3 mg/100 g, was attained within 40 h of storage (Fig. 2a). The total viable counts rapidly increased to 10^8 cfu/g within 8 h at 35 °C (Fig. 3a). *Enterobacteriaceae* appeared to be a predominant group during the course of storage at 35 °C (Fig. 3b). Proliferation of microbial flora corresponded with an increase in histamine level of Indian anchovy.

3.2. Isolation, confirmation, and identification of histamine-forming bacteria

Spoiled anchovy used for bacterial isolation contained 143.8 and 202.6 mg/100 g of histamine in the 1st and 2nd lot, respectively. One hundred and fifty three bacterial isolates were selected in the prescreening step. One hundred and sixteen isolates showed a positive reaction on Niven's medium in the screening step. Only 32 of 116 isolates were able to produce a wide range of histamine in HEB (Table 1).

Of the positive isolates obtained from PI agar, 75% were histamine formers producing histamine in the range of 3.8–116.6 mg/100 ml in HEB. Majority (75%) of the histamine formers produced high level of histamine, 104.1–116.6 mg/100 ml, in HEB. Selected isolates from VRBG agar showed 80% positive on the Niven's medium, but only 29% of the positive isolates were true histamine formers that produced histamine of 133.6–203.0 mg/100 ml (Table 1).

When the Niven's medium was used in the prescreening step, only 40% of the positive isolates were true histamine formers after confirming by culturing in HEB. Majority of histamine formers (82%) isolated from the Niven's medium produced high histamine of 72.1–153.9 mg/100 ml. Only non-vibrio colonies were found on TCBS agar. Almost 60% of the selected non-vibrio isolates showed a positive result on the Niven's medium, but histamine formation in the HEB was not detected. Thirty five colonies isolated from halobacterium medium were selected for screening using the Niven's medium, and 80% of these colonies showed positive results. All of them were Gram-positive. However, none was true histamine former when confirmed in HEB.

In this study, strong histamine formers, capable of producing more than 100 mg/100 ml of histamine, were isolated using both PI and VRBG agar, as well as Niven's medium. At least 25 histamine-forming isolates were strong histamine producers. Eleven and thirteen strong histamine-forming isolates were identified as *M. morganii* and *E. aerogenes*, respectively, while one isolate was identified as *P. vulgaris* (Table 1). These three species were also reported to exhibit strong histidine decarboxylase activity in various fish (Middlebrooks et al., 1988; Taylor, 1986). The histamine production capability of *M. morganii* and *E. aerogenes* was strain specific as seen by one strain from each species produced low level of histamine (Table 1). *Citrobacter youngae* produced a small amount of histamine (4.6 mg/100 ml), which has not been reported to be found in other fish species. Middlebrooks et al.

Table 1
Histamine-producing bacteria isolated from temperature-abused Indian anchovy

Bacterial strain	Range of histamine (mg/100 ml)	Number of isolate	Isolation medium
Morganella morganii	104.1–116.6 (110.8 ± 5.6)[a]	7	PI
M. morganii	133.6–203.0 (166.9 ± 28.4)[a]	4	VRBG
M. morganii	1.6–76.6	2	Niven
Proteus vulgaris	100.6	1	PI
Enterobacter aerogenes	115.0–134.5 (130.8 ± 12.9)[a]	13	Niven
E. aerogenes	4.9	1	PI
E. cloacae	1.1	1	Niven
Citrobacter youngae	4.6	1	PI
NI[b]	0.6	1	Niven
NI[b]	3.8	1	PI

[a]Mean ± standard deviation.
[b]Not identified.

(1988) showed that *M. morganii*, *P. vulgaris*, and *Pseudomonas putrefaciens* isolated from Spanish mackerel decomposed at 30 °C exhibited high histidine decarboxylase activity. However, either *Shewanella putrefaciens* (previously classified as *Pseudomonas putrefaciens*) or other *Pseudomonas* species could not be detected in Indian anchovy. *M. morganii* was the most prolific histamine producer isolated from decomposed tuna (López-Sabater et al., 1994b; Kim et al., 2000). High level of histamine produced by *M. morganii* has also been detected in Spanish salted anchovy (Rodríguez-Jerez et al., 1994), mackerel (Okuzumi et al., 1984), and sardine (Ababouch et al., 1991).

Based on our study, *M. morganii* was the prevalent species isolated using PI and VRBG agar. Other strong histamine formers, *E. aerogenes* and *P. vulgaris*, were isolated using the Niven's medium and PI agar, respectively (Table 1). Although the Niven's medium resulted in high rate of false-positive, strong histamine formers were successfully isolated from the medium. This was in contrast to the findings of Kim et al. (2001) who reported that only weak histamine formers were isolated from the Niven's medium.

3.3. Effects of temperature, NaCl, and pH on growth and histamine production of selected isolates

Selected isolates of *M. morganii*, *E. aerogenes*, and *P. vulgaris* obtained from decomposed Indian anchovy were investigated for some factors affecting their growth and histamine formation. The three representative strains grew well in HEB at 15, 25 and 35 °C with the maximum growth observed at 35 °C. *M. morganii* was the most prolific histamine former at 15 and 25 °C, while *E. aerogenes* produced the highest histamine at 35 °C ($p < 0.05$) (Fig. 4a). This corresponded to an increase in histamine found in anchovy stored at 15 °C (Fig. 2a). Histamine was not detected at 0 °C although the viable cell counts of all strains remained 10^8 cfu/ml. This would explain the low histamine level of anchovy stored in ice (Fig. 1a). Growth of the selected strains was limited at higher temperatures (45 and 55 °C). Consequently, no histamine was detected at high temperatures (Fig. 4a).

All strains were able to grow well at pH 5–7 with the number of viable cells was about 10^9–10^{10} cfu/ml at 35 °C for 18 h. However, histamine content of all strains was the highest at pH 5 and decreased as pH increased (Fig. 4b). These findings agree with Ababouch et al. (1991) reporting that histidine decarboxylase activity of *M. morganii* and *P. vulgaris* decreased as pH increased from 5 to 7. The high histamine production at low pH was likely due to high histidine decarboxylase activity at low pH. Tanase et al. (1985) also found that the enzyme purified from *M. morganii* exhibited the highest activity at pH 5 and its activity moderately decreased as pH increased to 8.0. Furthermore, bacteria were more strongly encouraged to produce histidine decarboxylase under acid condition, as a part of their defense mechanisms against the acidity (Santos, 1996).

The maximum histamine production of all strains was obtained in HEB medium containing 0.5% NaCl (Fig. 4c). *M. morganii*, *E. aerogenes*, and *P. vulgaris* produced high level of histamine (about 300 mg/100 ml) in the medium containing 5% NaCl, while negligible histamine was produced at 10% NaCl. Therefore, 10% NaCl can be used to control histamine level in Indian anchovy, especially on board when ice is limited. High salt content (20–25% NaCl) resulted in non-growth of the selected strains of *M. morganii*, *E. aerogenes*, and *P. vulgaris*. The most effective chemical for controlling histamine formation in many salted products has been thought to be NaCl (Hernández-Herrero et al., 1999; Kimura et al., 2001).

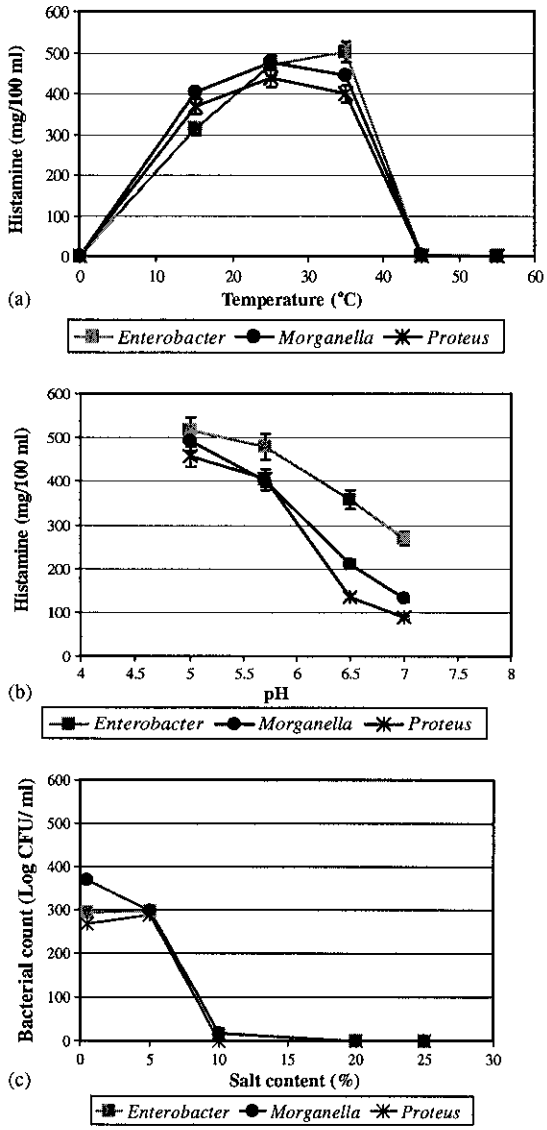

Fig. 4. Effects of temperature (a), pH (b), and NaCl concentration (c) on histamine-forming ability of selected bacterial strains isolated from Indian anchovy.

4. Conclusions

Indian anchovy is another fish species susceptible to high level of histamine when subjected to temperature abuse. Ice and cold storage (15 °C) could delay microbial growth, fish spoilage, and histamine accumulation. Histamine rapidly increased within 8 h when fish was kept at 35 °C. *M. morganii*, *E. aerogenes*, and *P. vulgaris* were prolific histamine formers isolated from decomposed Indian anchovy. Growth and histamine formation of the representative strains of the three species were minimal either at 0 °C or in the medium containing ⩾10% NaCl. Ice storage and/or salting of fish could be an effective means to control histamine level in Indian anchovy.

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