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Studies on Histamine Production Properties and Control of

Morganella psychrotolerans

**(*Morganella psychrotolerans* のヒスタミン産生特性と制御に
関する研究)**

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List of abbreviations and acronyms

AA	Acetic acid
ATP	Adenosine triphosphate
CA	Citric acid
CFSE	5-or 6-(N-Succinimidylloxycarbonyl) fluorescein 3',6'-diacetate
DNA	Deoxyribonucleic acid
DiSC ₃ (5)	3,3'-Dipropylthiadicarbocyanine iodide
EC	Epicatechin
ECG	Epicatechin-3-gallate
EDTA	Ethylenediaminetetraacetic acid
EGC	Epigallocatechin
EGCG	Epigallocatechin gallate
EtD-III	Ethidium Homodimer III
FDA	U.S. Food and Drug Administration
HDC	Histidine decarboxylase
HEPES	2-[4-(2-Hydroxyethyl)-1-piperaziny] ethanesulfonic acid
LVA	Levulinic acid
LA	Lactic acid
MA	DL-Malic acid
PAA	Peracetic acid
PBS	Phosphate-buffered saline
PB	Phosphate buffer
PCR	Polymerase chain reaction
PI	Propidium iodide
qRT-PCR	Quantitative real time-PCR
RNA	Ribonucleic Acid
TA	L-Tartaric acid

TSA	Tryptic soy agar
TSA-SC	Tryptic soy agar supplemented with 1.5 % sodium chloride
TSB	Tryptic soy broth
TSBS	Tryptic soy broth supplemented with sodium chloride
TSBH	Tryptic soy broth supplemented with L-histidine
SH	Sodium hypochlorite

Introduction

Histamine fish poisoning is a foodborne toxicity caused by ingesting high levels of histamine (Takahashi *et al.* 2015). The U.S. Food and Drug Administration (FDA) guideline for safe levels of histamine concentration in seafood is 50 mg/kg, while histamine concentration greater than 500 mg/kg is hazardous to humans (FDA 2011). Poisoning symptoms include rash, headache, nausea, diarrhea, and so on (Taylor 1986). Although many cases show mild symptoms, rare cases have shown a potential to threaten human life (D'Aloia *et al.* 2011; Sánchez-Guerrero *et al.* 1997). Histamine accumulation in food is caused by histamine-producing bacteria that produce histidine decarboxylase (HDC). This enzyme decarboxylates free histidine to histamine (Fig. 1) (Ferrario *et al.* 2012). And the enzyme can continue accumulation of histamine in seafood even after bacterial growth has stopped (Lehane and Olley 2000). Once histamine is formed in food, it cannot be destroyed by common food processing treatments such as heating and freezing (Etkind *et al.* 1987).

Histamine-producing bacteria are categorized into low and high histamine-producers. The bacteria producing more than 1000 mg/L of histamine in culture broth are classified as high histamine-producing bacteria (Bjornsdottir-Butler *et al.* 2018). Generally, mesophilic bacteria such as *Morganella morganii*, *Photobacterium damsela*, and *Klebsiella pneumoniae* are well documented as high histamine-producing bacteria in fishery products. Histamine formation of these mesophilic histamine-producers could

be prevented forming histamine by controlling the storage temperature and time during the transports and processing (Bjornsdottir-Butler *et al.* 2017). However, psychrotrophic histamine-producers such as some *Photobacterium* spp. are capable of producing histamine to a harmful level in seafood at refrigeration temperature, and the common cold chain cannot completely suppress histamine production by them. As psychrotrophic histamine-producers, *P. kishitanii* and *P. aquimaris* were recently isolated from fish (Torido *et al.* 2014), and *P. phosphoreum* caused histamine food poisoning incident in Japan (Kanki *et al.* 2004).

M. psychrotolerans is a novel psychrotolerant histamine-producer, that can grow at 0–2 °C. It's biochemical characteristics are very similar to those of *M. morganii* (Emborg *et al.* 2006). Previous studies have documented the poisoning incidents caused by *M. psychrotolerans* (Emborg and Dalgaard 2006; Emborg *et al.* 2005). Previous study suggested that *M. psychrotolerans* is commonly distributed through retail seafood (Table 1) and accumulates histamine in culture broth during low-temperature incubation (Kato *et al.* 2017).

In Chapter 1, the growth and histamine production abilities of *M. psychrotolerans*, *P. phosphoreum*, and *M. morganii* in canned tuna and broth culture under various environmental factors were compared. The activities of crude HDC and *hdc* gene expression levels were characterized under various environmental factors. And the inhibitory effect of the tea catechins against the HDC activity was also evaluated.

Sanitizers can disinfect foodborne pathogens effectively and affordably from seafood

surfaces, contributing to their high prevalence within the food industry (Shen *et al.* 2013; Rahman *et al.* 2011). Sodium hypochlorite (SH) is an effective disinfectant widely used to reduce microbial counts. However, the improper use of chlorine can create carcinogenic by-products and residues, such as chloroform and bromodichloromethane (Gil *et al.* 2009; Reckhow *et al.* 1990). Therefore, it remains necessary to find safer antimicrobial agents for food sanitization. Peracetic acid (PAA) is a known sanitizer used for processing water, fruits, and vegetables (Leggett *et al.* 2016; Ho *et al.* 2011; Hilgren and Salverda 2000; Lambert, *et al.* 1999). 15 ppm PAA caused *Vibrio parahaemolyticus* death in Luria-Bertani-3 % NaCl culture broth (Wong *et al.* 2018). Sheng *et al.* (2020) reported that 80 ppm PAA results in 1.8 log CFU/apple reduction of *Enterococcus faecium* after 2 min treatment. And 240 s of 50 ppm PAA treatment reduced the *Escherichia coli* under detectable level (< 10 CFU/g) on pangasius fillets (Thi *et al.* 2015). And, some studies have found that PAA by-products are safer than those produced during SH treatment (Mora, *et al.* 2018; Dell'Erba *et al.* 2007). However, limited studies are available on the sanitizing effects and mechanisms of PAA and SH on histamine-producing bacteria.

The objective of Chapter 2 was to compare the disinfection effects of PAA and SH on *M. psychrotolerans* *in vitro* and fish surface. And the antimicrobial mechanisms of PAA and SH were determined. This study might provide an insight into potential strategies for preventing *M. psychrotolerans*-related histamine food poisoning and useful information for choosing proper sanitizers for seafood processing.

Organic acids have been used for many years in the food industry. Most organic acids have been designated as Generally Recognized as Safe (GRAS) by FDA. Organic acid treatment showed well sanitizing effect on meat (Mohamed and Abdel-Naeem 2018), fish (Harada *et al.* 2017) and fresh vegetables (Zhao *et al.* 2019; Chen *et al.* 2019; Wang *et al.* 2018). Especially, AA, LA and CA are widely used in the food processing industry, because they are economic friendly and exhibit strong antimicrobial effects. And recently, the studies were focused on developing the novel health and nature potential sanitizer for concerns with consumer and environment. Effective antibacterial activity was observed for reduction of *E. coli* O157:H7, *Listeria monocytogenes* and *Salmonella* Enteritidis on lettuce under combined treatment of LA and hydrogen peroxide (Lin *et al.* 2002). Combination of LA and PAA showed a great disinfection ability for leafy green (Ho *et al.* 2011).

The actual antimicrobial activities and antibacterial mechanism of organic acids against *M. psychrotolerans* are not clear. In Chapter 3, the sanitizing effect of organic acids were evaluated and the mode of antimicrobial action of LA against *M. psychrotolerans* was physiologically determined. And combination of PAA and LA for reduction of *M. psychrotolerans* from fish surface were evaluated.

This study provides fully investigation on histamine production of *M. psychrotolerans* and would contribute to the development of the most suitable and effective strategies to reduce histamine poisoning and enhance seafood safety.

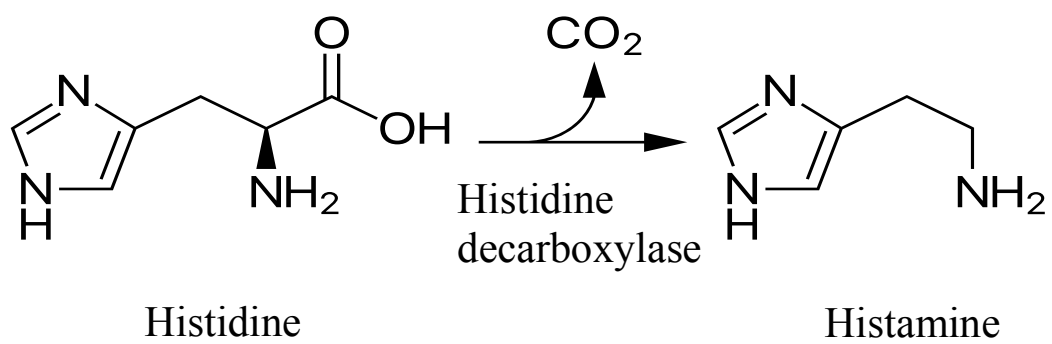


Fig. 1. Decarboxylation of histidine to histamine.

Table 1. Contamination status of *M. psychrotolerans* in various kinds of seafood

Seafood sample	Test sample number	Positive sample number (%)
Lean fish	98	31 (31.6)
White fish	56	35 (62.5)
Squid · Octopus	14	7 (50.0)
Shell	7	3 (42.9)
Prawn	8	2 (25.0)
Total	183	78 (42.6)

(Kato *et al.* 2017)

Chapter 1

Histamine production behaviors of a psychrotolerant histamine-producer,

***Morganella psychrotolerans*, under various environmental conditions**

The growth and histamine producing ability of *M. psychrotolerans* have not been studied well. This Chapter compared the growth and histamine accumulation of *M. psychrotolerans* with those of *P. phosphoreum* and *M. morganii* in canned tuna and culture broth at different pH, NaCl concentration and temperature. And crude HDC was extracted from the bacterial cells, the specificity of the HDC activity was investigated under various conditions. Also, the expression of the *hdc* gene of *M. psychrotolerans* and the inhibitory effect of tea catechins on the HDC activity were evaluated.

Section 1

Monitoring the growth and histamine producing ability of *M. psychrotolerans*

In this Section, effect of environmental conditions on the growth and histamine producing ability of histamine producers in canned tuna and culture broth was investigated.

Materials and methods

1-1-1. Bacterial strains and cultivation

M. psychrotolerans JCM 16473^T and *M. morganii* NBRC 3848^T were inoculated in TSB (BD, Franklin Lakes, NJ, USA) supplemented with 1% L-histidine (Wako Pure Chemical) and incubated at 25 and 30 °C (TSBH, pH 6.0), respectively. *P. phosphoreum* NBRC 103031^T was cultured in TSBH supplemented with 1.5 % NaCl (total NaCl concentration in medium: 2%) at 20 °C. The cultures were incubated for 24 h for the experiments described below.

1-1-2. Inoculation with bacteria to tuna samples

Canned tuna (Hagoromo Foods) was purchased from a local supermarket. A 100 g of tuna was aseptically put into a stomacher bag (Seward). The precultured bacteria were centrifuged (10,000×g, 2 min, 4 °C) and washed twice with PBS (pH 7.2). After

serial dilution with PBS, each bacterial suspension was inoculated to tuna samples (populations about 10^3 CFU/g), and the samples were stored at 4 or 20 °C for various durations. A 5 g of the sample was aseptically weighted in a stomacher bag, homogenized with 45 mL PBS at 200 rpm for 1 min, and used to determine total plate counts and histamine accumulation. The homogenized solutions were serially diluted with PBS and spread onto plates. *M. psychrotolerans* or *M. morganii* colonies were counted after incubating for 48 h at 25 or 30 °C on TSA (BD), respectively. *P. phosphoreum* colonies was counted after incubating for 48 h at 20 °C on TSA-SC agar. Histamine contents were determined with the histamine test kit (Kikkoman Biochemifa), according to the manufacturer's instructions.

1-1-3. Bacterial growth and histamine production under different environmental conditions in culture broth

Pre-cultured *M. psychrotolerans*, *M. morganii* and *P. phosphoreum* were washed and diluted with PBS. Then, 100 µL of the bacteria suspension was mixed with 9.9 mL TSBH, was adjusted to pH 5–8 using 1M NaOH or HCl. For different NaCl concentration, the 100 µL of the bacteria suspension was mixed with 9.9 mL TSBS (Table 1-1) supplemented with 1% L-histidine (NaCl concentration: 0–4 %, pH 6.0). The working culture (approximately about 3.2×10^5 CFU/mL of an initial inoculum size) was incubated at 4 or 20 °C. At each sampling time, the bacterial populations were

counted as describe above and the histamine concentration in the culture medium was determined.

Table 1-1. Composition of TSBS

Ingredients	
Tryptone (BD)	17 g
Phytone peptone (BD)	3 g
Glucose	2.5 g
NaCl (0, 2, 4 %)	0, 20, 40 g
Distilled water	1000 mL

Medium was autoclaved at 121 °C for 15 min

Results

1-1-1. Histamine accumulation in canned tuna

To evaluate the histamine production capabilities of *M. psychrotolerans* in canned tuna, I compared them with major mesophilic and psychrophilic histamine-producers, *M. morganii* and *P. phosphoreum*. Changes in viable cell counts and histamine concentration at 20 and 4 °C are shown in Fig. 1-1 and 1-2.

At 20 °C, the growth curves of three bacterial species showed similar trends. The viable cell count increased to more than 10^6 CFU/g after 12 h-incubation from the initial counts (10^3 - 10^4 CFU/g), and exceeded 10^8 CFU/g after 36 h-incubation (Fig. 1-1A). Histamine concentrations in the samples inoculated with *M. psychrotolerans* and *M. morganii* exceeded 1000 mg/kg after 36 h-incubation, and were 3000 mg/kg after 60 h-incubation. However, histamine concentration in samples with *P. phosphoreum* was less than 400 mg/kg after 60 h-incubation (Fig. 1-1B). When the tuna samples inoculated with the three bacteria were stored at 4 °C, the viable cell counts of *M. psychrotolerans* and *P. phosphoreum* were detected to be more than 10^6 CFU/g after 4 d-incubation, and reached to about 10^8 CFU/g after 8 d-incubation. However, *M. morganii* did not grow at 4 °C (Fig. 1-2A). Histamine production by *M. psychrotolerans* and *P. phosphoreum* were 1467, 2611 and 474, 793 mg/kg in canned tuna after 8 d-incubation and 10 d-incubation at 4 °C, respectively (Fig. 1-2B).

1-1-2. Effect of pH, NaCl concentration and temperatures on growth and histamine accumulation in culture broth

Growth and histamine formation at different initial pH and temperature are shown in Fig. 1-3 and 1-4. At 20 °C, the cell populations rapidly increased between 0–12 h and reached the maximum after 24 h-incubation, corresponding to the cell concentration between 9.6×10^7 and 2.2×10^9 CFU/mL. The maximum cell density of *M. psychrotolerans* and *M. morganii* were higher than that of *P. phosphoreum* in pH values. High levels of histamine (> 3100 mg/L) were accumulated by *M. morganii* under all pH conditions after 24 h-incubation. Moreover, the histamine was significantly produced by *M. psychrotolerans* for 36 h-incubation (> 2693 mg/L). The maximum histamine accumulation by *P. phosphoreum* was 2608 mg/L for 60 h-incubation at pH 6.

At 4 °C, the growths of *M. psychrotolerans* and *P. phosphoreum* were similar trends during the incubation periods, the cell populations showed a rapid increase at pH 6–8, and the viable cell counts reached around 10^9 CFU/mL for 8 d-incubation. However, significantly greater amount of histamine were produced by *M. psychrotolerans* than *P. phosphoreum* at 4 °C.

The histamine producers preferred acid conditions to the alkaline condition for histamine production. And histamine produced by *M. psychrotolerans* and *M. morganii* were greater than that by *P. phosphoreum* under all pH conditions.

The maximum of cell population was around 10^9 CFU/mL at 20 °C after 48 h-incubation and at 4 °C after 10 d-incubation (Figs. 1-5 and 1-6). And no difference in growth dynamics among *M. psychrotolerans*, *M. morganii* and *P. phosphoreum* in 2 % NaCl–TSBH at 20 °C (Fig. 1-5A, B, C). Histamine accumulated by *M. psychrotolerans* and *M. morganii* were greater than 5300 mg/L after 60 h-incubation at 20 °C. The maximum histamine accumulation by *P. phosphoreum* were 4659 mg/L after 60 h-incubation at 20 °C (Fig. 1-5a, b, c).

The growth rate of *P. phosphoreum* was greater than *M. psychrotolerans* at 4 °C, except *P. phosphoreum* did not grow without NaCl (Fig. 1-6A, B, C). Histamine produced by *M. psychrotolerans* were higher than that by *P. phosphoreum* at 4 °C under all salt concentration. Histamine accumulated by *M. psychrotolerans* at 4 °C in culture broth of any salt concentration exceeded 3890 mg/L (Fig. 1-6a, b, c).

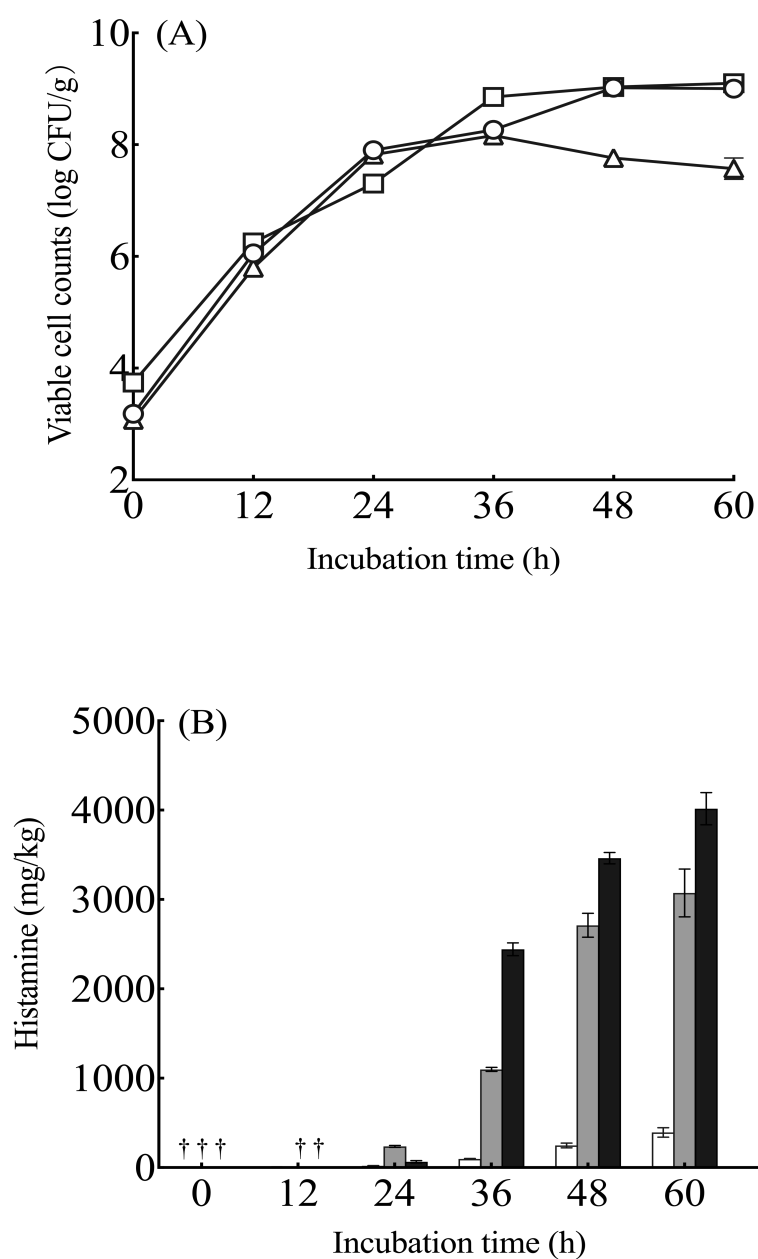


Fig. 1-1. The growth curve (A) and histamine accumulation (B) in canned tuna at 20 °C.

Symbols represent *M. psychrotolerans* (○), *P. phosphoreum* (Δ), and *M. morganii* (□), for viable counts. Columns represent *M. psychrotolerans* (gray), *P. phosphoreum* (white), and *M. morganii* (black) for histamine concentration. Data are shown as the mean $\pm$ standard deviation from three independent experiments. Dagger (†) means less than 4 mg/kg of histamine concentration.

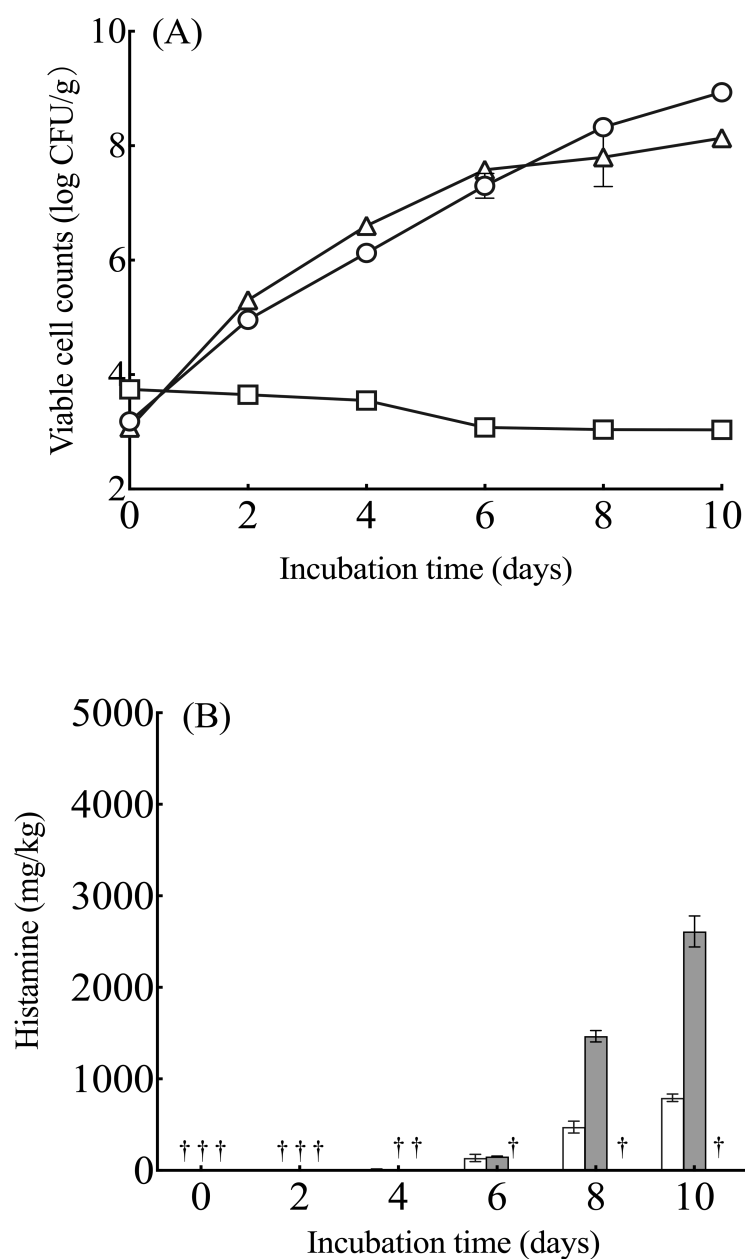


Fig. 1-2. The growth curve (A) and histamine accumulation (B) in canned tuna at 4 °C. Symbols represent *M. psychrotolerans* (○), *P. phosphoreum* (△), and *M. morganii* (□) for viable counts. Columns represent *M. psychrotolerans* (gray), *P. phosphoreum* (white), and *M. morganii* (black) for histamine concentration. Data are shown as the mean $\pm$ standard deviation from three independent experiments. Dagger (†) means less than 4 mg/kg of histamine concentration.

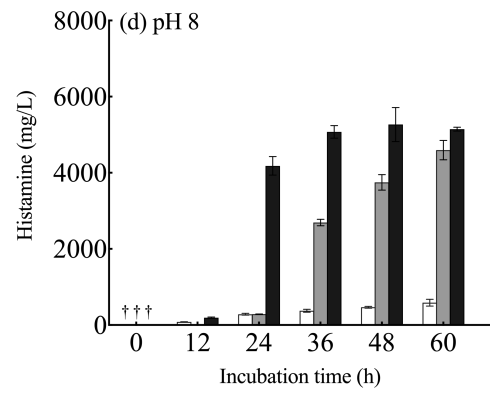
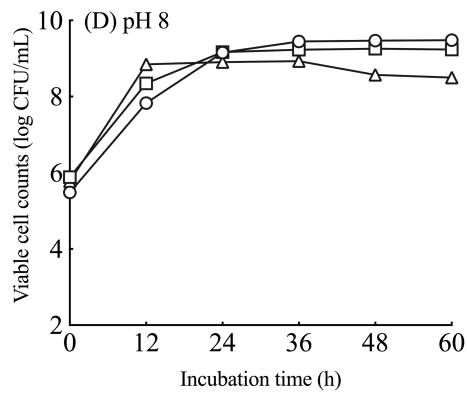
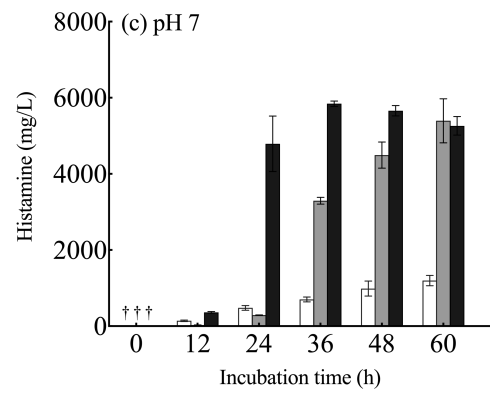
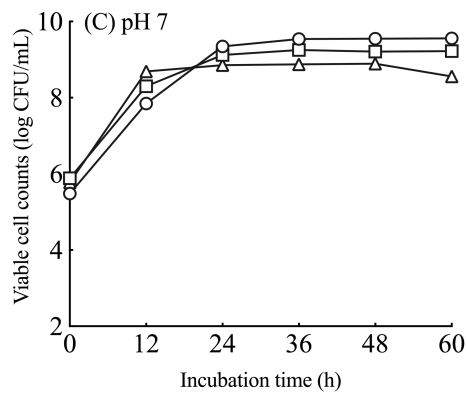
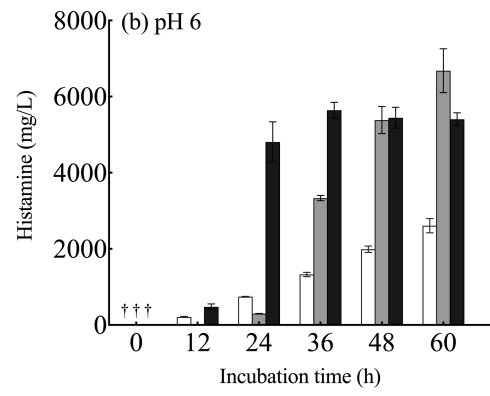
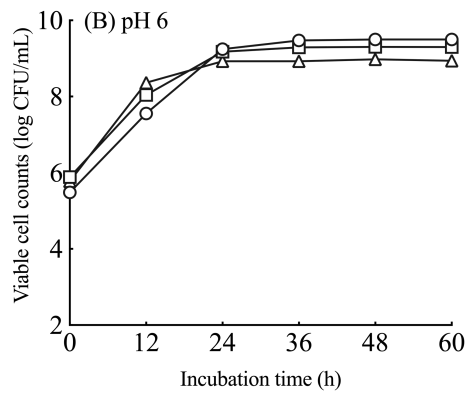
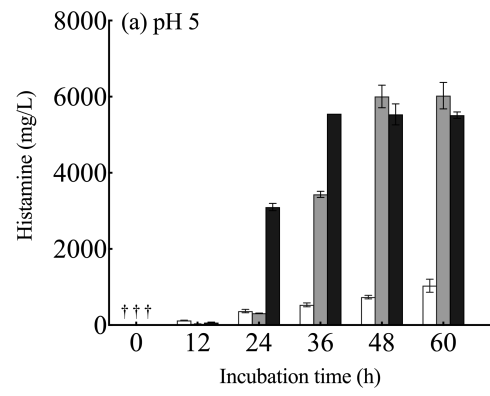
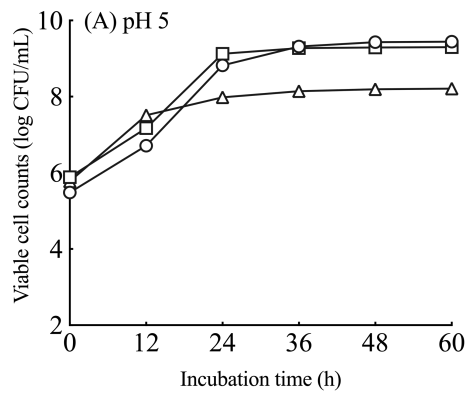


Fig. 1-3. The growth curve (A, B, C, D) and histamine accumulation (a, b, c, d) in TSBH under pH 5 - 8 at 20 °C.

Symbols represent *M. psychrotolerans* (○), *P. phosphoreum* (△) and *M. morganii* (□) for viable counts. Columns represent *M. psychrotolerans* (gray), *P. phosphoreum* (white), and *M. morganii* (black) for histamine concentration. Data are shown as the mean $\pm$ standard deviation from three independent experiments. Dagger (†) means less than 4 mg/L of histamine concentration.

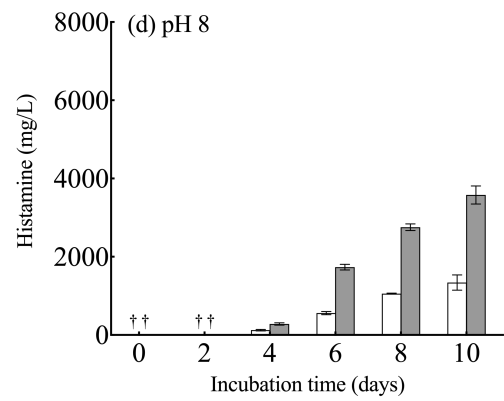
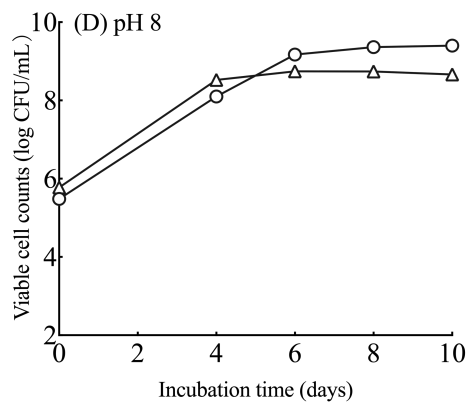
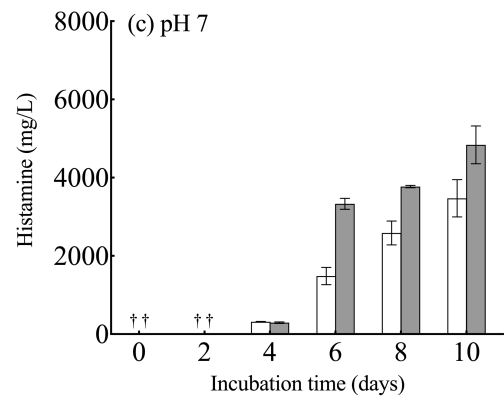
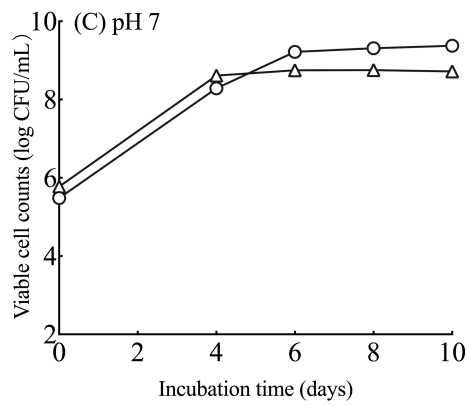
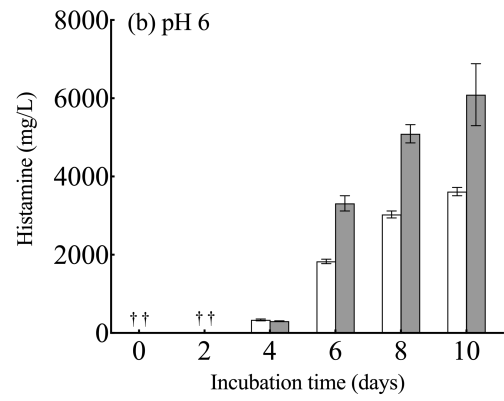
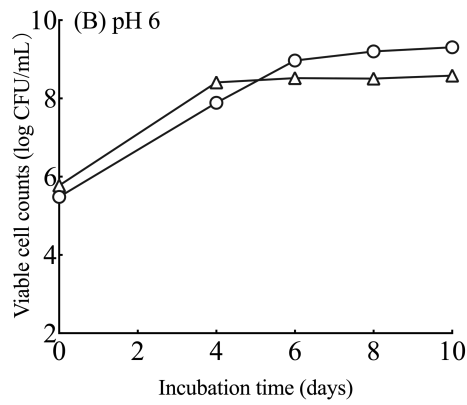
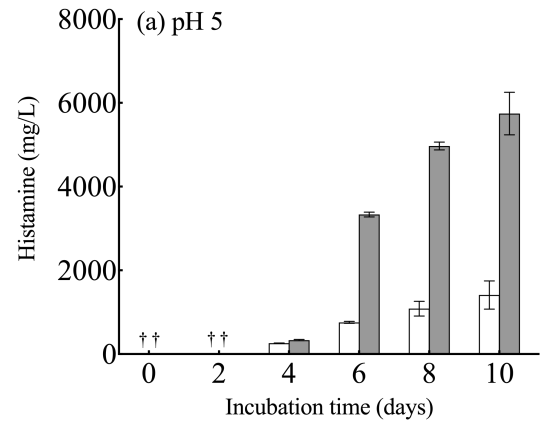
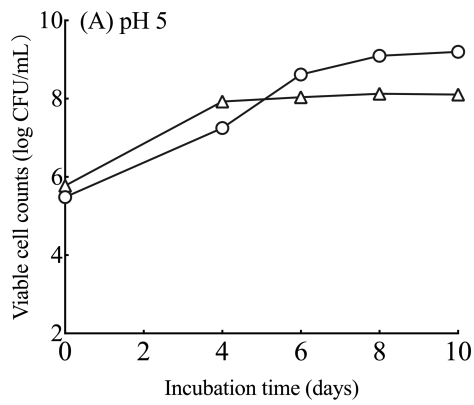


Fig. 1-4. The growth curve (A, B, C, D) and histamine accumulation (a, b, c, d) in TSBH under pH 5 – 8 at 4 °C.

Symbols represent *M. psychrotolerans* (○) and *P. phosphoreum* (△) for viable counts. Columns represent *M. psychrotolerans* (gray) and *P. phosphoreum* (white) for histamine concentration. Data are shown as the mean $\pm$ standard deviation from three independent experiments. Dagger (†) means less than 4 mg/L of histamine concentration.

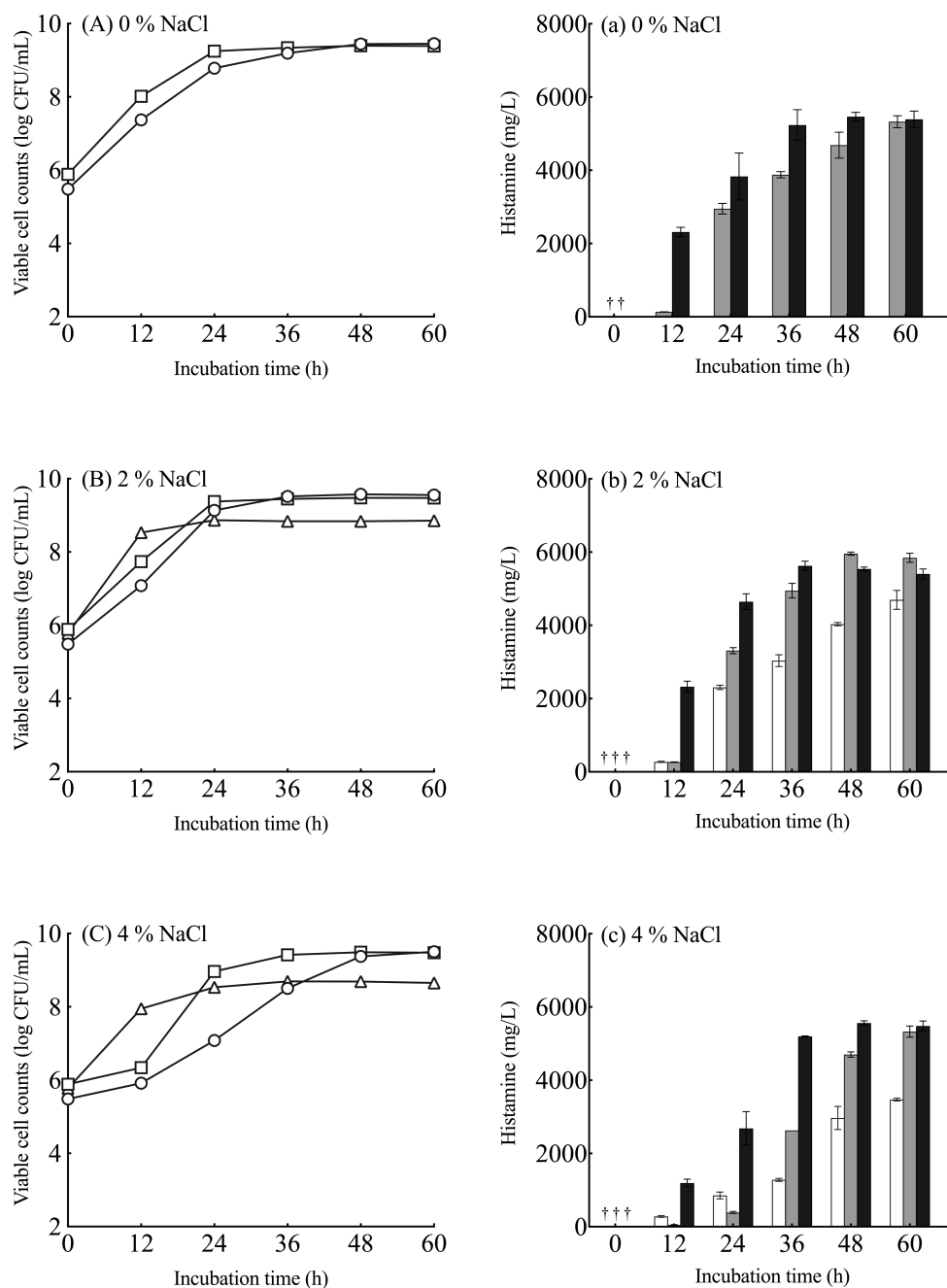


Fig. 1-5. The growth curve (A, B, C) and histamine accumulation (a, b, c) in TSBSH under NaCl (0 %, 2 %, 4 %) at 20 °C.

Symbols represent *M. psychrotolerans* (○), *P. phosphoreum* (△) and *M. morganii* (□), for viable counts. Columns represent *M. psychrotolerans* (gray), *P. phosphoreum* (white), and *M. morganii* (black) for histamine concentration. Data are shown as the mean $\pm$ standard deviation from three independent experiments. Dagger (†) means less than 4 mg/L of histamine concentration.

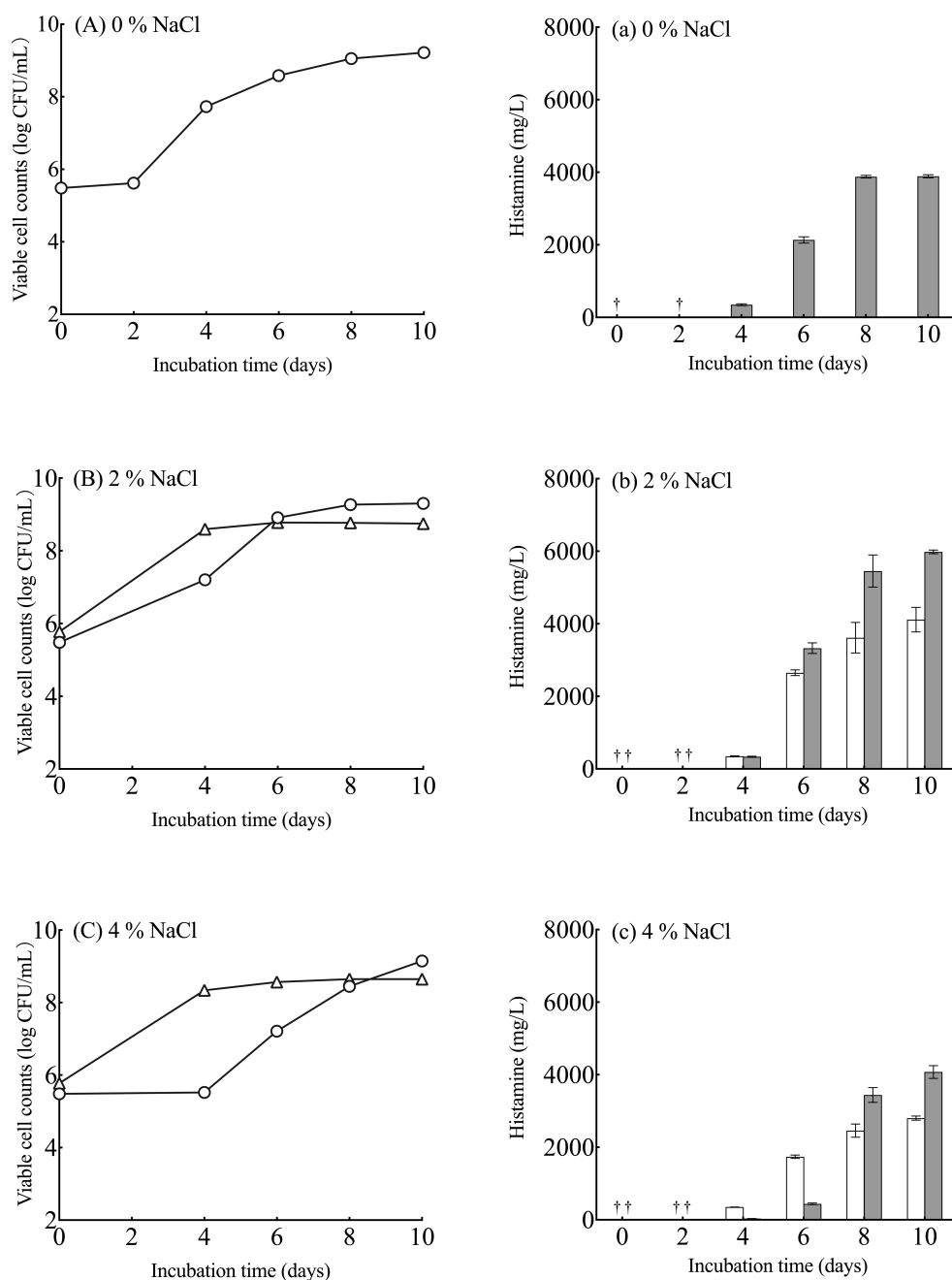


Fig. 1-6. The growth curve (A, B, C) and histamine accumulation (a, b, c) in TSBSH under NaCl (0 %, 2 %, 4 %) at 4 °C.

Symbols represent *M. psychrotolerans* (○) and *P. phosphoreum* (△), for viable counts. Columns represent *M. psychrotolerans* (gray) and *P. phosphoreum* (white) for histamine concentration. Data are shown as the mean $\pm$ standard deviation from three independent experiments. Dagger (†) means less than 4 mg/L of histamine concentration.

Section 2

Characterization of histidine decarboxylase (HDC) and *hdc* gene expression in

M. psychrotolerans

M. psychrotolerans showed high histamine producing ability under various environmental conditions in Section 1. In this Section, the specificity of crude HDC extracted from the bacterial cells and *hdc* gene expression were investigated under various environmental conditions. In addition, inhibitory effect of tea catechins on HDC activity was evaluated.

Materials and methods

1-2-1. Preparation of crude HDC and measurement of activity

The 5 mL of precultured bacterial cells were harvested by centrifugation (10,000×g, 5 min, 4 °C), and washed twice with McIlvaine buffer (0.01M citrate-0.02 M phosphate buffer, pH6.2). The bacterial cells were resuspended in 1 mL of the same buffer (approximately 3.2×10^9 CFU/mL in working buffer), and disrupted using zirconia beads (zircon prep mini, Nippon Genetics) and a Bead Beater (2,500 rpm, 15 min, CD-1000, EYELA). A cell-free supernatant was collected as a crude HDC solution by centrifugation (10,000×g, 15 min, 4 °C). The crude HDC solution (0.2 mL) was mixed with the reaction buffer (1.8 mL, 0.1 M citrate-0.2 M phosphate buffer) supplemented

with 1% L-histidine. After 1 h incubation, the reaction was terminated by heating at 100 °C for 15 min. The activity of HDC extracted from *M. psychrotolerans* was determined by checking the concentration of histamine in the solution at conditions ranging from pH 4–8 (30 °C), 10–50 °C (pH 6.2) and 0–5 % NaCl (pH 6.2). The crude HDC solution was directly used for the analysis.

1-2-2. Evaluation of inhibitory effect of tea catechins on HDC activity

0.4 mL of crude HDC solution were mixed with 0.4 mL reaction buffer (0.1 M citrate - 0.2 M phosphate buffer supplemented with 2 % L-Histidine monohydrochloride monohydrate, pH 6.0) and 0.2 mL tea catechins (final concentration: 1 mM; EGCG, purity 96.2 %; ECG, purity 92.1 %, Taiyo Kagaku; EGC and EC, purity ≥ 99.9 %, Nagara Science). EGCG and ECG were dissolved to 20 mM and 10 mM as the base solutions. Distilled sterile water was used as control and the activity of control groups was defined as 100 %. After incubation for 4 h at 30 °C, the reactions were stopped by heating at 100 °C for 15 min. HDC activity was determined by checking histamine concentration in the reaction solutions with the histamine test kit and the spike-and-recovery tests were also done for tea catechin.

1-2-3. Evaluation of *hdc* gene expression

Expression levels of *hdc* gene at different pH (5–8), NaCl concentration (0–4 %) and temperature levels (4 and 20 °C) were evaluated with quantitative RT-PCR (qRT-PCR). Firstly, the HDC amino acid sequences of Gram-negative histamine-producing bacteria were compared on database by a BLAST search (Fig. 1-7) (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) and then the *hdc* gene cluster of *M. psychrotolerans* was determined (Fig. 1-8) (<https://www.ncbi.nlm.nih.gov/orffinder/>). Depend on the HDC amino acid sequence and the *hdc* gene cluster, the coding *hdc* gene and reference gene (16S rDNA) primers were designed and showed in Table 1-2 (Ferrario *et al.* 2012; Bjornsdottir-Butler *et al.* 2016).

RNA was extracted using NucleoSpin RNA extraction kit (Macherey-Nagel) from *M. psychrotolerans* cells cultured at 4 °C for 6 d or 20 °C for 24 h. The concentration and purity (A_{260}/A_{280}) ratio of all RNA samples were checked with a spectrophotometer (GeneQuant pro). The RNA was stored at - 70 °C and subsequently used for cDNA synthesis. The cDNA was synthesized using RevertAid First strand cDNA Synthesis Kit (Fermentas, Thermo Fisher Scientific). The contamination of DNA residues was determined in RNA sample by a control reaction that had a cDNA synthesis without reverse transcriptase enzyme.

A 20 µL of reaction mixture for qRT-PCR contained 10 µL SsoAdvanced Universal SYBR Green Supermix (Bio-Rad), 1 µL cDNA, 3 µL primer mix (final concentration: 300 nM of each primer), and 6 µL water. The PCR conditions were an initial cycle at

95 °C for 45 s, which was followed by 40 cycles at 95 °C for 15 s, and 61 °C for 1 min in an ABI 7500 Real-Time PCR system (Applied Biosystems). For each target gene, the real-time PCR efficiency was calculated according to the equation $E = 10^{(-1/\text{slope})}$. The gene expression level was calculated by the method of Pfaffl (2001).

M.morganii_ACTT9237	MTLSINDQNKLD	AFWAYCVKNQYFNIGYPESADFDYT	NLERFLRFSINNCGDWGEYCNYL	60			
M.morganii_JCM1672	MTLSINDQNKLD	AFWAYCVKNQYFNIGYPESADFDYT	NLERFLRFSINNCGDWGEYCNYL	60			
M.morganii_O24	MTLSINDQNKLD	AFWAYCVKNQYFNIGYPESADFDYT	NLERFLRFSINNCGDWGEYCNYL	60			
M.psychrotolerans_GCSL-Mp20	MTLSINDQNKLD	AFWAYCVKNQYFNIGYPESADFDYT	NLERFLRFSINNCGDWGEYCNYL	60			
M.psychrotolerans_GCSL-Mp3	MTLSINDQNKLD	AFWAYCVKNQYFNIGYPESADFDYT	NLERFLRFSINNCGDWGEYCNYL	60			
M.psychrotolerans_GCSL-P101	MTLSINDQNKLD	AFWAYCVKNQYFNIGYPESADFN	YTNLERFLRFSINNCGDWGEYCNYL	60			
P.phosphoreum_ANT-2200	MTLSIENQNKLDE	FWAYCVKNQYFNIGYPESADFDYT	ILERFMRF	60			
P.phosphoreum_NBRC13896	MTLSIENQNKLDE	FWAYCVKNQYFNIGYPESADFDYT	ILERFMRF	60			
P.phosphoreum_YS4-7	MTLSIENQNKLDE	FWAYCVKNQYFNIGYPESADFDYT	ILERFMRF	60			
M.morganii_ACTT9237	LNSFDFEKEVMEYFADLFKIPFEK	SWGYVTNGGTEGNMFGCYLGREI	FPDGTLYYSKDT	120			
M.morganii_JCM1672	LNSFDFEKEVMEYFADLFKIPFEK	SWGYVTNGGTEGNMFGCYLGREI	FPDGTLYYSKDT	120			
M.morganii_O24	LNSFDFEKEVMEYFADLFKIPFEK	SWGYVTNGGTEGNMFGCYLGREI	FPDGTLYYSKDT	120			
M.psychrotolerans_GCSL-Mp20	LNSFDFEKEVMEYFADLFKIPFEK	SWGYVTNGGTEGNMFGCYLGREI	FPDGTLYYSKDT	120			
M.psychrotolerans_GCSL-Mp3	LNSFDFEKEVMEYFADLFKIPFEK	SWGYVTNGGTEGNMFGCYLGREI	FPDGTLYYSKDT	120			
M.psychrotolerans_GCSL-P101	LNSFDFEKEVMEYFADLFKIPFEK	SWGYVTNGGTEGNMFGCYLGREI	FPDGTLYYSKDT	120			
P.phosphoreum_ANT-2200	LNSFDFEKEVMEYFADLFKIPFEK	SWGYVTNGGTEGNMFGCYLGREI	FPDGTLYYSKDT	120			
P.phosphoreum_NBRC13896	LNSFDFEKEVMEYFADLFKIPFEK	SWGYVTNGGTEGNMFGCYLGREI	FPDGTLYYSKDT	120			
P.phosphoreum_YS4-7	LNSFDFEKEVMEYFADLFKIPFEK	SWGYVTNGGTEGNMFGCYLGREI	FPDGTLYYSKDT	120			
M.morganii_ACTT9237	YSVAKIVKLLRIKSQV	VESQPNGEIDYDDL	LMKKIAD	DKEAHP	IIIFANIGTTVRGAIDDI	A	180
M.morganii_JCM1672	YSVAKIVKLLRIKSQV	VESQPNGEIDYDDL	LMKKIAD	DKEAHP	IIIFANIGTTVRGAIDDI	A	180
M.morganii_O24	YSVAKIVKLLRIKSQV	VESQPNGEIDYDDL	LMKKIAD	DKEAHP	IIIFANIGTTVRGAIDDI	A	180
M.psychrotolerans_GCSL-Mp20	YSVAKIVKLLRIKSQV	VEAQPNGEIDYDDL	LMKKIAD	DKEAHP	IIIFANIGTTVRGAIDDI	T	180
M.psychrotolerans_GCSL-Mp3	YSVAKIVKLLRIKSQV	VEAQPNGEIDYDDL	LMKKIAD	DKEAHP	IIIFANIGTTVRGAIDDI	T	180
M.psychrotolerans_GCSL-P101	YSVAKIVKLLRIKSQV	VEAQPNGEIDYDDL	LMKKIAD	DKEAHP	IIIFANIGTTVRGAIDDI	A	180
P.phosphoreum_ANT-2200	YSVAKIVKLLRIKSQV	LDLSLPNGEIDYDDL	ISKIKQD	DEKHP	IIIFANIGTTVRGAIDDI	S	180
P.phosphoreum_NBRC13896	YSVAKIVKLLRIKSQV	LDLSLPNGEIDYDDL	ISKIKQD	DEKHP	IIIFANIGTTVRGAIDDI	S	180
P.phosphoreum_YS4-7	YSVAKIVKLLRIKSQV	LDLSLPNGEIDYDDL	ISKIKQD	DEKHP	IIIFANIGTTVRGAIDDI	H	180
M.morganii_ACTT9237	EIQKRLKAAGIKREDYY	LHADAALSGMILPFVD	DAQPFTFADGIDSIGVSGHKMIGSPI	P	240		
M.morganii_JCM1672	EIQKRLKAAGIKREDYY	LHADAALSGMILPFVD	DAQPFTFADGIDSIGVSGHKMIGSPI	P	240		
M.morganii_O24	EIQKRLKAAGIKREDYY	LHADAALSGMILPFVD	DAQPFTFADGIDSIGVSGHKMIGSPI	P	240		
M.psychrotolerans_GCSL-Mp20	EIQKRLKAAGIKREDYY	LHADAALSGMILPFVD	EPQAF	T	FADGIDSIGVSGHKMIGSPI	P	240
M.psychrotolerans_GCSL-Mp3	EIQKRLKAAGIKREDYY	LHADAALSGMILPFVD	EPQAF	T	FADGIDSIGVSGHKMIGSPI	P	240
M.psychrotolerans_GCSL-P101	EIQKRLKAAGIKREDYY	LHADAALSGMILPFVD	EPQAF	T	FADGIDSIGVSGHKMIGSPI	P	240
P.phosphoreum_ANT-2200	KIQAMIGELGIKREDYY	IHADAALSGMILPFVD	EPQG	FN	FADGIDSIGVSGHKMIGSPI	P	240
P.phosphoreum_NBRC13896	KIQAMIGELGIKREDYY	IHADAALSGMILPFVD	EPQG	FN	FADGIDSIGVSGHKMIGSPI	P	240
P.phosphoreum_YS4-7	KIQAMITELGIKREDYY	IHADAALSGMILPFVD	EPQG	FN	FADGIDSIGVSGHKMIGSPI	P	240
M.morganii_ACTT9237	CGIVVAKKENVDRI	ISVEIDYISAHDKTITGSRNGHTPLMLWEA	IRSHSAE	EWKRRIT	RS	300	
M.morganii_JCM1672	CGIVVAKKENVDRI	ISVEIDYISAHDKTITGSRNGHTPLMLWEA	IRSHST	EWKRRIT	RS	300	
M.morganii_O24	CGIVVAKKENVDRI	ISVEIDYISAHDKTITGSRNGHTPLMLWEA	IRSHST	EWKRRIT	RS	300	
M.psychrotolerans_GCSL-Mp20	CGIVVAKKENVDRI	ISVEIDYISAHDKTITGSRNGHTPLMLWEA	AVRAHS	FEDWKRRID	RS	300	
M.psychrotolerans_GCSL-Mp3	CGIVVAKKENVDRI	ISVEIDYISAHDKTITGSRNGHTPLMLWEA	AVRAHS	FEDWKRRIG	RS	300	
M.psychrotolerans_GCSL-P101	CGIVVAKKENVDRI	ISVEIDYISAHDKTITGSRNGHTPLMLWEA	AVRAHS	FEDWKRRIG	RS	300	
P.phosphoreum_ANT-2200	CGIVVAKKRNVD	AI	ISVEIDYISAHDKTITGSRNGHTPLMMW	CAVKSHSHADF	KRRIN	RS	300
P.phosphoreum_NBRC13896	CGIVVAKKRNVD	AI	ISVEIDYISAHDKTITGSRNGHTPLMMW	CAVKSHSHADF	KRRIN	RS	300
P.phosphoreum_YS4-7	CGIVVAKKRNVD	AI	ISVEIDYISAHDKTITGSRNGHTPLMMW	CAVKSHSHADF	KRRIN	RS	300
M.morganii_ACTT9237	DMAQYAVDRMQ	KAGINAWRNKNSITVVFP	CPSE	RVWREHCLATSG	DV	AHLITTAHHLDTA	360
M.morganii_JCM1672	DMAQYAVDRMQ	KAGINAWRNKNSITVVFP	CPSE	RVWREHCLATSG	DV	AHLITTAHHLDTA	360
M.morganii_O24	DMAQYAVDRMQ	KAGINAWRNKNSITVVFP	CPSE	RVWREHCLATSG	DV	AHLITTAHHLDTA	360
M.psychrotolerans_GCSL-Mp20	DMAQYAVDRMQ	KAGINAWRNKNSITVVFP	CPSE	RVWREHCLATSG	ND	AHLITTAHHLDTA	360
M.psychrotolerans_GCSL-Mp3	DMAQYAVDRMQ	KAGINAWRNKNSITVVFP	CPSE	RVWREHCLATSG	ND	AHLITTAHHLDTA	360
M.psychrotolerans_GCSL-P101	DMAQYAVDRMQ	KAGINAWRNKNSITVVFP	CPSE	RVWREHCLATSG	ND	AHLITTAHHLDTA	360
P.phosphoreum_ANT-2200	DLAQHAVQRLQ	TAGINAWCNKNSITVVFP	CPSE	AVWKKHCLATSG	GQ	AHLITTAHHLDTA	360
P.phosphoreum_NBRC13896	DLAQHAVQRLQ	TAGINAWCNKNSITVVFP	CPSE	AVWKKHCLATSG	GQ	AHLITTAHHLDTA	360
P.phosphoreum_YS4-7	DLAQHAVQRLQ	SAGINAWCNKNSITVVFP	CPSE	AVWKKHCLATSG	GQ	AHLITTAHHLDTA	360
M.morganii_ACTT9237	QIDKLIDDVI	ADFN	LHAA	-	-	378	
M.morganii_JCM1672	QIDKLIDDVI	ADFN	LHAA	-	-	378	
M.morganii_O24	QIDKLIDDVI	ADFN	LHAA	-	-	378	
M.psychrotolerans_GCSL-Mp20	QIDALIDDVI	ADHN	LHAA	-	-	378	
M.psychrotolerans_GCSL-Mp3	QIDALIDDVI	ADAK	LHAA	-	-	378	
M.psychrotolerans_GCSL-P101	QIDALIDDVI	ADAK	LHAA	-	-	378	
P.phosphoreum_ANT-2200	KVDALIDDVI	KDANGEMIA	IA	-	-	380	
P.phosphoreum_NBRC13896	KVDALIDDVI	KDANGEMIA	IA	-	-	380	
P.phosphoreum_YS4-7	KVDALIDDVI	KDANGETIA	IA	-	-	380	

Fig. 1-7. Alignment of histidine decarboxylase amino acid sequences of Gram-negative histamine producers.

Residues conserved in all sequences are shaded in dark blue, and residues conserved in more than 50% of sequences are shaded in light blue.

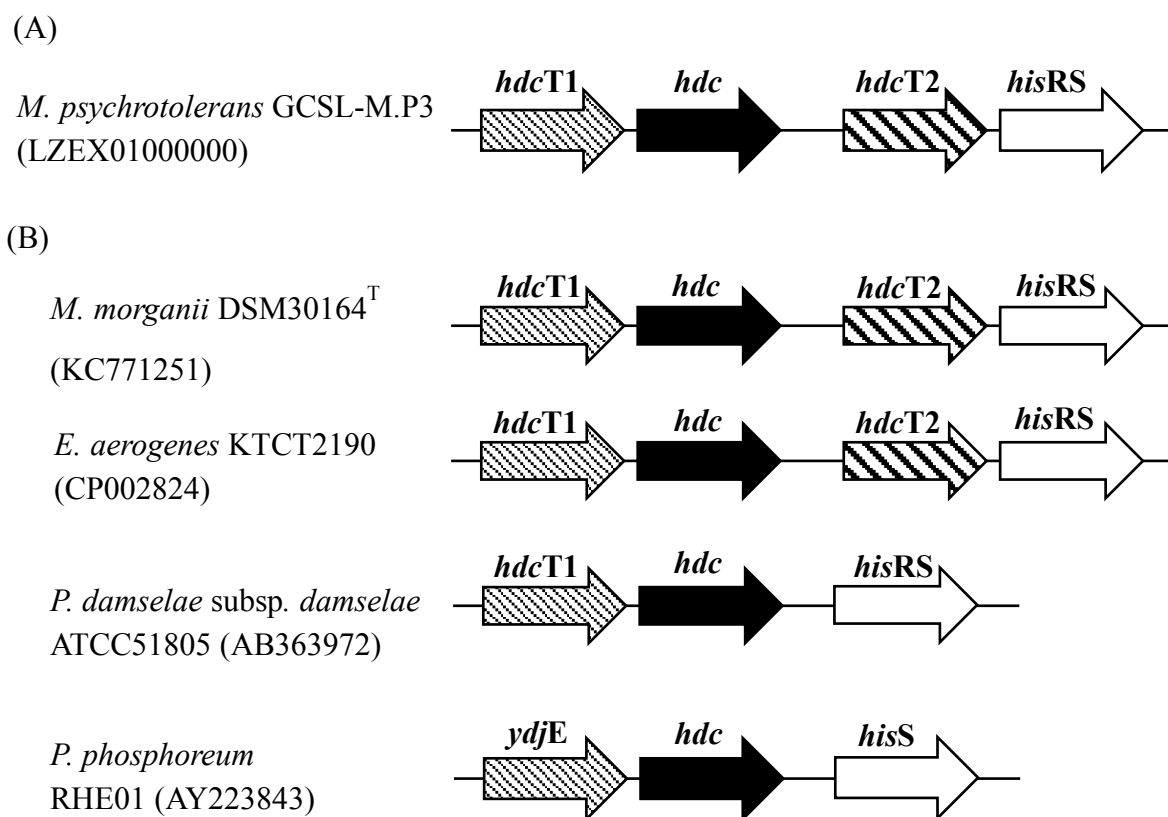


Fig. 1-8. Representation of histidine decarboxylase gene cluster of Gram-negative histamine producing bacteria.

Arrows represent coding genes: black, histidine decarboxylase (*hdc*); hatched, putative antiporters or amino acid permeases (*hdcT1*, *hdcT2*); white, histidyl - tRNA synthetase. (A) designed in this study; (B) from Ferrario *et al.* (2013).

Table 1-2. Primer sequences used for qRT-PCR experiments

Gene	Primer	Sequences (5'-3')
hdc	Hdc-MP-f	ATTCAATTGGTGTTTCCGGC
	Hdc-MP-r	TATGACCATTACGGGAACCG
16s rDNA	Mor-Ref-f	TTTCAGTCGGGAGGAAGGTG
	Mor-Ref-r	GGGGATTTCACATCTGACTYA

Results

1-2-1. HDC activity of *M. psychrotolerans* cell-free extracts under various conditions

The crude HDC was prepared as cell-free extract from *M. psychrotolerans* and its specificity was investigated under various conditions. Effects of temperature, pH, and NaCl concentration on the crude HDC are shown in Figs. 1-9 to 1-11. For effect of temperature, the crude HDC exhibited the most active at 30 °C, and it showed about two-fold higher activity than those at 10, 20, and 40 °C. However, the activity at 50 °C was 30% of the activity at 30 °C (Fig. 1-9). The HDC was greatly affected by the pH range from 4–8. The maximum activity was shown at pH 7 and the activities at pH 4, 5, 6, and 8 were 16%, 49%, 76% and 48%, respectively, compared with that at pH 7 (Fig. 1-10). And the HDC activity gradually decreased with increasing NaCl concentration and was highest in the absence of NaCl (Fig. 1-11).

1-2-2. Effect of tea catechins on HDC activity

Figure 1-12 shows the effect of tea catechins on the HDC activity of crude extract from *M. psychrotolerans*. EGCG and ECG inhibited HDC more effectively than EGC and EC at 1 mM. Notably, EGCG decreased the HDC activity to 9 % at 1 mM. The inhibitory effect was evaluated at different concentration of EGCG and ECG in Fig. 1-13. The HDC activity decreased with the increase in concentration of EGCG and ECG.

At the concentration range between 10^{-3} and 10^{-1} mM, the activity was gradually decreased, and the inhibitory effect of EGCG and ECG were similar. Then the activity was sharply decreased in the presence of 1 mM EGCG and ECG, and the inhibitory efficiency of EGCG was higher than ECG. The HDC activity decreased to almost zero when ECGG concentration increased to 2 mM, while the HDC still remained 29 % activity when ECG increased to 2 mM.

1-2-3. Effect of temperature, pH, and NaCl concentration on *hdc* gene expression

Levels of *hdc* gene expression at different temperature, pH, and NaCl concentration are shown in Figs. 1-14 and 1-15. The *hdc* gene expressions were also affected by the environmental pH. Expression levels decreased with a rise in pH, and the expression levels at pH 5 were 9.1-fold and 14.7-fold higher at 20 and 4 °C, respectively, than the levels at pH 8 (Fig. 1-14). The greatest expression of *hdc* was obtained when *M. psychrotolerans* cells were exposed to 4 % NaCl. The relative expression increased 6.2-fold and 4.5-fold at 20 and 4 °C, respectively. And at lower NaCl concentration, low levels of expression were detected (Fig. 1-15).

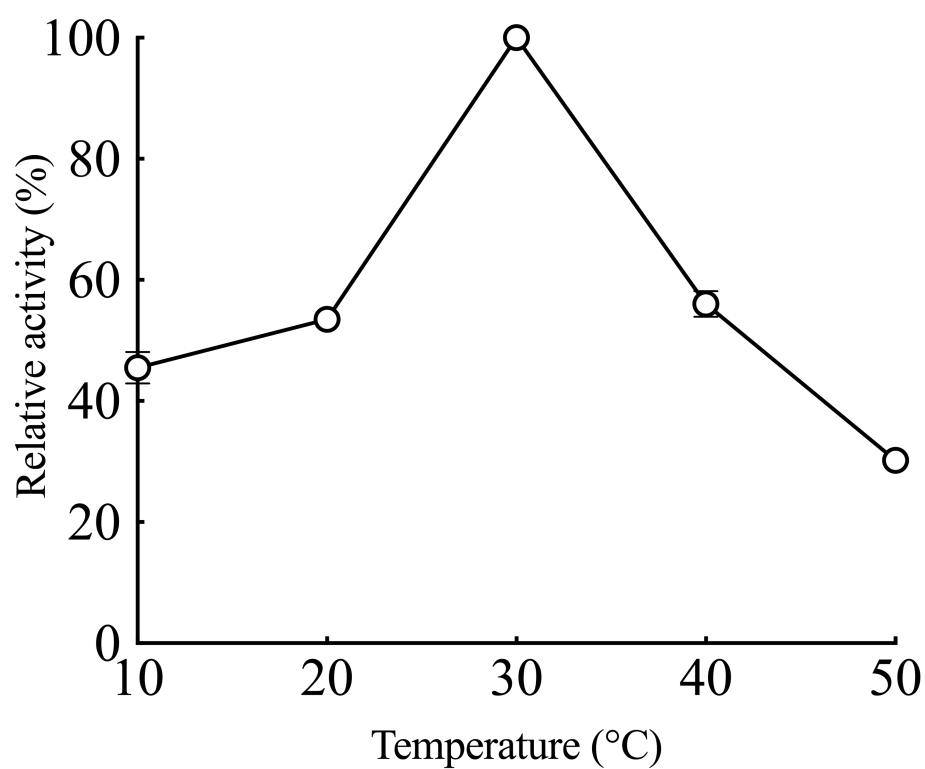


Fig. 1-9. Effect of temperature on histamine accumulation by the crude HDC of *M. psychrotolerans*.

Activities are expressed as relative values to the maximum HDC activity. Data are shown as the mean $\pm$ standard deviation from three independent experiments.

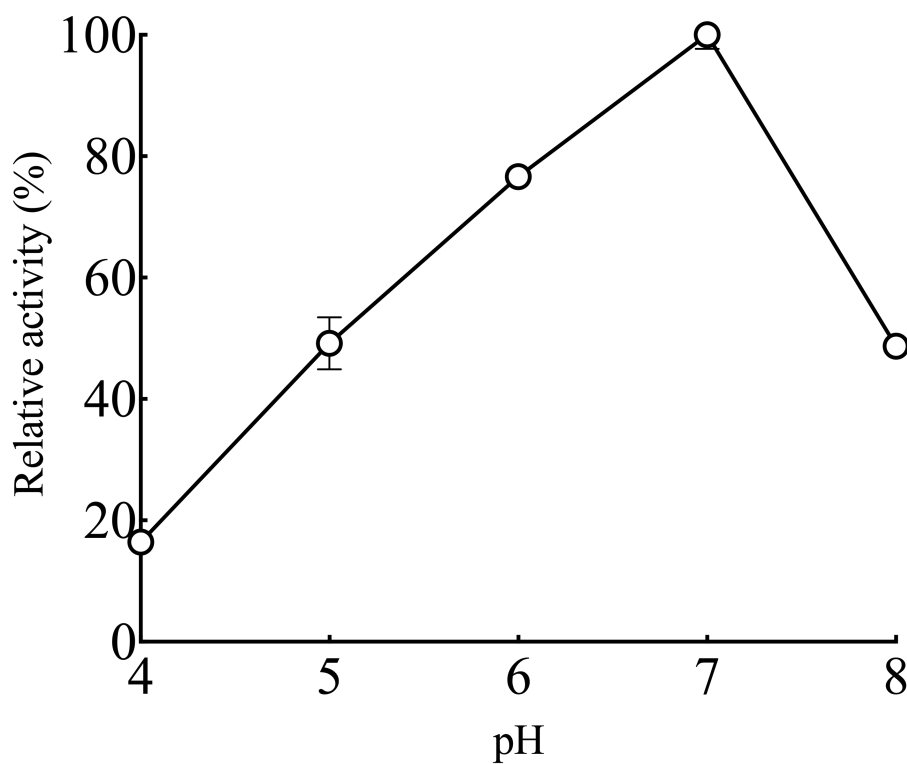


Fig. 1-10. Effect of pH on histamine accumulation by the crude HDC of *M. psychrotolerans*.

Activities are expressed as relative values to the maximum HDC activity. Data are shown as the mean $\pm$ standard deviation from three independent experiments.

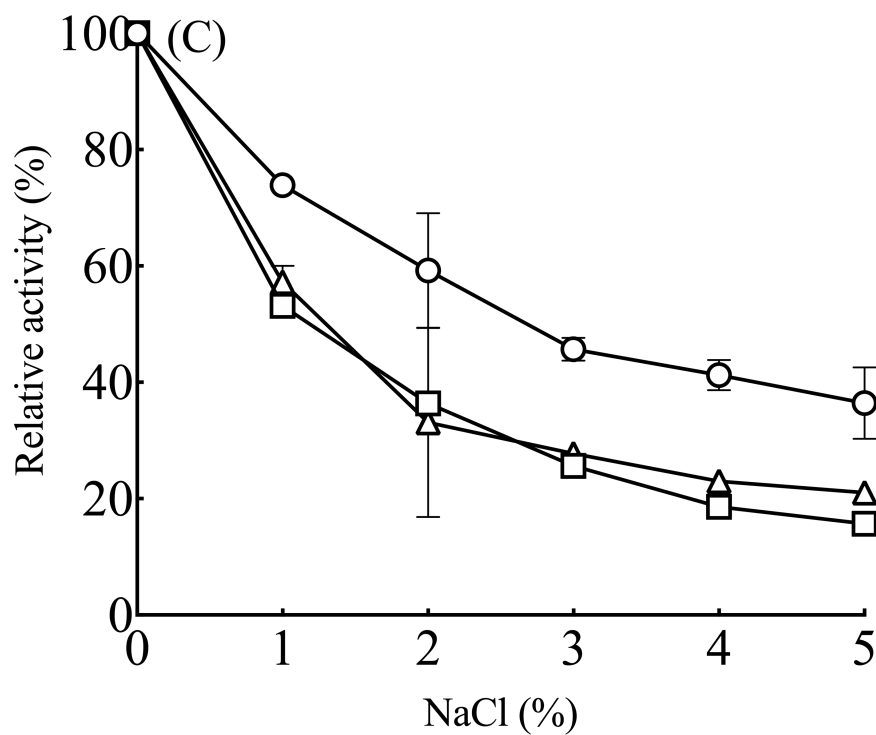


Fig. 1-11. Effect of NaCl concentration on histamine accumulation by the crude HDC of *M. psychrotolerans*.

Activities are expressed as relative values to the maximum HDC activity. Data are shown as the mean $\pm$ standard deviation from three independent experiments.

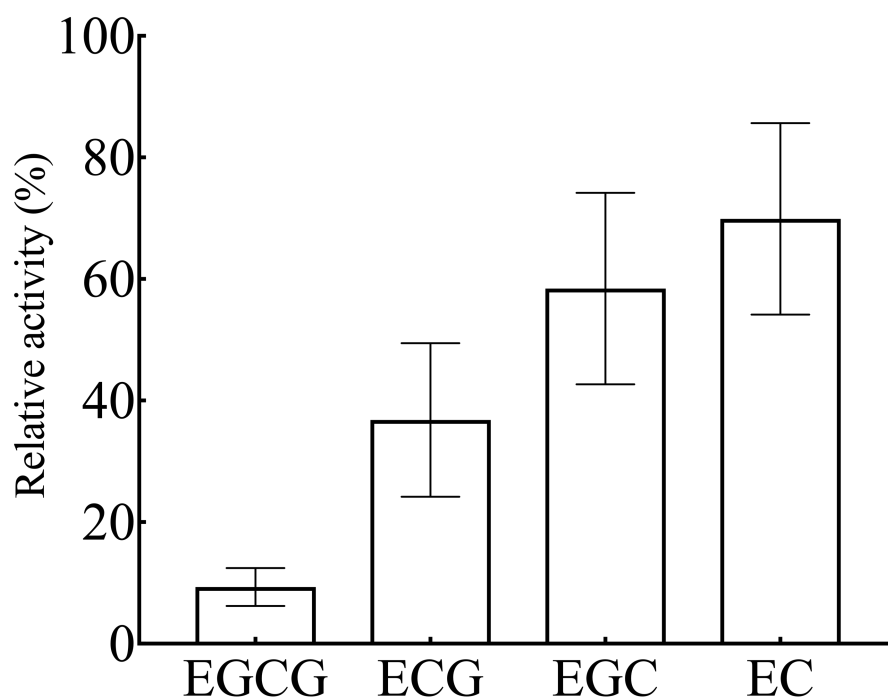


Fig. 1-12. Effect of tea catechins on HDC activity of *M. psychrotolerans* at 1 mM dose.

Activities are expressed as relative values to that of control (100 %). Catechins: EGCG, epigallocatechin gallate; ECG, epicatechin-3-gallate; EGC, epigallocatechin; EC, epicatechin. Data are shown as the mean $\pm$ standard deviation from three independent experiments.

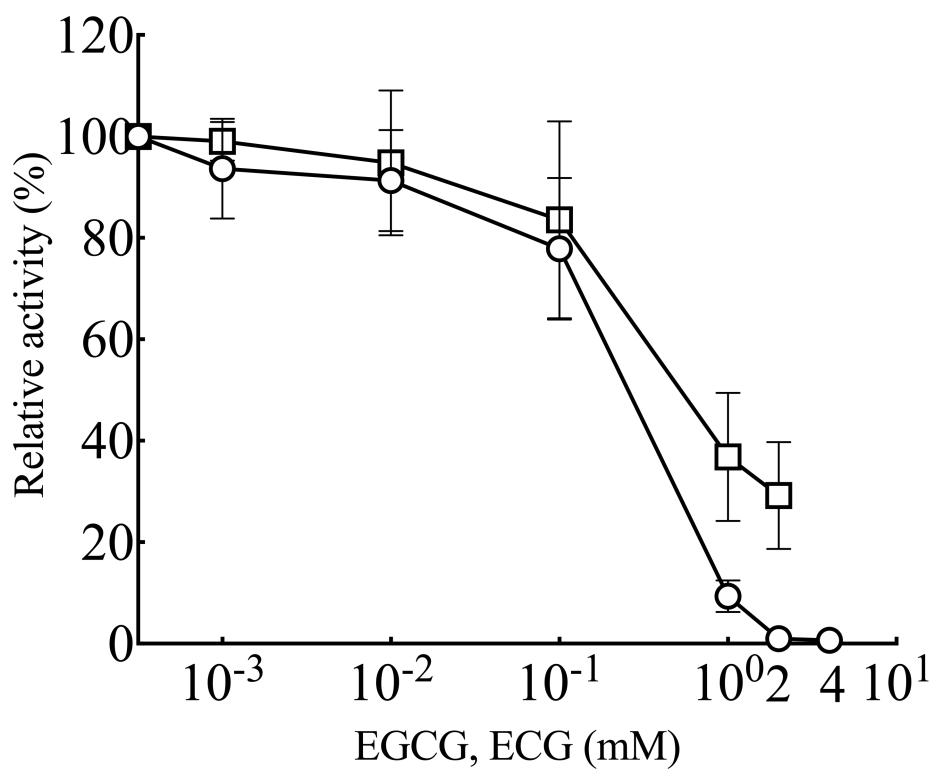


Fig. 1-13. Effect of EGCG (○) and ECG (□) concentration on HDC activity of *M. psychrotolerans*.

Activities are expressed as relative values to that of control (100 %). Data are shown as the mean $\pm$ standard deviation from three independent experiments.

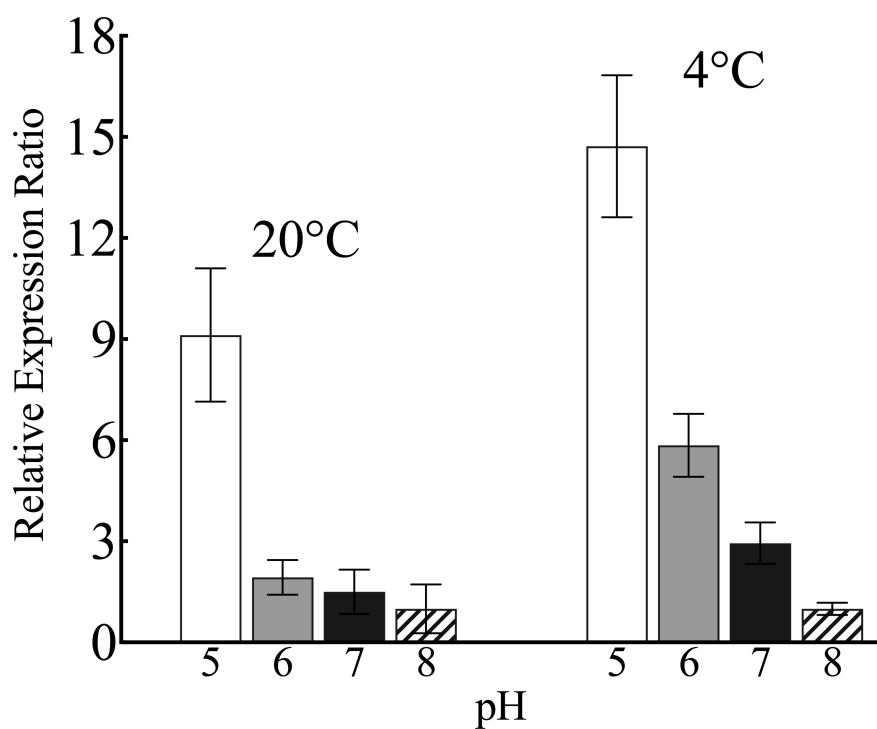


Fig. 1-14. Relative expression of histidine decarboxylase gene (*hdc*) of *M. psychrotolerans* at various pH.

Columns represent pH 5 (white), pH 6 (gray), pH 7 (black), and pH 8 (slashed). Data are shown as the mean $\pm$ standard deviation from three independent experiments.

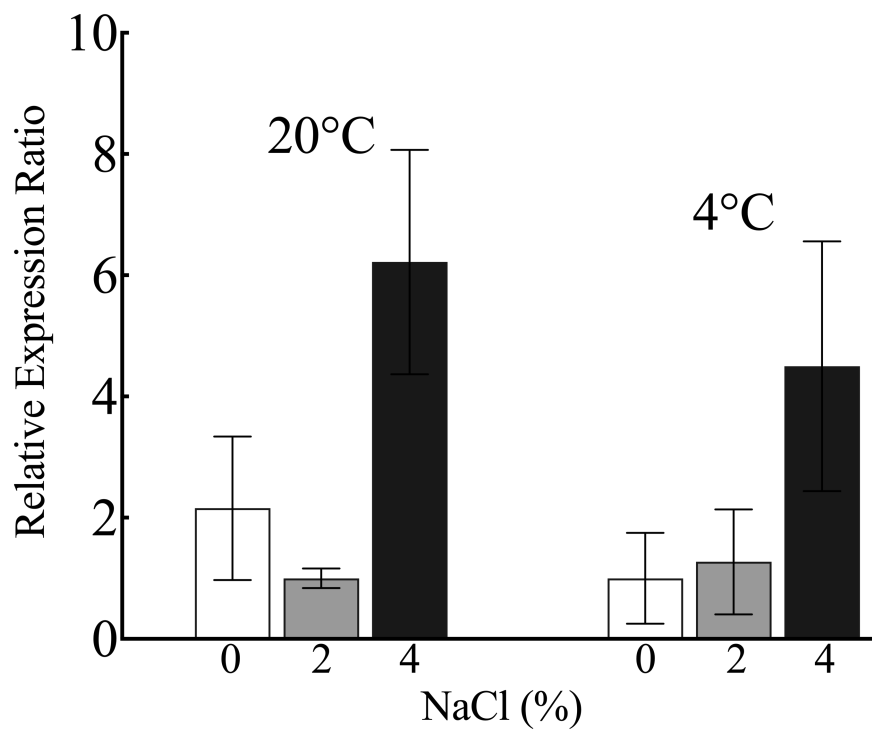


Fig. 1-15. Relative expression the histidine decarboxylase gene (*hdc*) of *M. psychrotolerans* at various NaCl concentration.

Columns represent 0 % NaCl (white), 2 % NaCl (gray), and 4 % NaCl (black). Data are shown as the mean $\pm$ standard deviation from three independent experiments.

Discussion

M. morganii and some *Photobacterium* species like *P. phosphoreum*, *P. kishitanii* and *P. aquimaris* are recognized as major mesophilic and psychrophilic histamine-producing bacteria (Kanki *et al.* 2004). The effects of environmental factors on histamine accumulation by psychrotolerant *M. psychrotolerans* have not been elucidated. Therefore, to avoid the histamine poisoning associated with seafood, in-depth studies on the relations between environmental factors and the histamine accumulation by *M. psychrotolerans* are necessary.

In Section 1, *M. psychrotolerans* and *M. morganii* produced similar amounts of histamine at room temperature (20 °C) during the incubation in canned tuna and various environmental conditions of culture broth. And, histamine produced by *M. psychrotolerans* were greater than that by *P. phosphoreum* in canned tuna and culture broths at 20 °C and 4 °C. *M. psychrotolerans* produced more than 1000 mg/kg of histamine at 4 °C after 8 d in canned tuna (Fig. 1-2B). Manthey-Karl *et al.* (2016) reported that about 700 mg/kg of histamine is accumulated by *P. phosphoreum* NBRC 13896 in tuna at 4 °C. *P. phosphoreum* NBRC 13896 was reported as a prolific histamine-producing bacteria at low temperatures (Bjornsdottir-Butler *et al.* 2018). Emborg & Dalgaard (2006) also reported that *M. psychrotolerans* could grow at pH 4.6–9.2 and at 2–35 °C, and did not require salt for its growth. In this study, *M. psychrotolerans* produced greater than 3500 mg/L of histamine under different environmental conditions in culture broth (Figs. 1-3 to 1-6). *Photobacterium* spp. as

marine bacteria require salt for growth, and the optimum condition for histamine production was shown at 1–4 % NaCl (Kimura *et al.* 2009). It was demonstrated that *M. psychrotolerans* could accumulate higher histamine than *P. phosphoreum* at 20 °C and 4 °C in different NaCl concentration of culture broth (Figs. 1-5 and 1-6).

In our previous study, *M. psychrotolerans* has been found from 40.6% of retail seafood in Japan (Kato *et al.* 2017). *M. morganii* and *M. psychrotolerans* exhibit very similar biochemical characteristics, and cannot be distinguished with the API50CH-E kit (BioMérieux, France), that is commonly used for rapid bacterial identification with biochemical indices (Emborg *et al.* 2006). Therefore, some of the isolates identified as *M. morganii* for a causative microorganism have been reasonably suspected to be *M. psychrotolerans*. NaCl concentration of the retail canned tuna used in this study was about $0.14 \pm 0.01\%$. The salt concentration of raw fish muscle is about 0.2-0.4% (Manthey-Karl *et al.* 2016; Geirsdottir *et al.* 2007; Thorarinsdottir *et al.* 2004). Therefore, a large percentage of seafood histamine poisoning outbreaks may be caused by *M. psychrotolerans*, which is highly capable of producing histamine in low NaCl conditions.

Although no significant differences among the three histamine producers were observed in the maximum density of cell counts under various conditions, the histamine accumulations were affected by environmental pH and NaCl content at both temperatures (4 and 20 °C). Namely, three bacterial species produced high amount of histamine at acidic and neutral pH conditions. These findings were consistent with the

results by Morii *et al.* (1994), who showed that the optimum culture pH for histamine production by *P. phosphoreum* was 5.5–6.5. And in this study, the histamine production at 4 % NaCl was lower than at 2 % NaCl for *M. psychrotolerans*. (Figs. 1-5 and 1-6). Increased salt concentration also reduced histamine accumulation in *Streptococcus thermophilus* PR160 (Rossi *et al.* 2011).

In order to control bacterial histamine accumulation, the enzyme HDC activity under various environmental conditions was investigated. The optimum temperature for the crude HDC from *M. psychrotolerans* was at 30 °C, and the crude HDC maintained 45% of its optimum activity at 10 °C (Fig. 1-9). Bjornsdottir-Butler *et al.* (2016b) reported that the optimum temperature of the HDC of *P. kishitanii* and *P. angustum* were at 30 °C. Morii *et al.* (1994) reported that the crude HDC from *P. phosphoreum* at 10 °C retained 10 % of activity at the optimum temperature. Thus, the HDC of *M. psychrotolerans* might be more adaptable to low temperature.

Maximum HDC activity of *M. psychrotolerans* was observed at pH 7 (Fig. 1-10). Similarly, HDC of other Gram-negative histamine producers, *M. morganii* (Tanase *et al.* 1985) and *Enterobacter aerogenes* (Zou *et al.* 2014), was optimal at pH 6.5. Morii *et al.* (1994) reported the crude HDC from *P. phosphoreum* exhibited no activity at pH 4. Although *M. psychrotolerans* did not grow at pH 4 in culture broth, its HCD activity at pH 4 showed 18% of that at pH 7 (Fig. 1-10). Hence, it was indicated that the histamine producing ability of *M. psychrotolerans* under low pH is greater than that of *P. phosphoreum*. The pH of fish muscle is 5.5 - 6.5 (Bjornsdottir-Butler *et al.* 2015).

Also, it is often adjusted with organic acids to inhibit growth of microorganisms. These findings suggested that *M. psychrotolerans* is more important than other psychrophilic histamine producers as a risk factor for histamine poisoning associated with seafood during low-temperature storage.

On the other hand, the HDC activity of *M. psychrotolerans* decreased at alkaline pH (pH 8) (Fig. 1-10). This corresponding with previous reports that HDC activity from *M. morganii* (Tanase *et al.* 1985), *P. phosphoreum* (Morii and Kasama 2004), and *S. thermophilus* (Tabanelli *et al.* 2012) were greatly reduced at pH 8. Bjornsdottir-Butler *et al.* (2015) have shown an excellent strategy for reducing histamine accumulation in fish muscle: pH elevation by trisodium phosphate treatment. In this study, it was suggested that the pH elevation could be one of the effective methods to suppress the histamine production by *M. psychrotolerans* in seafood as well.

The HDC activity were gradually affected with an increase in NaCl concentration (Fig. 1-11). Similar tendencies were reported for the HDC extracted from *M. morganii* and *P. phosphoreum*, and the HDC of *P. phosphoreum* was highly inhibited at 5 % NaCl concentration (Kanki *et al.* 2007; Morii and Kasama 2004).

Once the HDC enzyme was formed, it can continue to produce histamine even if the bacteria are not active. Moreover, HDC remains stable while in the frozen state and can be reactivated very rapidly after thawing (FDA 2011). Some studies reported that plant components could inhibit the activity of recombinant human HDC (Nitta *et al.* 2016; Nitta *et al.* 2007).

Tea catechins, the constituent of green tea polyphenols, can be classified into four major types including EGCG, EGC, EC and ECG. They have been shown strong antioxidant, antimicrobial, and antiangiogenic effects (Kitichalermkiat *et al.* 2019). Rodriguez-Caso *et al.* (2003) reported that tea catechins showed higher inhibitory activity against mammalian HDC than other food components they examined. In Section 2, the inhibition activity of tea catechins to the crude HDC of *M. psychrotolerans* was evaluated (Fig. 1-12). Polyphenols have been already reported as enzyme inhibitors because they can bind and aggregates proteins (Kitichalermkiat *et al.* 2019). Galloyl moiety of catechins would interact with polar groups of enzyme and elicit enzyme inhibition (Pal *et al.* 2014). The inhibition activity of EGCG was stronger than ECG, and decreased the HDC activity to almost zero at 2 mM dose (Fig. 1-13). These results were inconsistent with the report showed that ECG was more effective than EGCG on mammalian HDC (Nitta *et al.* 2007). This inconsistency might be due to the difference of target HDC. In addition, the higher inhibition activity of EGCG than other catechins might be related to the phenolic groups, and the eight phenolic groups of EGCG which serve as hydrogen donors might cause the higher binding efficiency than others (Pal *et al.* 2014).

The decarboxylation of histidine by histamine producers requires expression of *hdc* gene and synthesis of HDC enzyme in the cells. The *hdc* gene expression of *M. psychrotolerans* was greatly induced at acidic condition at both 4 and 20 °C (Fig. 1-14). This phenomenon in *M. psychrotolerans* agreed with other histamine-producing

bacteria. Many reports indicate that low pH was a necessary factor for *hdc* gene expression in *M. morganii* (Ferrario *et al.* 2013; Kimura *et al.* 2009), *P. iliopiscarium* (Takahashi *et al.* 2015), *P. damsela* subsp. *damsela* (Kimura *et al.* 2009), and *S. thermophiles* (Calles-Enríquez *et al.* 2010). The initial acidic to neutral pH enhances the *hdc* gene expression, forming HDC and histamine, which is excreted from the cells. That is, when the bacterial cells sense the acidic pH conditions, they begin to convert the histidine into histamine and CO₂. Thus, a large amount of H⁺ were eliminated and the bacteria could escape from acid stress through the decarboxylation of amino acid as a survival strategy (Ferrario *et al.* 2013; Kimura *et al.* 2009; Landete *et al.* 2008; Tanaka *et al.* 2008; Merrell and Camilli 2000).

Additionally, the maximum expression for the *hdc* gene of *M. psychrotolerans* was found at 4 % NaCl (Fig. 1-15). This results did not correspond to the histamine accumulation tendency in culture broth, which was decreased at 4 % NaCl. It might be due to the histidine decarboxylase cluster of pathways composed of *hdcT1* (putative histidine/histamine antiporters), *hdc*, *hdcT2* (putative histidine/histamine antiporters), and *hisRS* (histidyl-tRNA synthetase) (Fig. 1-8), and it was suggested that other genes were also related to incorporation of histidine and excretion of histamine for histamine synthesis in bacterial cells (Ferrario *et al.* 2013; Kimura *et al.* 2009). Moreover, it might be due to the inhibitory effect of high NaCl concentration on HDC activity.

In this Chapter, the histamine production behaviors of *M. psychrotolerans* under various environmental conditions were revealed. Characteristics of the crude HDC and

hdc gene expression level of *M. psychrotolerans* were also investigated under different conditions. The results indicate that *M. psychrotolerans* has a higher capacity to produce histamine toxicologically even at low temperature (4 °C), high salt concentration (4 % NaCl), and over broad pH conditions (pH 4–8). And the crude HDC maintained its activity under a wide range of environmental conditions. The *hdc* gene expression explained might provide understanding on the environmental conditions that affect the histamine production in *M. psychrotolerans*. Furthermore, effect of tea catechins on the HDC activity was evaluated. EGCG was found to have an inhibitory effect on HDC activity. In this Chapter, it was comprehensively confirmed that *M. psychrotolerans* have high histamine producing ability even at low temperature. Therefore, EGCG is expected as an HDC inhibitor for reducing histamine accumulation in seafood, which is commonly stored at low temperature.

Chapter 2

Sanitizing efficacy and antimicrobial mechanism of peracetic acid (PAA) and sodium hypochlorite (SH) on *Morganella psychrotolerans*

Chapter 1 described that the *M. psychrotolerans* could produce high levels of histamine, which causes the human illness due to histamine food poisoning. Sodium hypochlorite (SH) is the most commonly used disinfectant in food processing. But as a disadvantage, SH significantly loses effectiveness in the presence of organic materials. Chlorine may lead to formation of hazardous by-products, which is a threat to human health. Compared with SH, peracetic acid (PAA) has useful disinfectant properties, such as a broad-antimicrobial spectrum, rapid bactericidal action, high fusibility in water, and low toxicity at practical dose. PAA as a cleaning disinfectant on washing seafood is not well investigated right now. In this Chapter, the efficacy of PAA against *M. psychrotolerans* and the antibacterial action was attempted to clarify.

Materials and methods

2-1. Cultivation of bacterial strain

M. psychrotolerans JCM 16473^T cells were cultured in TSB at 25 °C for 24 h. The cultures (approximately 10⁹ CFU/mL) were centrifuged at 6,000 × g for 10 min at 4 °C. The harvested cell pellets were washed twice with PBS (pH 7.2) and then subjected to different treatment described below.

2-2. Evaluation of bacterial disinfection with PAA and SH *in vitro*

Composition of the peracetic acid (PAA) sanitizer, TEC P-10 (Table 2-1) (Adeka Clean Aid) used in this study was shown in Table 2-1. The PAA concentration used in this thesis refers to the concentration of peracetic acid in the sanitizer. SH was purchased from Wako Pure Chemical, and available chlorine concentration of SH was assayed with iodometric method described by Saputro *et al.* (2011). The concentration of SH in the text means the available chlorine concentration.

The washed bacterial cells were resuspended in PBS at the inoculum size of about 10⁹ CFU/mL and 9 mL of bacterial suspension was mixed with 1 mL PAA (10, 20, and 40 ppm) and SH (10, 20, 40, and 50 ppm). Sterile deionized water was used as a control. After the treatment for 1, 3, or 5 min, 0.1 mL of treated suspension was transferred and immediately mixed with 0.9 mL inactivator solution (Table 2-2). The neutralized

samples were then serially diluted (1:10) with PBS (pH 7.2) and 0.2 mL from each dilution were spread on plates of TSA supplemented with 0.2 % sodium pyruvate. The survivor colonies of *M. psychrotolerans* were counted after incubation at 25 °C for 72 h.

2-3. Evaluation of bacterial disinfection with PAA and SH on fish surface

Fresh Pacific sauries (*Cololabis saira*) placed on ice were purchased from a local supermarket in Japan and immediately brought to the laboratory for freezing at -70 °C. Each whole saury was initially put in tap water for 30-40 min until thawing and washed three times with 9-fold distilled water. The saury was then submerged in the *M. psychrotolerans* culture suspension (1:9 w/v, about 10⁹ CFU/mL) for 5 min. The inoculated sauries were air-dried in a biological safety cabinet for 12 min (initial inoculum size: about 10⁶ CFU/cm²). The samples were then dipped for 1 or 5 min into 9-fold (w/v) volumes of the sanitizer solutions; 80 ppm PAA, 100 ppm SH and deionized water as control. This was followed by immediately dipping the sauries in deionized water (1:9, w/v) to remove the sanitizers. The saury surfaces were wiped with a 10 mL dilution attached swab test (Kanto Chemical). The 0.1 mL of sample solutions were spread and incubated on Niven's agar (Table 2-3) (Niven *et al.* 1981) supplemented with 0.2% sodium pyruvate at 25 °C for 72 h. Formed colonies with purple surroundings on the Niven's agar were counted as histamine-producing bacteria, *M. psychrotolerans*.

2-4. Scanning Electron Microscopy (SEM) observation

The morphology of *M. psychrotolerans* cells, treated as described above, was observed by SEM. The neutralized cells were centrifuged ($3,000 \times g$, 5 min, 4 °C) and washed three times with 0.2 M phosphate buffer (PB, pH 7.4). The cells were then fixed with 2% glutaraldehyde–PB (2 h, room temperature), washed, resuspended in the same PB and placed on a Sempore ($\phi 0.6 \mu\text{m}$, JEOL) as a 100 μL droplet. After washing three times with PB, the samples were dehydrated in a graded series of ethanol (50-100 %). The cells were future replaced by *t*-butyl alcohol (Wako Pure Chemical) for 30 min and lyophilized. Samples were the sputtered with Pt-Pd and observed by SEM (JSM-6010LA, JEOL).

2-5. Evaluation of membrane integrity of bacterial cell

The cells were stained, after disinfectant treatment, with a Bacteria Live/Dead Staining Kit (Promokine, PromoCell). The DMAO (green fluorescence) and EtD-III (red fluorescence) nucleic acid dyes were mixed with 0.85% NaCl to obtain the dye mixture, followed by adding 1 μL mixture dye to a 100 μL cell suspension and incubating for 15 min at room temperature in dark. Then, 5 μL of the stained bacterial cell suspension was put on a glass slide and immediately visualized through epifluorescence microscopy (BX 51-34, Olympus). Green and red fluorescence

indicates cells that have intact and damaged membranes, respectively. Fluorescence images were processed using the Image J software package (Schneider *et al.* 2012).

2-6. Evaluation of chromosomal DNA integrity of bacteria

The bacterial DNA were extracted, from disinfection and control groups described in 2-2, with a Genomic DNA kit (Macherey-Nagel) according to the manufacturer's instructions. Extracted DNA was then separated by gel electrophoresis (1% agarose). After ethidium bromide staining, DNA band on the agarose gel was visualized with an UV transilluminator (STAGE-1000, AMZ).

Table 2-1. Composition of the peracetic acid (PAA) sanitizer, TEC P-10

Component	Composition (%)
CH_3COOOH	9–10
H_2O_2	19–21
CH_3COOH	15–17
H_2O	52–57

The peracetic acid sanitizer, TEC P-10 was provided by Adeka Clean Aid Co.

Table 2-2. Composition of inactivator solution for the sanitizers

Ingredients	
Lecitin, from soybean (Wako Pure Chemical)	10 g
Polyoxyethylene (20) sorbitan monooleate	30 g
L-histidine	1 g
Tryptone (BD)	1 g
Na ₂ S ₂ O ₃ ·5H ₂ O	20 g
Distilled water	1000 mL

Medium was autoclaved at 121°C for 1 min

Table 2-3. Composition of Niven's agar

Ingredients	
Tryptone (BD)	5 g
Yeast extract (BD)	5 g
CaCO ₃	1 g
L-histidine	18.5 g
NaCl	5 g
Bromocresol purple	0.06 g
Agar	20 g
Distilled water	1000 mL
	(pH 5.1)
Medium was autoclaved at 121°C for 15 min	

Results

2-1. Sanitizing effect on *M. psychrotolerans*

The antimicrobial effect of PAA and SH on *M. psychrotolerans* was investigated *in vitro* (Fig. 2-1). For treatment with PAA, the bacterial cell counts were significantly decreased from 8.4×10^8 to 4.4×10^4 CFU/mL at 10 ppm within 1 min, and 20 ppm PAA had potential to decrease population of *M. psychrotolerans* to undetectable levels ($< 5 \times 10^1$ CFU/mL) for 5 min (Fig. 2-1A). Treatment with SH resulted in 1.2 and 5.2 log CFU/mL reduction at 10 and 20 ppm for 5 min, respectively ($p \leq 0.05$). And for use of 40 ppm SH for 5 min, the survival population of *M. psychrotolerans* was below the detection level (Fig. 2-1B). Moreover, for the treatment with 40 ppm PAA (Fig. 2-1A) or 50 ppm SH (Fig. 2-1B) for 1 min, *M. psychrotolerans* was reduced to below the detection limit.

2-2. Sanitizing effect on *M. psychrotolerans* from saury surface

The antimicrobial effect of PAA and SH on *M. psychrotolerans* from the saury surface is shown in Fig. 2-2. The population of *M. psychrotolerans* on saury surface about 10^6 CFU/cm² before treatment by sanitizer. The control groups led to 0.5 log CFU/cm² reduction of *M. psychrotolerans* for 1 min. And treatment with 80 ppm PAA and 100 ppm SH at 1 min resulted in 1.4 and 1.3 log CFU/cm² reduction, respectively.

The extension of washing time improved the reduction of *M. psychrotolerans* from saury surface, and the viable cell count was decreased 2.23 and 1.99 log CFU/cm² by washing with 80 ppm PAA and 100 ppm SH at 5 min, respectively.

2-3. Morphological property of *M. psychrotolerans* cells treated with the sanitizers

The effect of the sanitizers on cell morphology was observed with SEM. The SEM image showed a smooth and intact surface of control cells (Fig. 2-3A). After treatment with 20 ppm PAA for 5 min, the surfaces of cells had no clear differences from control cells (Fig. 2-3B). Whereas, after treatment with 80 ppm PAA for 5 min, there were some corrugated cells (Fig. 2-3C). SH caused cells irregular alterations, some dimples on the cells were observed after treatment with 40 ppm SH for 5 min (Fig. 2-3D), and some debris were observed after treatment with 100 ppm SH for 5 min (Fig. 2-3E). These observations suggested that PAA and SH treatment destroyed outer membrane of *M. psychrotolerans* cell and the damaged level might be related to the concentration of the sanitizer.

2-4. Integrity of cell membrane of *M. psychrotolerans* cells treated with sanitizers

Cytoplasmic membrane integrity of the bacterial cells was observed with Bacteria Live/Dead Staining kit. Two fluorescent probes, EtD-III (red) and DMAO, are used to distinguish membrane damaged cells from intact cells in the kit. DMAO is a membrane-

permeable green fluorescent dye with intact membranes, and EtD-III is considered to be able to bind to nucleotides in cells with damaged cytoplasmic membranes and emits red fluorescence (Sawant *et al.* 2016). *M. psychrotolerans* cells treated with water (control) emitted green fluorescence, and the cells treated with PAA and SH showed red fluorescence (Fig. 2-4). This suggests that the cells membranes lost integrity by PAA and SH treatment.

2-5. Integrity of chromosomal DNA of *M. psychrotolerans* cells treated with sanitizers

Changes in chromosomal DNA of bacterial cells treated with the sanitizers were analyzed with the agarose gel electrophoresis as shown in Fig. 2-5. The DNA extracted from cells of the control and the group treated with PAA and SH displayed a bright single band on the agarose gel. Additionally, an increase in PAA and SH concentration showed no particular difference in the DNA band profiles. This result indicates that PAA kills *M. psychrotolerans* cells without damaging the bacterial DNA.

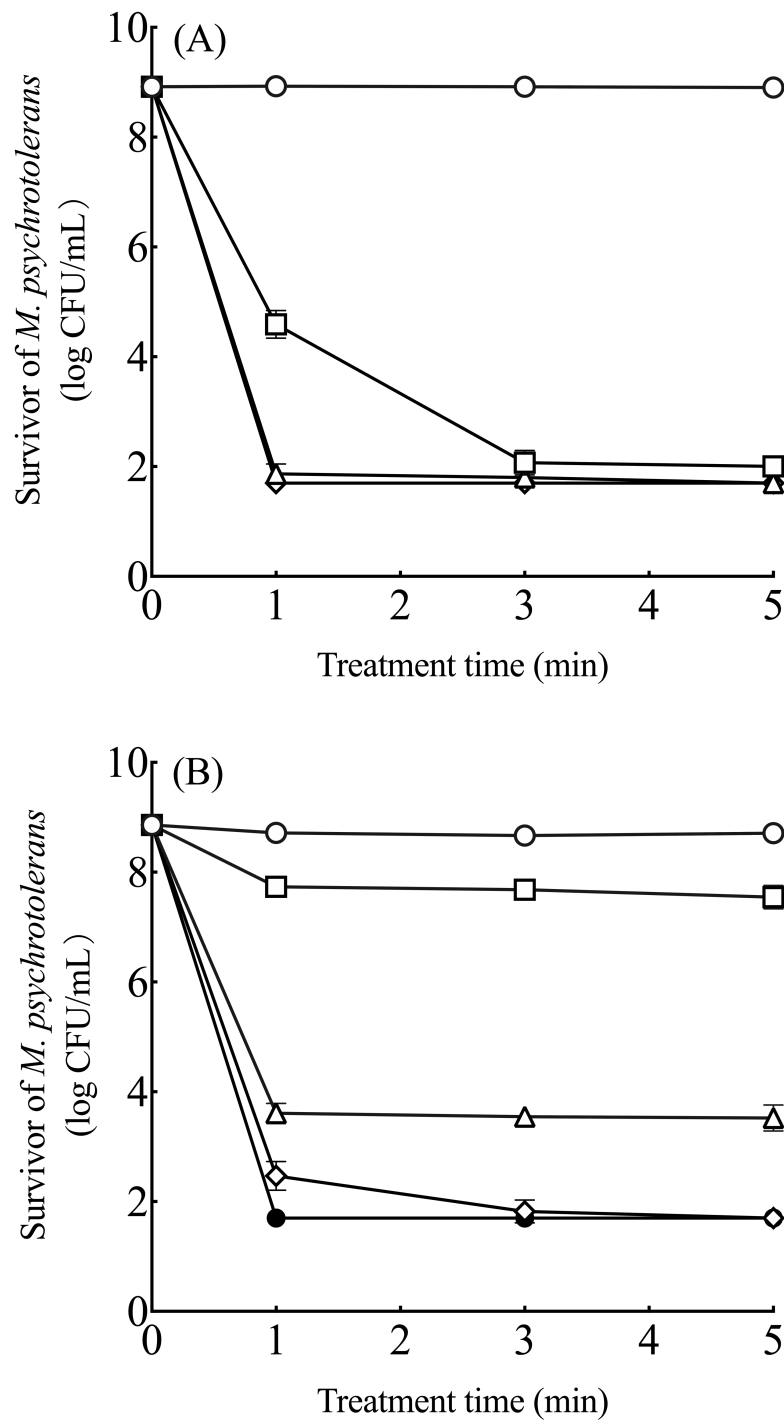


Fig. 2-1. Survival curves of *M. psychrotolerans* under treatment with PAA (A) and SH (B) *in vitro*.

Symbols represent control (○), 10 ppm (□), 20 ppm (△), 40 ppm (◇), and 50 ppm (●). The minimum detection level was 5×10^1 CFU/mL. Data are shown as the mean $\pm$ standard deviation from three independent experiments.

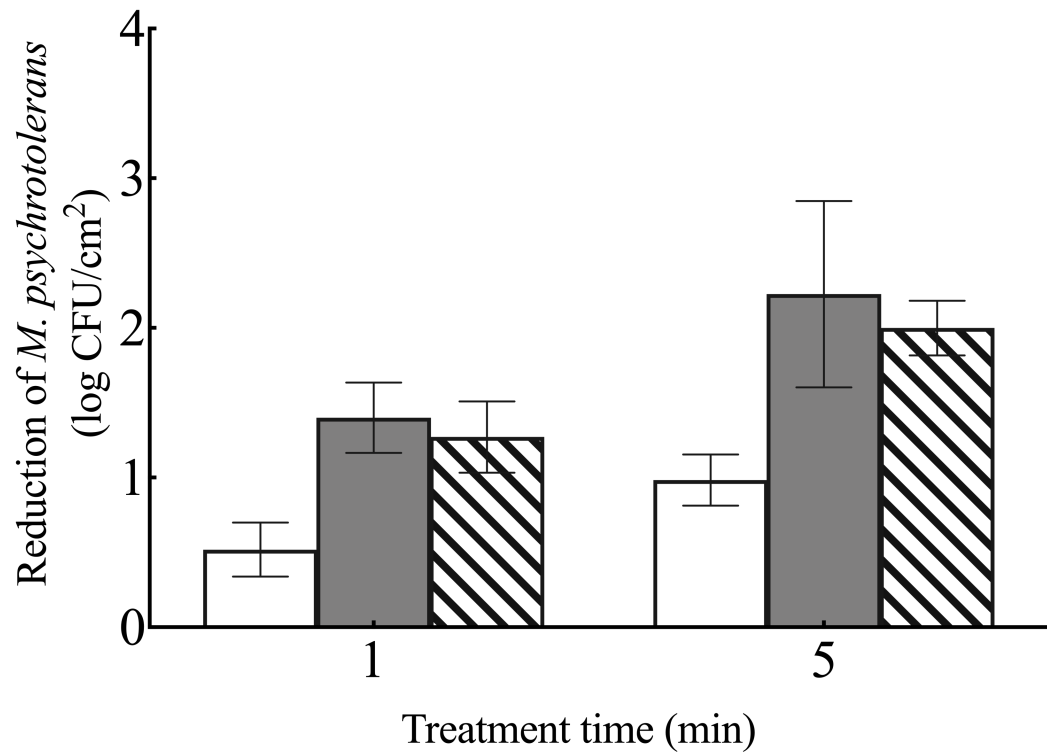


Fig. 2-2. Reduction of viable cell counts of *M. psychrotolerans* from saury surface after washing with sanitizers for 1 and 5 min.

Columns represent control (white), 80 ppm PAA (gray) and 100 ppm SH (slashed). Data are shown as the mean $\pm$ standard deviation from three independent experiments.

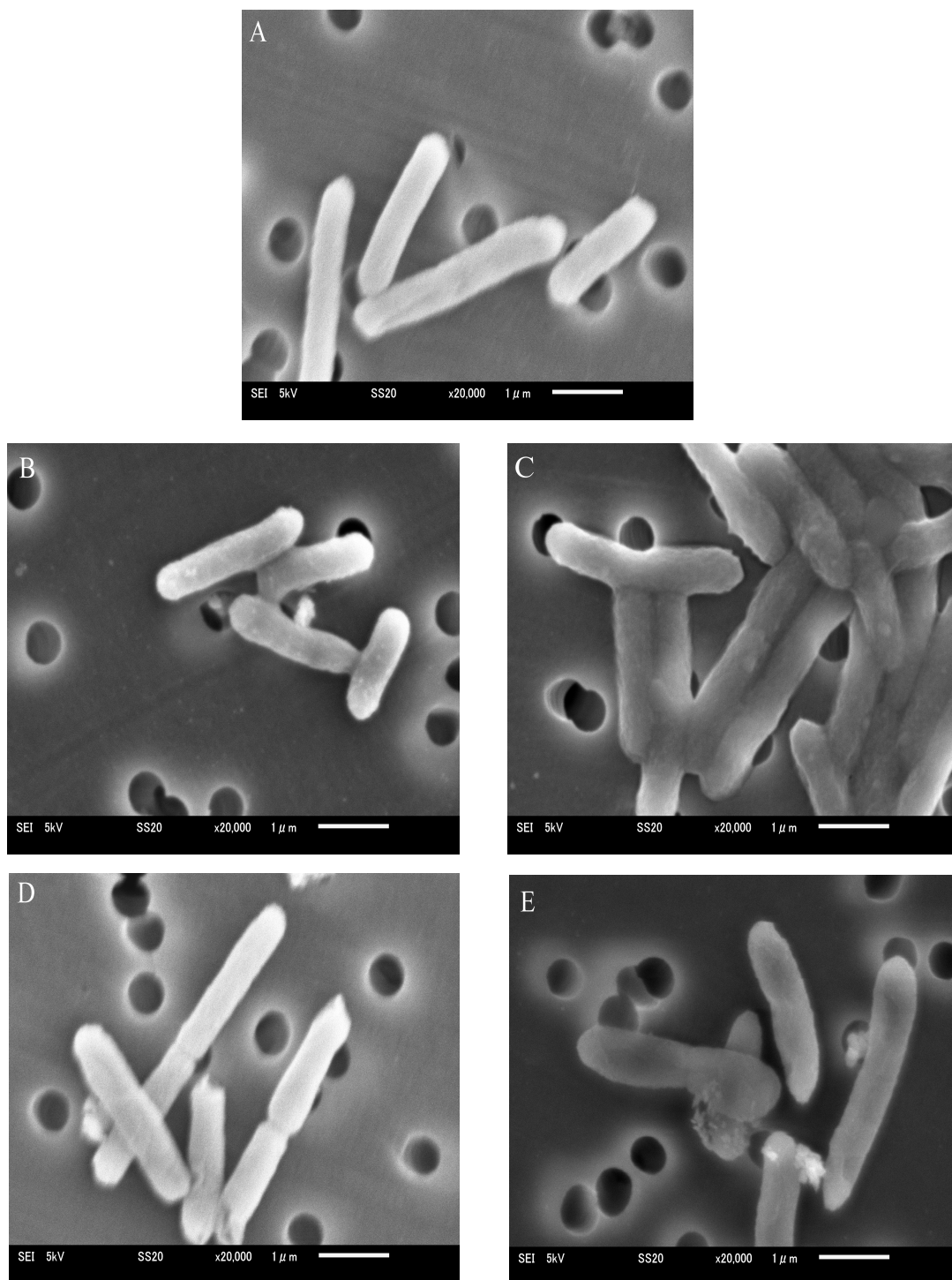


Fig. 2-3. Scanning Electron Microscope (SEM) images of *M. psychrotolerans* cells treated with PAA and SH for 5 min.

Images (sanitizers): A, control; B, 20 ppm PAA; C, 80 ppm PAA; D, 40 ppm SH; E, 100 ppm SH.

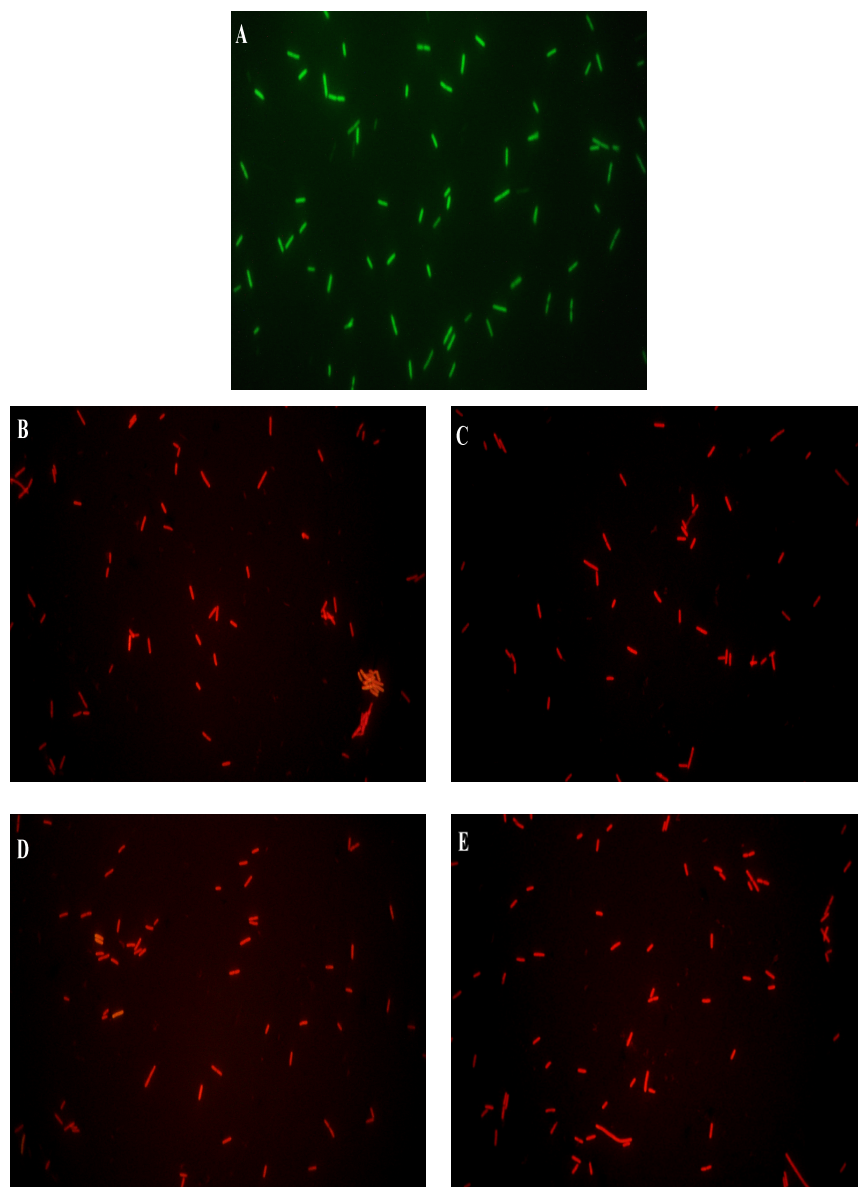


Fig. 2-4. Epifluorescence microscopy images for Live/Dead staining of *M. psychrotolerans* cells after treated with PAA and SH for 5 min.
 Images (sanitizers): A, control; B, 20 ppm PAA; C, 80 ppm PAA; D, 40 ppm SH; E, 100 ppm SH. Green and red fluorescence indicates cells of intact and damaged membranes, respectively.

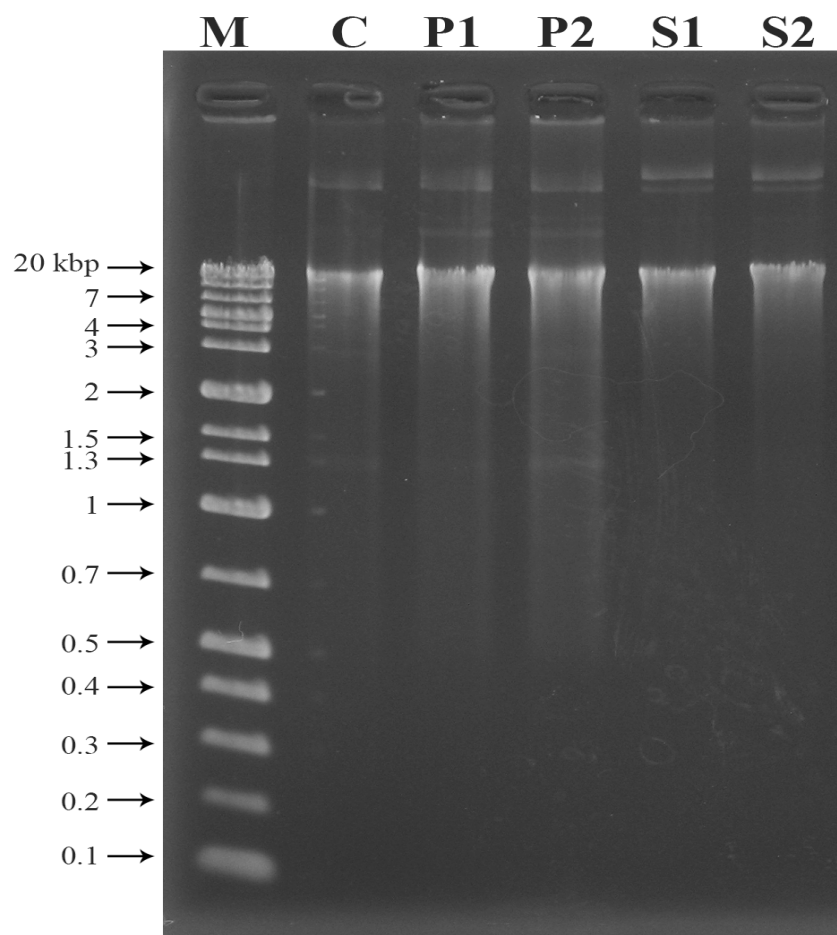


Fig. 2-5. Agarose gel electrophoretic patterns of chromosomal DNA of *M. psychrotolerans* cells treated with PAA and SH for 5 min.

Lanes: M, nucleotide size marker; C, control; P1, 20 ppm PAA; P2, 80 ppm PAA; S1, 40 ppm SH; S2, 100 ppm SH.

Discussion

Disinfection is common within the food industry. Our previous study revealed that *M. psychrotolerans* has been found to distribute in retail seafood in Japan (Kato *et al.* 2017). Considering the significant role of *M. psychrotolerans* in histamine accumulation and subsequent histamine poisoning, appropriate sanitization is required industry-wide to enhance seafood safety. SH (50–100 ppm) is the conventional sanitizer used to disinfect fish fillets (Thi *et al.* 2013). Also, for the sensory properties of fish it can be altered by washing in 150 ppm PAA (Thi *et al.* 2015). PAA was approved as a cleaning disinfectant in meat, poultry, fruits, and vegetables at concentrations of 220 ppm, 220 ppm, and 80 ppm, respectively (FDA, 1999). In this study, the sanitizing effect and mechanism of PAA and SH was firstly compared at appropriate concentration for food processing against *M. psychrotolerans*. Treatment with 20 ppm PAA for 5 min eliminated *M. psychrotolerans* under detectable levels, whereas 40 ppm SH was required to achieve undetectable levels of *M. psychrotolerans* with 5 min of treatment (Fig. 2-1). Treatment with 80 ppm PAA for 5 min was lethal to *E. coli* O157:H7 (Rodgers *et al.* 2004). And 15 ppm PAA resulted in *Vibrio parahaemolyticus* death in Luria-Bertani broth containing 3 % NaCl (Wong *et al.* 2018). Additionally, the antibacterial efficacy of PAA and SH rose as treatment concentration increased. The population of *M. psychrotolerans* reached undetectable levels with 1 min of treatment with either 40 ppm PAA or 50 ppm SH (Fig. 2-1). Similar study suggested that

treatment at 30 ppm SH for 1 min exerted significant antibacterial effects against *Staphylococcus aureus* (Ujimine *et al.* 2017).

The challenge test for saury-washing indicated that PAA displayed an effective antimicrobial activity (Fig. 2-2), which corresponded to the *in vitro* results (Fig. 2-1). The fish flesh is free of bacteria when fish alive, and the histamine-producing bacteria are mainly distributed on the skin, gills, and gut (Phuvasate and Su 2010). After death, the fish's defense mechanisms no longer inhibit bacterial growth in the muscle tissue and then the histamine-producing bacteria start to grow. Fish processing also can cause contamination of flesh with histamine-producing bacteria (Phuvasate and Su 2010). Therefore, the reduction of histamine-producing bacteria from skin would extend fish shelf life. The washing treatment with 80 ppm PAA and 100 ppm SH for 5 min resulted in 2.23 and 1.99 log CFU/cm² reduction, respectively (Fig. 2-2). Sheng *et al.* (2020) reported that 80 ppm PAA resulted in 1.8 log CFU/apple reduction of *Enterococcus faecium* after 2 min treatment. Moreover, soaking vegetables in 30 ppm PAA for 10 min reduced *E. coli* counts by 4.39 and 2.33 log CFU/g on tomato and lettuce, respectively (Keeratipibul *et al.* 2011). Use of 50 ppm SH resulted in 6.9 log CFU reduction of *E. coli* from chicken carcasses (Russell and Axtell 2005). Park *et al.* (2012) reported that PAA could replace SH, because it was more effective than SH at equivalent concentration against *E. coli* O157:H7, *Salmonella* Typhimurium, and *Listeria monocytogenes*. Similarly, this study showed that PAA was effective than SH on the reduction of *M. psychrotolerans* from the saury surface. And Lee and Huang

(2019) demonstrated that the washing by PAA generated much less by-products than washing by SH. The results indicated that PAA might be a better sanitizer than SH for fish processing.

Since PAA is produced by a reaction between hydrogen peroxide and acetic acid, the PAA sanitizer is commonly a mixture containing acetic acid and hydrogen peroxide. The disinfectant activity of PAA should be due to its oxidation potential and it can also release protons (to lower pH) and undissociated acid that cause the intercellular pH to drop. Both of the two functions will cause metabolic disturbance within the cell, resulting in cell death. (Kitis, 2004). Zhang *et al.* (2018) reported that PAA and SH disrupted cell wall integrity to inactivate microbes. This was consistent with the observations as presented in Figs. 2-3 and 2-4. SEM images showed that PAA and SH damaged the cell-cortex of *M. psychrotolerans* and created other changes as the sanitizer concentration was increased. Additionally, SEM images revealed that treatment with PAA and SH created cell surface irregularities and partial grooves with the damage. These become increasingly apparent as sanitizer concentration increased (Chino *et al.* 2017; Ujimine *et al.* 2017; Park *et al.* 2013; Mustapha and Liewen 1989) . However, the significant damage was not observed to *M. psychrotolerans* cell morphology, especially with the 5 min treatment of 20 ppm PAA. This observation was consistent with other paper reporting no significant damage of cell surface treated with low or medium levels of free chlorine (Cho *et al.* 2010). Additionally, it was reported

that the effects of PAA sanitizer on bacterial cell morphology was mitigated when the treatment concentration used was lower than 60 mg/L (Zhang *et al.* 2019).

Two fluorescent probes, EtD-III and DMAO, were used to distinguish membrane damaged cells from intact cells in Live/Dead Staining kit. When *M. psychrotolerans* cells were treated with the PAA and SH, the cell membranes obviously lost integrity (Fig. 2-4). Changes in the permeability and integrity of bacterial cell membrane may be caused by many factors, such as inactivation of enzyme and changes in membrane potential (Joux and Lebaron 2000). The fluorescence dyes of SYTO 9 and PI are also used to indicate the cell membranes damage. SYTO 9, a green fluorescent nucleic acid dye could penetrate both intact and damaged cells' membranes, while PI, a red fluorescent nucleic acid dye could only penetrate the damaged cell membrane, showing red fluorescence (Zhao *et al.* 2019). SYTO 9 and PI staining of *E. coli* treated with SH revealed red fluorescence, signifying cell membrane damage (Park and Kim 2018). SYTO 9 and PI staining of *S. aureus* SA1 and *L. monocytogenes* after PAA treatment also revealed cell membrane damage (Lee *et al.* 2016).

From a genetic perspective, the PAA treatment did not lead to chromosomal DNA damage (Fig. 2-5). This is inconsistent with report by Ujimine *et al.* (2017) who found the DNA in *S. aureus* cells after treating with higher concentration than 3.1 ppm SH was undetected on the agarose gel, due to DNA degradation. However, the results in this study are consistent with a report that DNA in *C. jejuni* and *C. coli* cells were not affected by treatment with 4000 ppm SH (Hatanaka *et al.* 2020). These conflicts might

be related to differences in the efficiency of sanitizers used, the target microorganism, sanitizer concentration, and treatment time (Zoellner *et al.* 2018). Also, PAA exerted an antimicrobial effect on *E. coli* TOP10 without affecting the plasmid DNA (Zhang *et al.* 2019). Overall, the observation from this study suggests that the antimicrobial mechanisms of PAA and SH include damage of outer membrane, changes of cell cytoplasmic membrane, but not related to damage to the chromosomal DNA.

In this Chapter, it was revealed that PAA and SH had significant sanitizing effect against *M. psychrotolerans* *in vitro* and on saury surface. It was also suggested that PAA could become a potential sanitizer to replace SH in seafood processing. The PAA showed highly lethal damage to the bacterial cells than SH. The membrane permeability and morphologies of *M. psychrotolerans* cells showed changes by treatment with PAA and SH. Further, the PAA and SH treatment did not cause the chromosomal DNA damages in *M. psychrotolerans* cells. In Chapter 1, a large amount of histamine accumulation by *M. psychrotolerans* was observed, when the viable count of *M. psychrotolerans* reached 10^7 CFU/mL or CFU/g. The highest population reduction of *M. psychrotolerans* was observed in washing with PAA from the saury surface. Therefore, the results suggested that treatment with PAA during the fish washing process could extend the shelf life of stored fish for improving fish safety. PAA as a cleaning disinfectant for washing seafood has not been allowed in Japan and China. The findings of this Chapter can provide a useful information for improving seafood

safety and developing effective strategies to reduce the risk of histamine poisoning caused by *M. psychrotolerans*.

Chapter 3

Sanitizing efficacy of organic acid on reduction of *M. psychrotolerans*

The PAA showed good efficacy on the reduction of *M. psychrotolerans* in Chapter 2. However, customer demands natural, healthy, safe products, and environment-friendly processing steps in recent years. Natural and safer sanitizer should be developed in response to the customer's demands. Organic acids have been designated as generally recognized as safe (GRAS) materials for food products by the FDA. And the organic acid treatments are cheap, fast, simple, and also have good efficiency to improve safety and quality of food. In this Chapter, the antimicrobial activity and the mode of action of organic acids against *M. psychrotolerans* were investigated. Also, combination of PAA and LA also proposed as a novel sanitizing method against *M. psychrotolerans* on surface of fish.

Materials and methods

3-1. Cultivation of bacterial strains

M. psychrotolerans JCM 16473^T was cultured in TSB at 25 °C for 24 h. *M. morganii* NBRC 3848^T and *Enterobacter aerogenes* NCTC 10006^T were cultured in TSB at 30 °C for 24 h. *P. damsela* JCM 8968 and *P. phosphoreum* NBRC 103031^T were cultured in TSB supplemented with 1.5 % NaCl at 25 °C and 20 °C for 24 h, respectively. The bacterial cultures were centrifuged at 10,000 × g for 2 min at 4 °C. And then, the harvested cell pellets were washed twice with 0.01 M PBS (pH 7.2) for subjecting to experiments described below.

3-2. Evaluation of antibacterial activity of organic acids

The antibacterial activity of organic acids was tested against some histamine-producing species *in vitro*. The washed bacterial cells were resuspended (about 6×10⁸ CFU/mL) in 0.01 M PBS and mixed with different concentration of organic acids (Table 3-1). Sterile deionized water was used as a control. After treatment for 1, 3, or 5 min, 0.1 mL of treated suspension immediately mixed with 0.9 mL of 0.1 M PBS (pH 7.2) to neutralize organic acid. The neutralized samples were then serially diluted (1:10) with 0.01 M PBS (pH 7.2) and 0.1 mL from each dilution were spread on plate of TSA

supplemented with 0.2 % sodium pyruvate. The survivor colonies of *M. psychrotolerans*, *M. morganii*, and *E. aerogenes* were counted after incubation at 25 °C and 30 °C for 72 h. The counts of *P. phosphoreum* and *P. damsela* were determined with plate culture on TSA-SC at 20 °C and 25 °C for 72 h, respectively.

3-3. Measurement of membrane potential

Exponential growth phase cells of *M. psychrotolerans* were prepared, and the bacterial cells were washed by 5 mM HEPES buffer. Then, it was suspended in 5 mM HEPES buffer supplemented with 5 mM glucose and 0.2 mM EDTA. The membrane potential was measured using the fluorescence probe DiSC₃(5) (TCI) (Liu *et al.* 2016). DiSC₃(5) was added to the cell suspensions to final concentration of 0.5 µM, and held for 15 min at room temperature. 100 µL of the cell suspensions was poured into black microplate wells (AS ONE), and then 100 µL of 2 % LA, 1 % LA, and 2 % AA solutions were added to the wells. 1 µM valinomycin and 1 µM nigericin (Wako Pure Chemical) were used as positive and negative controls, respectively. Valinomycin depolarizes cell membrane. Nigericin as the negative control has little effect on cell membrane charge. The fluorescence intensity was measured at excitation and emission wavelengths of 622 and 670 nm, respectively (Infinite 200 microplate reader, Tecan).

3-4. Measurement of intracellular pH

The determination of intracellular pH in bacterial cells was based on the method of Breeuwer *et al.* (1996). Briefly, the cells were harvested by centrifugation at 4°C and 6,000×g for 5 min, washed and resuspended in 5 mM EDTA-50 mM potassium HEPES buffer (pH 8.0) to OD_{660nm} of 0.6. The cells were stained with 0.3 µM CFSE (Dojindo) and incubated for 20 min at 30 °C. Then, the cells were washed twice with 10 mM MgCl₂-50 mM potassium phosphate buffer (pH 7.0) and resuspended in 10 mM glucose-10 mM MgCl₂-50 mM potassium phosphate buffer (pH 7.0), and incubated for 30 min at 30°C.

After incubation, the cells were washed twice with 50 mM phosphate buffer (pH 7.0) and resuspended in different pH buffer for the calibration curve (different pH buffer: 50 mM glycine, 50 mM citric acid, 50 mM Na₂HPO₄, 50 mM KCl adjusted with NaOH/HCl, pH 1–8). The intracellular pH (pH_{in}) and extracellular pH (pH_{out}) were equilibrated by adding 1 µM valinomycin or 1 µM nigericin.

To measure the intracellular pH after treated, LA and AA (sterile deionized water as control) were added to stained cells after the cells were washed with 50 mM phosphate buffer (pH 7.0). The fluorescence intensities at emission 525 nm were measured at excitation 490 nm and 440 nm using a spectrofluorophotometer (RF6000, Shimadzu). The pH_{in} was determined from ratio of fluorescence intensity (pH-sensitive) at excitation 490 nm to that (pH-insensitive) at excitation 440 nm. To eliminate the background, the cell mixture was filtered through 0.22 µm filter for fluorescence

measurement, and the fluorescence of the filtrate was subtracted from that of cell mixture.

3-5. Measurement of intracellular and extracellular ATP

The late exponential growth phase of bacterial cells was washed and resuspended in 0.01 M PBS buffer (pH 7.2). The cell suspensions were exposed to LA and AA solutions for 1, 3 or 5 min at room temperature. Then, the cells were harvested by centrifugation at 4°C and 10,000×g for 2 min. The cell pellets were washed and resuspended in 0.01 M PBS buffer (pH 7.2) for measuring the intracellular ATP by bioluminescence using CheckLite 250 kit and Lumitester C-110 (Kikkoman). For assaying the extracellular ATP, the cell suspensions were mixed with 9-fold volumes of 0.1 M PBS for neutralization after the treatment, and centrifuged at 4°C and 6,000×g for 2 min, then, the supernatant were measured by CheckLite 250 plus kit and Lumitester C-110. Calibration curves for intracellular and extracellular ATP were prepared according to the manufacturer's instructions.

3-6. Evaluation of sanitizing effect on saury surface

The sanitizing effect of LA, PAA and LA combined with low concentration of PAA on *M. psychrotolerans* from saury surface was determined as described in 2-3. After frozen saury was thawed, saury was washed three times with 9-fold volumes of distilled

water and then submerged in *M. psychrotolerans* culture suspension (1:9 w/v, about 10^9 CFU/mL) for 5 min. The inoculated sauries were air-dried in a biological safety cabinet for 12 min (initial inoculum size: about 6 log CFU/cm²). The samples were then dipped for 1 or 5 min into 9-fold (w/v) volumes of the following sanitizer solutions: 10 ppm PAA, 2 % LA, and PAA (10 ppm) combined with LA (2 %). Deionized water was used as control. The saury surfaces were then wiped with a 10 mL dilution attached swab test. *M. psychrotolerans* populations were determined with plate count on Niven's agar at 25 °C for 72 h.

Results

3-1. Effects of organic acid on reduction of *M. psychrotolerans* *in vitro*

In vitro disinfectant effects of different organic acid treatments against *M. psychrotolerans* are shown in Table 3-1. The treatment with 2 % TA and 2 % CA for 5 min reduced the bacterial populations by 0.71 and 0.88 log CFU/mL, respectively. For treatment with 2 % AA and 2 % LA for 5 min, the bacterial populations were decreased by 6.4 and 8.7 log CFU/mL, respectively. 2 % LA treated group showed a notable reduction by 8.7 log CFU/mL even for 1 min treatment. 1 % LA and 0.5 % LA groups also showed significant reduction for 5 min treatment. The antibacterial effects of all organic acids used in this study can be ordered as 2 % LA > 1 % LA > 0.5 % LA > 2 % AA ≥ 2 % CA ≥ 2 % LVA ≥ 2 % MA ≥ 2 % TA (Table 3-1).

For LA was confirmed to have higher antibacterial effect on *M. psychrotolerans*, effect of LA against other histamine-producing bacteria was also evaluated. The efficacies of LA on reduction of *M. morganii*, *E. aerogenes*, *P. damsela*, and *P. phosphoreum* are shown in Fig. 3-1. 2% LA treatment significantly decreased the population of all histamine-producing bacteria to undetectable level (<10² CFU/mL) within 3 min.

3-2. Membrane potential of *M. psychrotolerans* treated with lactic acid (LA) and acetic acid (AA)

Membrane potential ($\Delta\psi$) of *M. psychrotolerans* cell was determined with a $\Delta\psi$ -sensitive fluorescence probe, DiSC₃(5). DiSC₃(5) accumulates in intact cells and becomes self-quenched. If cell membranes were damaged by antimicrobial compounds, DiSC₃(5) was released from the membranes, resulting in an increase in fluorescence (Moon and Huang 2019). As shown in Fig. 3-2, the fluorescence intensities rapidly increased after treatment with 1-2% LA and 2% AA on *M. psychrotolerans* cells. This indicates that membrane depolarization on the bacterial cell was led by LA and AA treatments.

3-3. Intracellular pH (pH_{in}) of bacteria treated with LA and AA

Calibration curve of Ratio_{490/440} vs. intracellular pH was fitted by an equation with correlation coefficient of over 0.95 as shown in Fig. 3-3A. This indicates that CFSE as a fluorescence probe is stable and applicable to measure pH_{in} of *M. psychrotolerans* under the experimental condition.

A clear change in pH_{in} was found after treatment with 1-2% LA and 2% AA (Fig. 3-3B). The pH_{in} in control was 6.7. The LA- and AA-treatments caused a significant drop in pH_{in} of *M. psychrotolerans* within 1 min. After then, the pH_{in} showed no change. Actually, the pH_{in} after 2 % AA treatment dropped from 6.7 to 4.1, and the pH_{in} after

1 % and 2 % LA treatments dropped to 3.8 and 3.5, respectively.

3-4. Effect of LA and AA on intracellular and extracellular ATP

Changes in intracellular and extracellular ATP of *M. psychrotolerans* during LA and AA treatments were shown in Fig. 3-4. The intracellular ATP in *M. psychrotolerans* was gradually decreased during treatment with LA throughout the exposure time. And the intracellular ATP greatly decreased at higher LA concentration (2% LA). After exposure to 2 % LA for 5 min, the level of intracellular ATP was decreased from 384 nmol/L to 50 nmol/L. For 2 % AA treatment, the intracellular ATP level slightly decreased from 384 nmol/L to 367 nmol/L for 1 min and then it was kept for 5 min (Fig. 3-4A). For the extracellular ATP, the control group was around 0.3 nmol/L. Treatment with 1 % LA and 2 % LA caused extracellular ATP level to increase to 4.27 nmol/L and 5.26 nmol/L at 1 min, respectively (Fig. 3-4B). The results suggested that the intracellular ATP consumption rate was higher than the leakage of ATP out of the cells.

3-5. Effect of sanitizer on *M. psychrotolerans* populations from saury surface

Figure 3-5 shows the sanitizing effect of LA and PAA treatment on *M. psychrotolerans* populations from saury surface. The populations of *M. psychrotolerans* showed reduction of around 0.66, 1.49, 2.87 and 1.88 log CFU/cm² after treatments for 1 min with 10 ppm PAA, 2 % LA, 2 % LA-10 ppm PAA combination and 200 ppm

PAA, respectively. Especially, the drastic reduction of *M. psychrotolerans* population occurred in 2 % LA-10 ppm PAA combination group, which showed 3.4 log CFU/cm² reduction after 5 min-treatment. This result was similar to the reduction obtained for treatment with 200 ppm PAA for 5 min.

Table 3-1. Reduction of *M. psychrotolerans* population with organic acid treatment *in vitro* (log CFU/mL)

Treatment	Treatment time (min)		
	1	3	5
2 % TA	0.22±0.03	0.38±0.07	0.88±0.09
2 % MA	0.27±0.13	1.56±0.58	8.11±0.98
2 % CA	0.31±0.13	0.48±0.06	0.71±0.12
2 % LVA	0.27±0.19	1.23±0.27	4.31±0.10
2 % AA	0.39±0.11	2.07±1.01	6.42±0.58
2 % LA	8.73±0.29	8.73±0.04	8.73±0.04
1 % LA	3.47±0.09	8.73±0.04	8.73±0.04
0.5 % LA	2.44±0.72	7.83±0.91	8.68±0.12

TA: L-tartaric acid; MA: DL-malic acid; CA: citric acid; LVA: levulinic acid; AA: acetic acid; LA: lactic acid

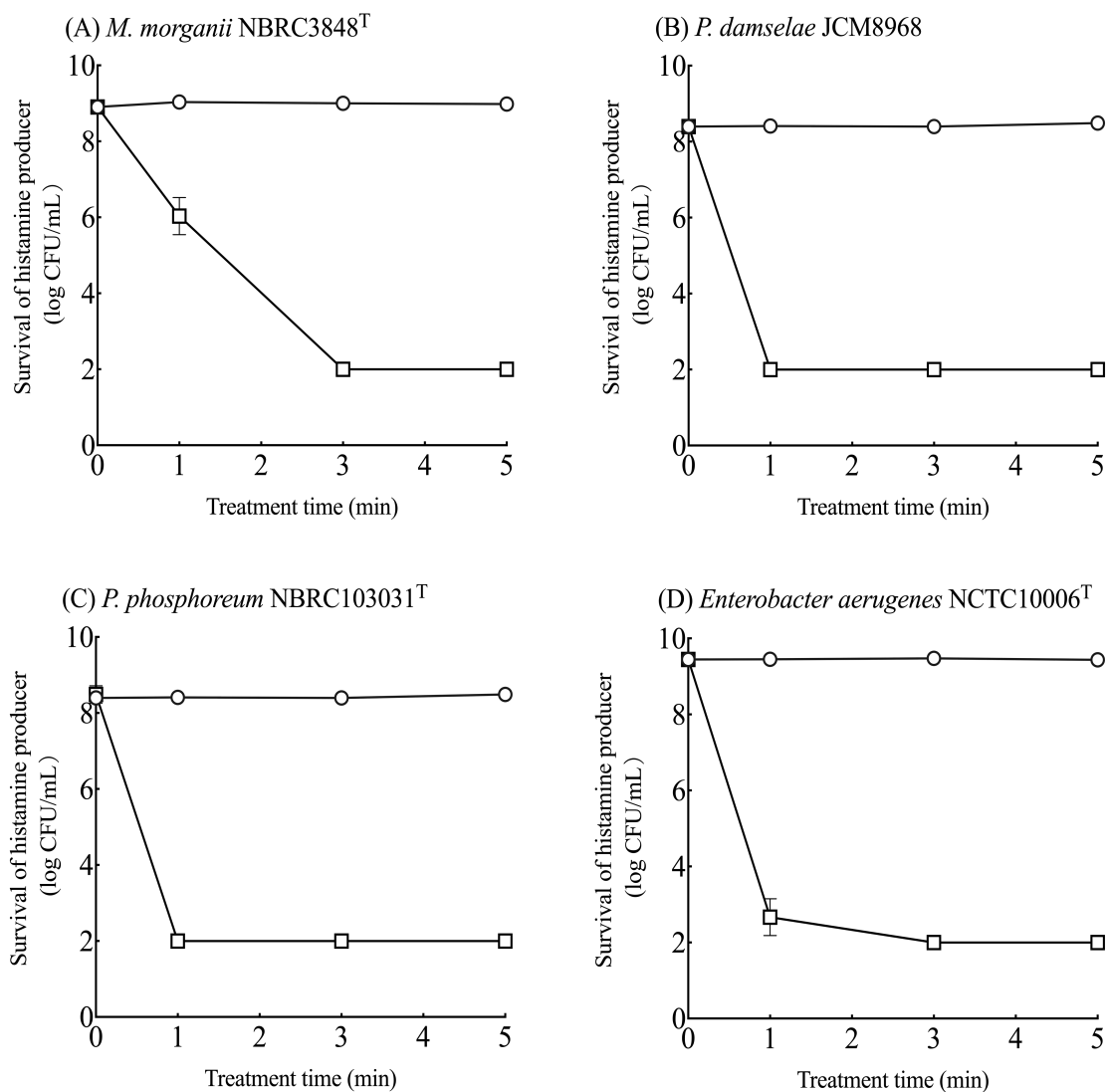


Fig. 3-1. Survival curves of histamine-producers (A, *M. morganii* NBRC3848^T; B, *P. damsela* JCM8968; C, *P. phosphoreum* NBRC103031^T; D, *Enterobacter aerogenes* NCTC10006^T) under treatment with 2 % LA *in vitro*.

Symbols represent control (○) and LA-treated (□). The minimum detection level was 10² CFU/mL. Data are shown as the mean ± standard deviation from three independent experiments.

Table 3-2. Chemical properties of organic acids used in this study

Organic acid	pH	pKa	Total concentration (mol/L)	Undissociated acid concentration (mol/L)	Undissociated acid fraction ^a (%)
2% TA	2.58	pKa ₁ 2.98 pKa ₂ 4.34	0.132	0.094	71.2
2 % MA	2.82	pKa ₁ 3.4 pKa ₂ 5.2	0.148	0.117	79.1
2 % CA	2.76	pKa ₁ 3.1 pKa ₂ 4.7 pKa ₃ 6.4	0.102	0.070	68.4
2 % LVA	3.41	pKa 4.59	0.012	0.011	93.8
2 % AA	3.35	pKa 4.75	0.346	0.333	96.2
2 % LA	2.92	pKa 3.86	0.225	0.199	88.4
1 % LA	3.11	pKa 3.86	0.113	0.093	82.3
0.5 % LA	3.39	pKa 3.86	0.006	0.004	74.7

^aThe undissociated concentration of organic acids, dibasic acid, and tribasic acid calculated by the equations:

$$\text{Monobasic acid: } [AH] = C_a \times [H^+] / ([H^+] + Ka)$$

$$\text{Dibasic acid: } [AH] = C_a \times [H^+]^2 / ([H^+]^2 + Ka_1[AH] + Ka_1 Ka_2)$$

$$\text{Tribasic acid: } [AH] = C_a \times [H^+]^3 / ([H^+]^3 + Ka_1[AH]^2 + Ka_1 Ka_2 [AH] + Ka_1 Ka_2 Ka_3)$$

where, [AH]: undissociated concentrations, C_a : total acid concentration (Giannuzzi & Zaritzky 1996).

^bUndissociated acid fraction (%) = (Undissociated acid concentration /total concentration)×100

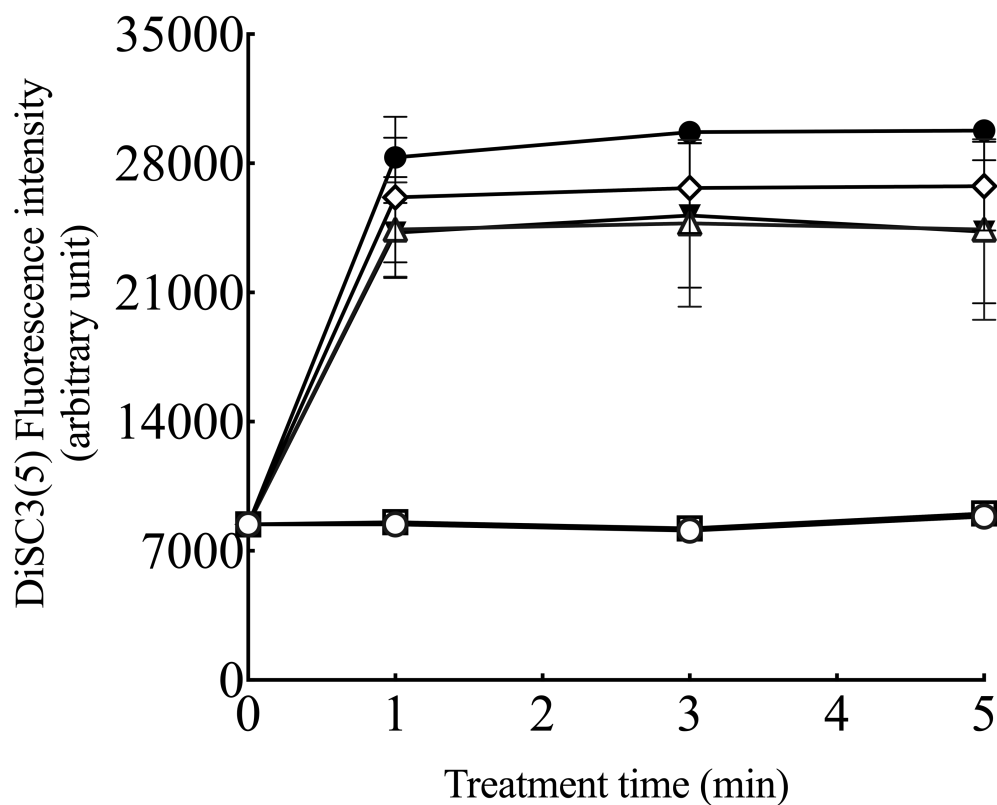


Fig. 3-2. Changes in the membrane potential in *M. psychrotolerans* cells treated with LA and AA.

Symbols represent control (sterile deionized water) (○), negative control (□), positive control (●), 2 % LA (△), 1 % LA (▼), and 2 % AA (◇). Data are shown as the mean $\pm$ standard deviation from three independent experiments.

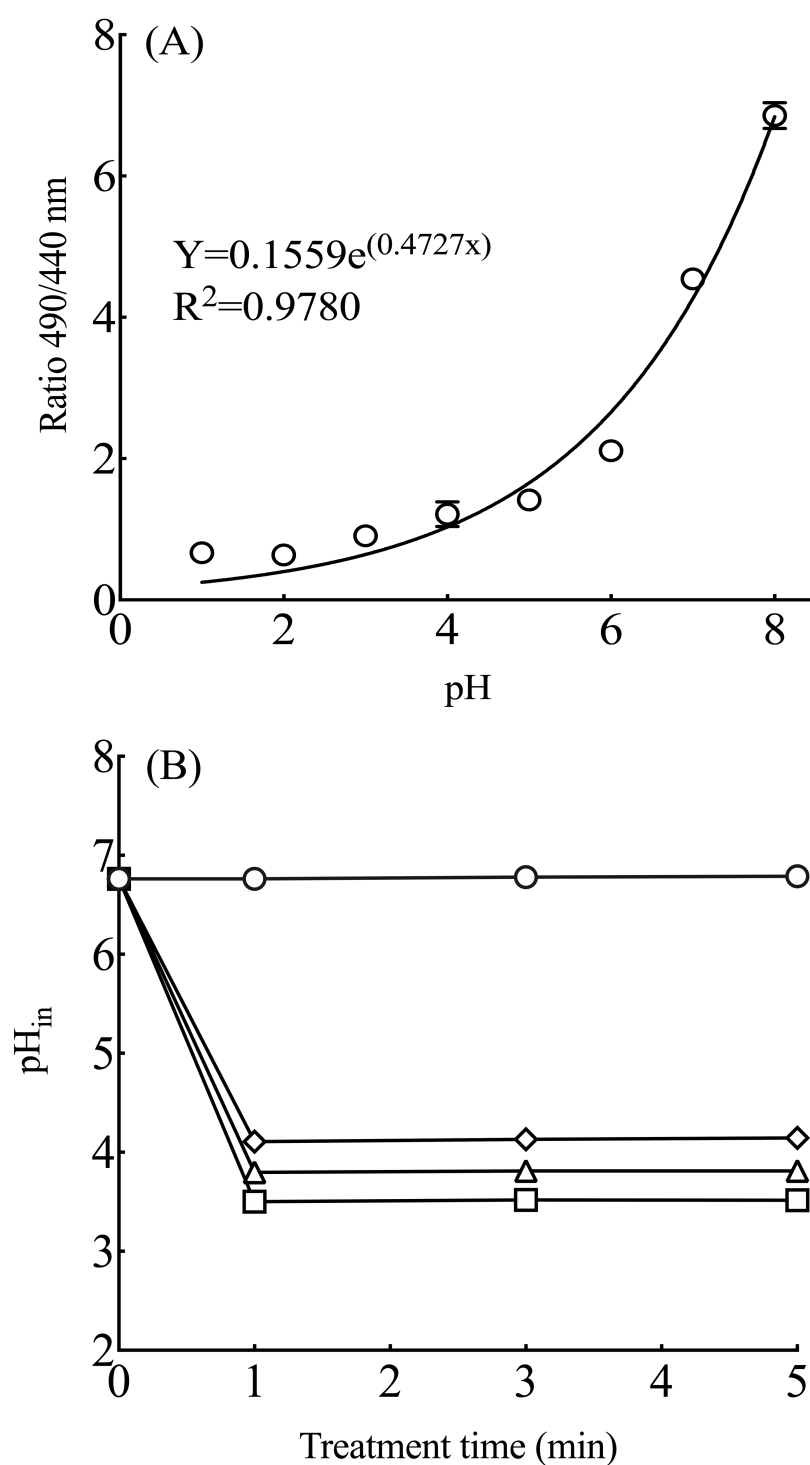


Fig. 3-3. The calibration curves of intracellular pH (A) and effect of LA and AA on the intracellular pH of *M. psychrotolerans* (B).

Symbols represent control (○), 2 % LA (□), 1 % LA (△), and 2 % AA (◇). Data are shown as the mean $\pm$ standard deviation from three independent experiments.

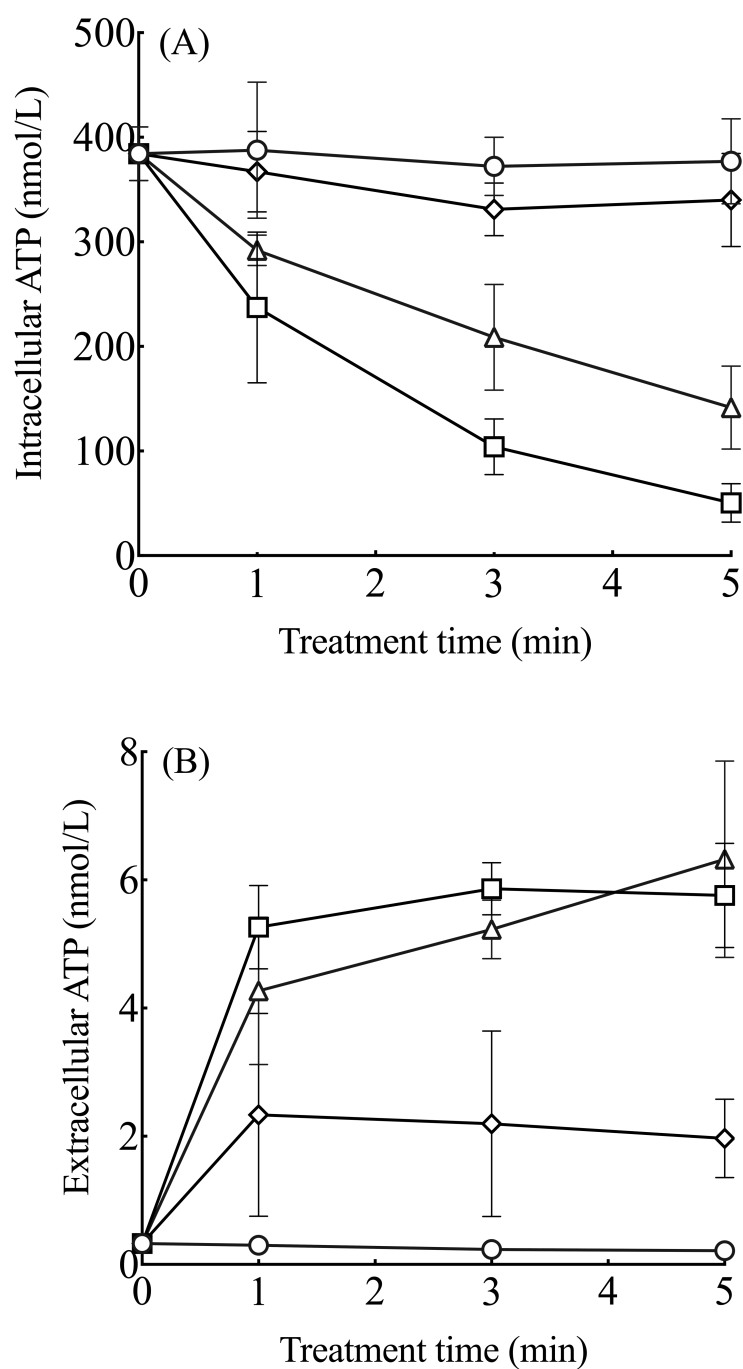


Fig. 3-4. Effects of LA and AA on intracellular (A) and extracellular ATP (B) of *M. psychrotolerans*.

Symbols represent control (○), 2 % LA (□), 1 % LA (△), and 2 % AA (◇).

Data are shown as the mean $\pm$ standard deviation from three independent experiments.

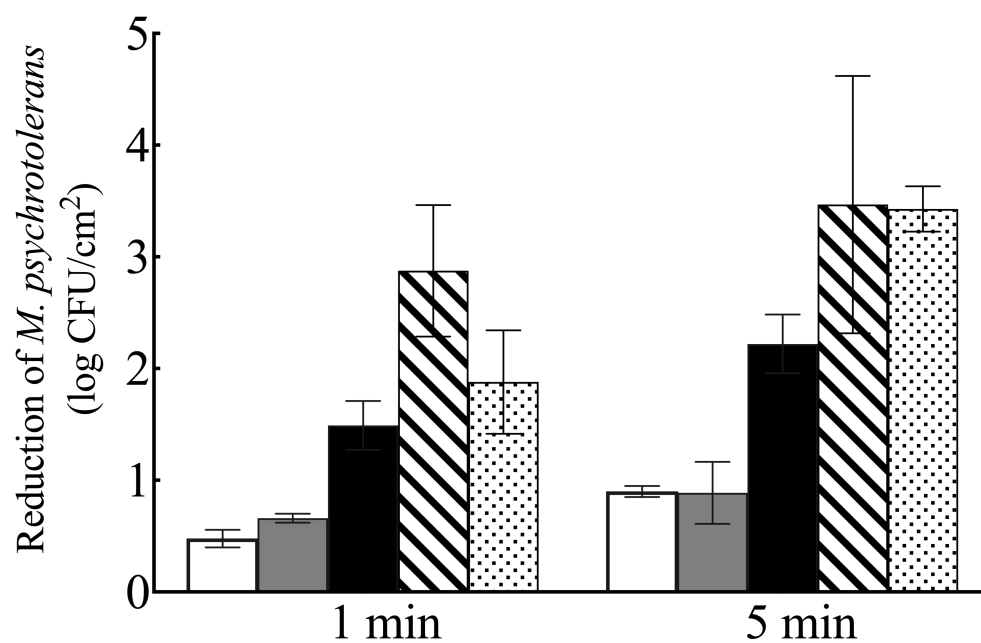


Fig. 3-5. Reduction of viable cell counts of *M. psychrotolerans* on saury surfaces after washing with sanitizers for 1 and 5 min.

Columns: white, control; gray, 10 ppm PAA; black, 2 % LA; slashed, 2 % LA - 10 ppm PAA; dotted, 200 ppm PAA. Data are shown as the mean $\pm$ standard deviation from three independent experiments.

Discussion

Organic acids have long been used to preserve foods and enhance food flavor due to their effectiveness as well as growing demand for natural and healthy preservative food products. In this study, the antimicrobial activity of organic acids against *M. psychrotolerans* was evaluated. 2 % LA showed an effective bactericidal activity against *M. psychrotolerans* (Table 3-1) and other histamine producer strains (Fig. 3-1). Especially, the viable cell counts of *M. psychrotolerans* sharply decreased to an undetectable level within 1 min by 2 % LA treatment. In previous papers, *L. innocua* populations were sharply decreased 5.69 log CFU/mL by 3 min treatment with 2 % LA (Chen *et al.* 2019), and 1 % LA could reduce *Enterobacter cloacae* cell counts by more than 2 log CFU/mL for 5 min (Liu *et al.* 2018). Results in this study also showed that *M. psychrotolerans* populations were reduced by 6.42 log CFU/mL after 2 % AA treatment for 5 min (Table 3-1).

E. coli could be inactivated by treating with 2 % LA for 30 min, and the antimicrobial mechanism of LA was associated with damage or disruption of cell membrane and cellular proteins, which causes a loss of cell function (Wang *et al.* 2015). However, the mechanism of rapid bactericidal action of LA against *M. psychrotolerans* has not been understood. Therefore, the changes in cell membrane potential, intracellular pH, intracellular and extracellular ATP were investigated to elucidate the strong antimicrobial mode of action of LA.

Resting membrane potential is important for the living bacteria cells (Shi *et al.*

2016a). LA and AA caused cell membrane depolarization for *M. psychrotolerans* (Fig. 3-2). This phenomenon was also found for *E. cloacae* using phenyllactic acid (Liu *et al.* 2018). Silverman *et al.* (2003) suggested that the cell depolarization might occur due to release of ions. The cell depolarization increased to similar levels by treatment with different concentrations of LA. *M. psychrotolerans* population was not rapidly decreased after 2 % AA treatment for 1 min, whereas AA is capable of rapid depolarization of the cytoplasmic membrane. These results suggested that membrane depolarization might not be a major bactericidal issue of organic acid. However, it was reported that depolarization could affect bacterial metabolism and cause the antimicrobial compounds more easily to insert into the cell (Moon and Huang 2019).

The intracellular pH (pH_{in}) is an important index of many cell metabolism processes such as DNA transcription, enzyme activities and protein synthesis (Kastbjerg *et al.* 2009). The pH_{in} of 2 % LA treated cells was lower than that of 2 % AA and 1 % LA (Fig. 3-3). Similarly, lipoic acid induced reduction of pH_{in} in *Cronobacter sakazakii* (Shi *et al.* 2016b). In general, organic acids exhibit stronger antimicrobial activity when environmental pH was lower. The pK_a of LA and AA are about 3.8 and 4.8, respectively. At the pK_a , the undissociated and dissociated molecules are equal in the solution. The pH of LA and AA solutions used in this study were lower than pK_a (Table 3-2). The undissociated acids can permeate through the cell membrane and dissociate in the cytoplasm and it will cause the intracellular pH rapidly to decrease (Lund *et al.* 2014).

After treatment with LA and AA, the intracellular ATP decreased. However, a higher decrease rate was observed under LA treatment than AA treatment at the same dose concentration. Moreover, the decreasing rate of intracellular ATP was greater than the increasing rate of extracellular ATP in LA treated groups. This phenomenon was also enhanced at higher LA concentration (Fig. 3-4). This might suggest that the ATP hydrolysis was faster than the ATP synthesis rate during LA treatment, or the ATP could not release from pores formed by LA treatment as implied by Turgis *et al.* (2009). Moreover, the bacteria need to spend ATP to maintain the homeostasis by pumping out dissociate organic acids, and if the dissociated organic anions are not excreted or consumed, it will cause the cells death (Cheng *et al.* 2015).

In this Chapter, LA showed dramatic efficacy on reduction of *M. psychrotolerans*. pH_{in} is an important indicator for individual bacterial health. Although the percentage of undissociated AA was greater than LA, the pH_{in} after treatment with AA was lower than LA (Table. 3-3) in this study. And it was found that decreasing rate of the intracellular ATP by treatment with AA was slower than LA. Therefore, it is hypothesized that the rapid lethal activity of LA was due to quick drop of intracellular pH and consumption of large amounts of ATP, which caused *M. psychrotolerans* cells to be unable to recover after LA treatment. However, the intracellular ATP was not significantly decreased by AA treatment, which might be involved in recovery of *M. psychrotolerans* cells.

In order to meet healthy, economic, and environmental protection demand, the

sanitizer of PAA or chlorite combined with organic acids has been used in vegetables and fruits in recent years. Rahman *et al.* (2011) reported that combination of 1 % CA and alkaline electrolyzed water reduced the populations of *L. monocytogenes* and *E. coli* O157:H7 by 3.97 log CFU/g and 4 log CFU/g from carrots, respectively. And 2 % LA combined with 5 ppm ozone showed a reduction of 2.8 log CFU/g of *L. monocytogenes* from blueberries after spray wash for 5 min (Tadepalli *et al.* 2019). However, combination of PAA and organic acids on seafood industry has not been reported. Of organic acids tested in this study, LA showed the most effective bactericidal activity against *M. psychrotolerans* (Table 3-1). Therefore, the sanitizing effect of combination of LA and PAA were investigated on saury surface. LA-PAA enhanced the reduction efficacy against *M. psychrotolerans* on saury surface, and reduction of *M. psychrotolerans* population with the combination of 2 % LA and 10 ppm PAA was similar to washed with 200 ppm PAA for 5 min (Fig. 3-5). This is so that the antimicrobial efficiency of PAA was enhanced with lowering pH by adding LA at low pH values, and LA caused the changes on the cell membrane (e.g. cell membrane potential) that allows more PAA to penetrate into cells (Silveira *et al.*, 2015). For this synergistic effect of LA and PAA, it was probable that the safety of seafood could be improved by the introduction of the combination of LA-PAA sanitizing strategy to the processing.

General discussion

Seafood has been increased to consume in past decades due to rich in nutritional composition (Seves *et al.* 2016). With expanding global trade, the risks and consequences of foodborne disease outbreaks increase. Currently, numerous outbreaks of foodborne illnesses associated with histamine-producing bacteria, *Vibrio* spp, *L. monocytogenes*, and *Clostridium botulinum* have been reported in food.

Histamine food poisoning is a major worldwide safety concern surrounding seafood consumption. The accumulation of histamine in seafood by histamine producing bacteria such as *M. psychrotolerans*, *M. morganii*, *P. phosphoreum* and *P. damsela* (Kato *et al.* 2017; Bjornsdottir-Butler *et al.* 2016b) at inappropriate storage or processing steps. Some reports have already shown the linked cases of histamine poisoning with seafood contaminated with *M. psychrotolerans* (Dalgaard *et al.* 2008; Emborg and Dalgaard 2006; Emborg *et al.* 2005). However, the histamine producing ability of *M. psychrotolerans* has not been well investigated.

In Chapter 1, the interest has been focused on the histamine production capacity of *M. psychrotolerans* under different environmental conditions, then the specificity of its crude HDC was screened under different conditions. And the expression of histidine decarboxylase gene, *hdc*, was also investigated to understand the characteristic of histamine accumulation by *M. psychrotolerans*.

Histamine concentration greater than 500 mg/kg is hazardous to humans (FDA 2011).

Although the low temperature and high salt concentration retarded the growth rate of *M. psychrotolerans*, the high level of histamine (>2611 mg/kg or 3500 mg/mL) was still accumulated by *M. psychrotolerans* under all the environmental conditions tested in canned tuna and culture broth (Figs. 1-1 to 1-6). Refrigeration temperature has been demonstrated as the most effective method to prevent histamine food poisoning in seafood (Phuvasate and Su 2010). The present study indicates that *M. psychrotolerans* can grow and accumulate histamine in seafood during storage even at refrigeration temperature. And once the histamine accumulates in seafood, there is a danger for histamine poisoning (Nitta *et al.* 2016). Hence, it is necessary to investigate the HDC characteristics to inhibit HDC activity. This study showed that the optimal temperature, pH, and NaCl concentration for histamine production by crude HDC of *M. psychrotolerans* were 30 °C, pH 7 and 0 % NaCl, respectively (Figs. 1-9 to 1-11).

As a means of inhibiting HDC activity other than the control of these conditions, the efficacy of tea catechins was evaluated. Of 4 major types of tea catechins, EGCG exhibited the highest inhibitory activity against *M. psychrotolerans* HDC (Fig 1-12). When EGCG concentration increased to 2 mM, the HDC of *M. psychrotolerans* almost lost the activity (Fig. 1-13). Nitta *et al.* (2007) also reported EGCG possessed inhibitory activity against recombinant human HDC. EGCG would be expected as an HDC inhibitor for preventing histamine food poisoning. Further research will be needed to clarify the relationship between chemical structure and inhibitory activity of catechins. It may also be necessary to confirm the effects of tea catechin on the growth and cell

functions including HDC-producing ability of histamine-producing bacteria.

From a genetic perspective, the expression of histidine decarboxylase gene *hdc* of *M. psychrotolerans* was induced by low pH conditions in this study (Fig. 1-14). All the findings in Chapter 1 confirmed that *M. psychrotolerans* had high histamine producing ability even at refrigeration temperature. And the previous study suggested that *M. psychrotolerans* is commonly distributed through retail seafood (Kato et al. 2017). Therefore, it has probable reason to believe that *M. psychrotolerans* is an important species causing histamine poisoning at low temperature.

Consequently, development of a suitable disinfection means is important for control of the growth and histamine formation of *M. psychrotolerans* in seafood. PAA has been suggested as an alternative sanitizer for chlorine (Van Haute et al. 2015) and it has been used as a disinfectant for washing fruits and vegetables (González-Aguilar et al. 2012). FDA has already approved to use PAA in seafood industry in USA. However, it has not been allowed to use for seafood in Japan and China. Hence, the supporting data for PAA application in seafood industry is needed. In Chapter 2, the sanitizing effect of PAA against *M. psychrotolerans* was compared to SH, which is a disinfectant commonly used in food industry. 20 ppm PAA and 40 ppm SH could similarly reduce the populations of *M. psychrotolerans* below the detection level by the treatment for 5 min *in vitro* (Fig. 2-1). For challenge test on fish (saury) surface, the treatment with 80 ppm PAA and 100 ppm SH for 5min showed about 2.23 and 1.99 log CFU/cm² reduction of *M. psychrotolerans*, respectively (Fig. 2-2). Therefore, PAA treatment for

washing fish surface will be an alternative method to SH treatment. Washing with 80 ppm PAA and 200 ppm SH for 2 min reduced the same levels of *L. innocua* populations from strawberry (Nicolau-Lapeña *et al.* 2019). Furthermore, 80 ppm PAA treatment showed effectiveness in reducing *Campylobacter jejuni* from chicken breast and it affected the chlorine resistant *C. jejuni* strain (Chen *et al.* 2020). These results proved that PAA also was a suitable alternative sanitizer to chlorine in the food processing industry. Meanwhile, the mode of antimicrobial action of PAA and SH was considered to damage of cell membrane and cell surface (Figs. 2-3, 2-4). However, both antimicrobial substances did not damage chromosomal DNAs in a practical concentration (Fig. 2-5).

In addition, concerns for consumer's demand for safer and natural additives have resulted in developing the potential sanitizing strategy. Organic acids are designated by the FDA as generally recognized as safe (GRAS) and organic acid treatments have shown clear antimicrobial efficiency against food pathogens (Mani-López *et al.* 2012). Therefore, a combination of organic acids and redox potential sanitizers was popular in recent years, and their combined use was better than the use of each compound alone (Ho *et al.* 2011).

First, the antimicrobial activity of organic acids against *M. psychrotolerans* was investigated in Chapter 3. LA showed an effective antimicrobial activity than AA against *M. psychrotolerans* (Table 3-2). 0.5 % LA showed more effectiveness on reduction in *E. coli* O157:H7 and *L. monocytogenes* population than 0.5 % AA from

leaves (Chhetri *et al.* 2019). Similarly, 1 % LA exhibited good disinfection effects than 1 % AA in reduction of the viable counts of aerobic bacteria, molds and yeasts on fresh-cut lettuce (Wang *et al.* 2018). Susceptibility of bacteria to the action of organic acids action might be related to undissociated species of acid, microbial tolerance to acid and physiological state of the test bacteria (Chhetri *et al.* 2019). Treatment with 1 % LA inactivated *E. cloacae* to minimum detection level within 15 min (Liu *et al.* 2018) and *E. coli* population was decreased to undetectable level by 2 % LA within 30 min (Wang *et al.* 2015). Viable cell counts of *M. psychrotolerans* decreased to undetectable level by treatment with 2 % LA for 1 min in this study. Mani-López *et al.* (2012) suggested that the rapid decrease in cell populations was not associated with acid adaptation. To elucidate the reason of strong antimicrobial effect of LA to *M. psychrotolerans*, changes in cell membrane potential, intracellular pH and ATP by LA treatment were investigated. The strong antimicrobial activity of LA might be related to sharp drop of the intracellular pH and ATP after LA treatment, and their values in *M. psychrotolerans* cells were decreased lower than those in the cells treated by AA (Figs. 3-3 and 3-4). Moreover, the cell membrane depolarization was detected in *M. psychrotolerans* after exposure to LA and AA (Fig. 3-2). Therefore, it was hypothesized that the rapid bactericidal effect of LA was not related to the membrane depolarization but it might be related to quick acidification of cytoplasm with subsequent uncoupling of energy production and regulation, and the dissociated acid anion accumulated to toxic levels (Mani-López *et al.* 2012). PAA sanitizer is a mixture containing acetic acid and

hydrogen peroxide, and has typical pungent odor and taste. However, LA showed the most effective bactericidal activity against *M. psychrotolerans* than AA (Table 3-1). The combination of LA and low concentration of PAA was proposed in this study. Combination of 2 % LA and 10 ppm PAA exhibited a good efficiency on the reduction of *M. psychrotolerans* population from saury surface, and the reducing of *M. psychrotolerans* population from saury surface by 5 min treatment showed a similar effect to 200 ppm PAA (Fig. 3-5). Previously a combined effect of PAA and LA for washing vegetables was also reported. PAA and LA have a surprising synergistic antimicrobial activity, and they also exhibited a reduction of cross-contamination in washing water (Ho *et al.* 2011). The enhanced antimicrobial efficacy of combined group might be due to action of LA for lowering pH and causing changes in bacterial cell membrane (e.g. cell membrane potential) that allows more PAA to penetrate into cells (Silveira *et al.*, 2015). These results will provide alternative insights into the choice of a proper processing sanitizer in seafood industry. Further research will be needed to observe the bacterial and physico-chemical qualities of seafood after LA-PAA washing during storage.

This study could help to understand the histamine producing ability of *M. psychrotolerans* and prevent the outbreak of histamine poisoning related to the psychrotrophic bacteria in seafood.

Summary

Histamine poisoning is a major seafood-borne disease, primarily caused by ingestion of seafood accumulating large amounts of histamine. *M. psychrotolerans* as a psychrotrophic histamine producer can form histamine even at 0 °C. In this study, histamine production properties and control strategies of *M. psychrotolerans* were investigated.

In Chapter 1, a comprehensive investigation has been conducted the histamine production ability of *M. psychrotolerans* in canned tuna at 20 °C and 4 °C and culture broth under pH 5–8 or NaCl 0–4 % at 20 °C and 4 °C. It was confirmed that *M. psychrotolerans* had high histamine-producing ability, especially showed high ability even at low temperature. Expression of histidine decarboxylase gene *hdc* of *M. psychrotolerans* was induced at low pH conditions. The optimal temperature, pH, and NaCl concentration for histamine production of crude HDC extracted from *M. psychrotolerans* cells were 30 °C, pH 7 and 0 % NaCl, respectively. The activity of the crude HDC at 10 °C retained 45% of the activity at 30 °C. Compared four kinds of tea catechins, EGCG exhibited the highest inhibitory effect on *M. psychrotolerans* HDC. 2 mM EGCG almost completely inactivated the crude HDC.

In Chapter 2, the sanitizing effect of PAA on *M. psychrotolerans* was compared to SH. 20 ppm PAA and 40 ppm SH could similarly reduce the populations of *M. psychrotolerans* below the detection level by the treatment for 5 min *in vitro*. And

treatment with 80 ppm PAA and 100 ppm SH treatment for 5min showed about 2.23 and 1.99 log CFU/cm² reduction of *M. psychrotolerans*, respectively. PAA showed to be more effective on reduction of *M. psychrotolerans* from saury surface than SH and it was proposed that PAA could be a better sanitizer for worldwide seafood processing to replace SH. The mode of antimicrobial action of PAA was related to damage the cell membrane and not to the DNA.

In Chapter 3, organic acids as natural antimicrobials were also evaluated for the antimicrobial activity against *M. psychrotolerans*. The antimicrobial activity of LA was greater than other organic acids against *M. psychrotolerans*. Moreover, 2 % LA showed significant antimicrobial activity against histamine producers *in vitro*. The antimicrobial action against *M. psychrotolerans* was related to depolarization of cell membrane potential, decrease in intracellular ATP and sharp decline in intracellular pH. The combination of 2 % LA and 10 ppm PAA had similar effect to 200 ppm PAA for reducing *M. psychrotolerans* population from saury surface at 5 min.

This study devises strategies for reducing histamine poisoning from the control of *M. psychrotolerans* growth and its HDC activity. In conclusion, potential HDC inhibitors such as EGCG and combined sanitizer of PAA and LA would be expected to prevent histamine poisoning and ensure safety of seafood during storage and processing.

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research.

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