Histamine production behaviors of a psychrotolerant histamine-producer, *Morganella psychrotolerans,* in various environmental conditions

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Acknowledgements

This work was supported by JSPS KAKENHI (Grant Number JP17K07931).

Compliance with Ethical Standards

Conflict of Interest

The authors declare that they have no conflict of interest.

Abstract

Histamine food poisoning is a major safety concern related to seafood consumption worldwide. *Morganella psychrotolerans* is a novel psychrotolerant histamine-producer. In this study, the histamine production behaviors of *M. psychrotolerans* and two other major histamine-producers, mesophilic *Morganella morganii* and psychrotrophic *Photobacterium phosphoreum,* were compared in seafood products, and histamine accumulation by *M. psychrotolerans* was characterized at various pH and temperature levels in culture broth. The growth of *M. psychrotolerans and P. phosphoreum* increased similarly at 4°C in canned tuna, but *M. psychrotolerans* produced much higher levels of histamine than *P. phosphoreum*. Histamine accumulation by *M. psychrotolerans* was induced at lower environmental pH condition at 4 and 20 °C. The optimal temperature and pH for producing histamine by crude histidine decarboxylase of *M. psychrotolerans* was 30 °C and pH 7, respectively. The activity of the crude HDC extracted from *M. psychrotolerans* cells at 10 °C retained 45% of the activity at 30°C. Histidine decarboxylase gene expression of *M. psychrotolerans* was induced by low pH conditions. These results suggest that *M. psychrotolerans* are also a very important histamine-producer leading to histamine poisoning associated with seafood below the refrigeration temperature.

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Introduction

Histamine fish poisoning is a foodborne toxicity caused by ingesting high levels of histamine [1]. 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 [2]. Poisoning symptoms include rash, headache, nausea, diarrhea, and so on [3]. Although many cases show mild symptoms, rare cases have shown a potential to threaten human life [4, 5]. Histamine accumulation in food is caused by histamine-producing bacteria that produce histidine decarboxylase (HDC), an enzyme that decarboxylates free histidine to histamine [6]. Once histamine is formed in food, it cannot be destroyed by common food processing treatments such as heating and freezing [7].

Histamine-producing bacteria are categorized into low and high histamine-producers. The bacteria producing more than 1,000 mg/L of histamine in culture broth are classified as high histamine-producing bacteria [8]. Generally, mesophilic bacteria such as *Morganella morganii*, *Photobacterium damselae*, and *Klebsiella pnuemoniae* are well documented as high histamine-producing bacteria in fishery products. These mesophilic histamine-producers will prevent forming histamine by controlling the storage temperature and time during the transports, processing [9]. However, psychrotrophic histamine-producers such as some *Photobacterium* spp. capable of produce histamine to a harmful level in seafood at

refrigeration temperature, and normal cold chain cannot completely suppress histamine production by them. *P. kishitanii* and *P. aquimaris* were recently isolated from fish [10], and *P. phosphoreum* caused histamine food poisoning incident in Japan [11].

M. psychrotolerans is a novel psychrotolerant histamine-producer, that can grow at 0–2 °C. Its biochemical characteristics are very similar to those of *M. morganii* [12]. Previous studies have documented the poisoning incidents caused by *M. psychrotolerans* [13, 14, 15]. In our previous study, we suggested that *M. psychrotolerans* is commonly distributed through retail seafood and accumulates histamine in culture broth during low-temperature incubation [16].

In this study, we compared the histamine production abilities of *M. psychrotolerans, P. phosphoreum,* and *M. morganii* in canned tuna and investigated the effect of environmental factors (pH and temperature) on histamine production by *M. psychrotolerans* in culture broth. The activities of crude HDC and *hdc* gene expression levels were also characterized.

Materials and methods

Bacterial strains and growth conditions

M. psychrotolerans JCM 16473^T and *M. morganii* NBRC 3848^T were inoculated in tryptic soy broth (TSB, BD, Franklin Lakes, NJ) supplemented with 1% L-histidine (Wako Pure Chemical Industries, Osaka, Japan) at 25 and 30 °C (TSBH, pH 6.0), respectively. *P. phosphoreum* NBRC 13896 was cultured in TSBH supplemented with 1.5% NaCl (Final

NaCl concentration in medium: 2%) at 25 °C. The cultures were incubated for 24 h for the experiments described below.

Inoculation with bacteria to tuna samples

Canned tuna (Hagoromo Food co. Ltd., Shizuoka, Japan) was purchased from a local supermarket. A 100 g of tuna was aseptically put into a stomacher bag (Seward Ltd., Worthing, U.K.). The precultured bacteria were centrifuged (10,000×*g*, 2 min, 4 °C) and washed twice with phosphate buffered saline (PBS, pH 7.2). After serial dilution by PBS, each bacterial suspension was inoculated to tuna samples (populations about 3 log CFU/g), and the samples were stored at 4 or 20 °C. A 5 g of the sample was aseptically weighted in a stomacher bag, homogenized in 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 tryptic soy agar (TSA, BD, Franklin Lakes, NJ), respectively. *P. phosphoreum* colonies was counted after incubating for 48 h at 25 °C on TSA-SC agar (TSA supplemented with 1.5% NaCl). Histamine contents were determined with the histamine test kit (Kikkoman Biochemifa Company, Tokyo, Japan), according to the manufacturer's instructions.

***M. psychrotolerans* growth and histamine production under different environmental conditions**

Pre-cultured *M. psychrotolerans* was washed and diluted with PBS. Then, 100 μL of the bacteria suspension was mixed with 9.9 mL TSB supplemented with 1% L-histidine (TSBH, pH 5 - 8, adjusted with 1M NaOH or HCl). The working culture (approximately about 5.5 log CFU/mL of an initial inoculum size) was incubated at 4 or 20 °C. At each sampling time, the bacterial population and the histamine concentration in the culture medium were determined.

Activity of Cell-free crude HDC under different environmental conditions

The 5 mL of precultured *M. psychrotolerans* 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 9.51 log CFU/mL in working buffer), and disrupted using zirconia beads (zircon prep mini, Nippon Genetics Co. Ltd., Tokyo, Japan) and a Bead Beater (2,500 rpm, 15 min, CD-1000, EYELA, Tokyo, Japan). 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, 0.1M citrate-0.2 M phosphate buffer) supplemented with 1% L-histidine. After 1 h incubation, the reaction terminated by heating at 100°C for 5 min. The activity of crude HDC was determined by checking the concentration of histamine in the solution at conditions ranging from pH 4-8 (30 °C) and 10-50 °C (pH 6.2). The crude HDC solution was directly used for the analysis of extraction.

M. psychrotolerans hdc gene expression by quantitative RT-PCR

Gene expression levels of *hdc* at different pH (5 - 8) and temperature levels (4 or 20 °C) were quantified by quantitative RT-PCR (qRT-PCR). The *hdc* and reference gene (16S rDNA) primers are shown in Table 1. Each primer was designed according to previously reported nucleotide sequences [6, 17]. RNA extraction was extracted using NucleoSpin RNA extraction kit (Macherey-Nagel GmbH, Germany) 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, UK). 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 Inc. USA). 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 reaction mixture for qRT-PCR contained 10 µL SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad Laboratory Inc., Hercules, CA, USA), 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, Foster City, CA, USA). For each target gene, the real-time PCR efficiency was calculated according to the equation $E = 10^{(-1/slope)}$. The gene expression level was calculated by the PfaffI method [18].

Statistical analysis

All the experiments were carried out in triplicate. The mean values and standard deviation were calculated from the triplicate results. The statistical analyses were performed using Tukey's *post hoc* range method. Significance level was based on 5% ($P < 0.05$). All statistical analyses were done using RStudio Desktop (RStudio Desktop, Inc., Boston, MA).

Results

Histamine accumulation in canned tuna

To evaluate the histamine production capabilities of *M. psychrotolerans* in canned tuna, we compared them with major mesophilic and psychrophilic histamine-producers, *M. morganii* and *P. phosphoreum.* Changes in viable cell counts and histamine concentration at 4 and 20 °C are shown in Fig. 1. At 20 °C, the growth curves of the three bacteria showed similar trends. The numbers of the viable cell count increased to more than 6 log CFU/g after 12 h from the initial counts, and exceeded 8 log CFU/g after 36 h (Fig. 1-A). Histamine concentrations in the samples inoculated with *M. psychrotolerans* and *M. morganii* exceeded 1,000 mg/kg after 36 h, and were 3,000 mg/kg after 60 h. However, histamine concentration in samples with *P. phosphoreum* was less than 400 mg/kg after 60 h (Fig. 1-B). 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 6 log CFU/g after 4 d, and reached to about 8 log CFU/g after 8 d. However, *M. morganii* did not grow at 4 °C (Fig. 1-C). Histamine production by *M. psychrotolerans* and *P. phosphoreum* were 1,467, 2,611

and 556, 883 mg/kg in canned tuna after 8 d and 10 d at 4 °C, respectively (Fig. 1-D).

Effect of pH and temperature on *M. psychrotolerans* growth and histamine accumulation in culture broth

The *M. psychrotolerans* growth and histamine accumulation at different pH and temperature levels are shown in Fig. 2. At 20 °C, the cell populations rapidly increased to approximately 9 log CFU/mL at pH 6, 7, and 8 after 24 h. The viable cells also reached 9 log CFU/mL at pH 5 after 36 h (Fig. 2-A). High levels of histamine (> 2,600 mg/L) were produced by *M. psychrotolerans* under all pH conditions after 36 h (Fig. 2-B). At 4 °C, the cell populations showed a rapid increase at pH 6 - 8, and the viable cell counts reached to 9 log CFU/mL after 6 d (Fig. 2-C). Significant histamine accumulations (> 1,700 mg/L) were observed after 6 d at pH 5, 6, and 7 (Fig. 2-D). The histamine concentrations increased with decreasing pH at both pH conditions (after 48 h and 8 d incubated at 20 °C and 4 °C, respectively).

Effect of temperature and pH on cell-free crude HDC activity

Effects of temperature and pH on crude HDC extracted from *M. psychrotolerans* cells are shown in Fig. 3. The results showed that the crude HDC exhibited the most active at 30 °C, and it showed about two-fold higher compared with those at 10, 20, and 40 °C. However, the HDC activity at 50 °C was 30% of the activity at 30 °C (Fig. 3-A). Also, crude HDC activity was greatly affected by the environmental pH. The maximum activity was shown at pH 7.

147 The HDC activities at pH 4, 5, 6, and 8 were 16%, 49%, 76% and 48%, respectively,
148 compared with that at pH 7 (Fig. 3-B).
149
150 **Effect of pH and temperature on *hdc* gene expression levels**
151 Changes of *hdc* gene expression at different pH and temperature levels are shown in Fig. 4.
152 The *hdc* gene expressions were also affected by the environmental pH. Expression levels
153 decreased with increasing pH, and the expression levels at pH 5 were 9.1-fold and 14.7-fold
154 higher at 20 and 4 °C, respectively, than the levels at pH 8.
155
156 **Discussion**
157 *M. morganii* and some of *Photobacterium spp.* like *P. phosphoreum*, *P. kishitanii* and *P.*
158 *aquimaris* are recognized as major mesophilic and psychrophilic histamine-producing
159 bacteria [11, 19], respectively. The effects of environmental factors on histamine
160 accumulation by psychrotolerant *M. psychrotolerans* have not been studied well yet.
161 Therefore, to avoid the histamine poisoning associated with seafood, in-depth studies on the
162 relations between environmental factors and the histamine accumulation by *M.*
163 *psychrotolerans* are needed. In this study, *M. psychrotolerans* and *M. morganii* produced
164 similar amounts of histamine at room temperature (20 °C) during the incubation. In addition,
165 *M. psychrotolerans* produced greater amount of histamine than *P. phosphoreum* at 20 °C (Fig.
166 1-B). Moreover, *M. psychrotolerans* produced higher amount of histamine (more than 1,000
167 mg/kg at 8 d) than *P. phosphoreum* (about 556 mg/kg) at low temperature (4 °C) (Fig. 1-D). *P.*

168 *phosphoreum* NBRC 13896 has reported that be classified as *P. kishitanii*, a more prolific
169 histamine producing bacteria at low temperatures [8]. And Bjornsdottir-Butler [8] reported
170 that *P. kishitanii* produced ≤ 1545 mg/L histamine in LSW-70 with 1% L-histidine at 20 °C
171 after 48 h. However, *M. psychrotolerans* produced ≥ 3700 mg/L histamine in TSBH at 20 °C
172 after 48 h in our study (Fig. 2-B). *Photobacterium spp.* as the marine bacteria, it requires salt
173 for growing, and the optimum activity for producing histamine was shown at 1-4% NaCl
174 concentration [20, 21]. On the contrary, Emborg *et al* [12] have reported *M. psychrotolerans*
175 can grow at pH 4.6 - 9.2, temperature 2 - 35 °C, and does not require salt for its growth. The
176 NaCl concentration of the canned tuna used in this study was about 0.14±0.01% (Data not
177 shown). The salt concentration of common raw fish muscle is about 0.2-0.4% [22, 23, 24].
178 This could be a reason that *M. psychrotolerans* showed higher ability for producing histamine
179 than *P. phosphoreum* NBRC 13896 in this study. In our previous study, *M. psychrotolerans*
180 has been found from 40.6% retail seafood in Japan [16]. *M. morganii* and *M. psychrotolerans*
181 exhibit very similar biochemical characteristics, and cannot be distinguished with the
182 API50CH-E kit (BioMérieux, France), commonly used for rapid bacterial identification [12].
183 Therefore, some of the isolates identified as *M. morganii* for a causative microorganism on
184 histamine outbreaks associated with seafood have been reasonably suspected to be *M.*
185 *psychrotolerans.*

186 Although no big differences were observed in the growth curves of *M. psychrotolerans*
187 under various pH conditions, the histamine accumulations were greatly affected by
188 environmental pH at both temperatures (Fig. 2). Namely, *M. psychrotolerans* produced high

amount of histamine at low environmental pH conditions (Fig. 2-B and D). These findings were consistent with the results found by Torres et al [25], who showed that the histamine production by *M. morganii* was reduced at higher pH than 6.6. Morii et al [21] have also reported the optimum culture pH for histamine production by *P. phosphoreum* to be 5.5 - 6.5.

Maximum crude HDC activity was observed at pH 7 (Fig. 3-B). Similarly, the HDC activity of other gram-negative histamine-producers, *M. morganii* [26] and *E. aerogenes* [27], was optimal at pH 6.5. The crude HDC activity of *M. psychrotolerans* at pH 4 was 18% of the activity at pH 7 (Fig. 3-B). However, the expression level of the *hdc* gene in *M. psychrotolerans* cells at low pH condition was much higher than that at neutral pH condition (Fig. 4). The decarboxylation of histidine by histamine-producers requires *hdc* gene expression and HDC enzyme synthesis in the cells. Moriii [21] reported the crude HDC from *P. phosphoreum* cells exhibited no activity at pH 4. Although *M. psychrotolerans* did not grow at pH 4 in culture broth, its crude HCD still showed an activity (Fig. 3-B). Hence, it indicates that the histamine-producing ability of *M. psychrotolerans* under low environmental pH is higher than that of *P phosphoreum*. The optimum temperature for the *M. psychrotolerans* crude HDC was at 30 °C, and the crude HDC maintained 45% of its optimum activity at 10 °C (Fig. 3-A). Bjornsdottir-Butler et al. [20] reported that the optimum activity of the HDC of *P. kishitanii* and *P. angustum* were at 30 °C. And Moriii [21] reported that the crude HDC from *P. phosphoreum* at 10 °C retained 10 % activity of the optimum temperature. The pH of fish muscle is 5.5 - 6.5 [25, 28]. Also, it is often adjusted with organic acids to inhibit the growth of microorganisms. From these findings, it can be argued 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. However, the crude HDC activity of *M. psychrotolerans* decreased at alkaline pH (pH 8) (Fig. 3-B). Corresponding with previous studies reported that the HDC activity from *M. morganii* [26], *P. phosphoreum* [29], and *Streptococcus thermophilus* [30] were greatly reduced at pH 8. Bjornsdottir-Butler et al [28] have shown an excellent strategy for reducing histamine accumulation in fish muscle: pH elevation by trisodium phosphate treatment. From the results of this study, pH elevation can be one of the effective methods to suppress the histamine production by *M. psychrotolerans* in seafood as well.

The *hdc* gene expression of *M. psychrotolerant* was greatly induced at acidic conditions at both 4 and 20 °C (Fig. 4). This phenomenon in *M. psychrotolerans* agrees with other histamine-producing bacteria. Many reports indicate that low pH is a necessary factor for inducing *hdc* gene expression in *M. morganii* [31, 32], *P. iliopiscarium* [1], *P. damselae* subsp. *damselae* [32], and *S. thermophiles* [33]. Histidine decarboxylase cluster of *M. morganii* is composed of *hdcTl* (putative histidine/histamine antiporters), *hdc*, *hdcT2* (putative histidine/histamine antiporters), and *hisRS* (histidyl-tRNA synthetase), suggesting that bacterial cells incorporate extracellular histidine and excrete histamine to extracellular environment [31, 32]. The initial acidic to neutral pH enhances the *hdc* gene expression, forming HDC and histamine, which is excreted from the cells. The bacterial cells sense the acidic pH conditions, which can turn the histidine over to histamine and CO_2. Thus, a large of H+ were eliminate and the bacteria could escape from acid stress through the decarboxylation

reaction of the amine as a survival strategy [31, 32, 34, 35, 36].

This study is the first report on the histamine production behaviors of *M. psychrotolerans* in various environmental conditions and characterization of the crude HDC activity and *hdc* gene expression level of *M. psychrotolerans*. Our results indicate that *M. psychrotolerans* has a higher capacity to produce histamine toxicologically in seafood under low temperature than *P. phosphoreum.* Results of this study could be useful for improving seafood safety and developing effective strategies to reduce a risk of histamine poisoning by *M. psychrotolerans*. However, further studies are needed to construct suitable strategies, especially for disinfection and suppression of *M. psychrotolerans* growth in seafood.

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382

383 **TABLE AND FIGURE LEGENDS**

384

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

386

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

388 4 °C (C, D). Symbols represent *Morganella psychrotolerans* (◯), *M. morganii* (▢), and

389 *Photobacterium phosphoreum* (△) for viable counts. Bars represent *M. psychrotolerans* (gray), *P.*

390 *phosphoreum* (white), and *M. morganii* (black) for histamine concentration. Dagger (†) indicates

391 histamine concentration less than 4 mg/kg. Means with different letters within the same incubation

392 time indicate significantly difference ($p<0.05$).

393

394 Fig. 2. The growth curve (A, C) and histamine production (B, D) by *M. psychrotolerans* at 20 °C (A, B)

395 and 4 °C (C, D). Symbols represent pH 5 (◯), pH 6 (▢), pH 7 (△), and pH 8 (◇) for viable counts.

396 Bars represent pH 5 (white), pH 6 (gray), pH 7 (black), and pH 8 (slash) for histamine concentration.

397 Dagger (†) means less than 4 mg/kg of histamine concentration. Means with different letters within the

398 same incubation time indicate significantly difference ($p<0.05$).

399

400 Fig. 3. The effects of temperature (A) and pH (B) on histamine accumulation by the cell-free crude

401 histidine decarboxylase of *M. psychrotolerans*. Means with different letters within the same

402 temperature and pH indicate significantly difference ($p<0.05$).

403

404 Fig. 4. Relative expression of the histidine decarboxylase gene of *M. psychrotolerans* under various pH

405 conditions. Bars represent pH 5 (white), pH 6 (gray), pH 7 (black), and pH 8 (slash). Means with

406 different letters within the same temperature indicate significantly difference ($p<0.05$).

407

Fig. 1 Wang et al.

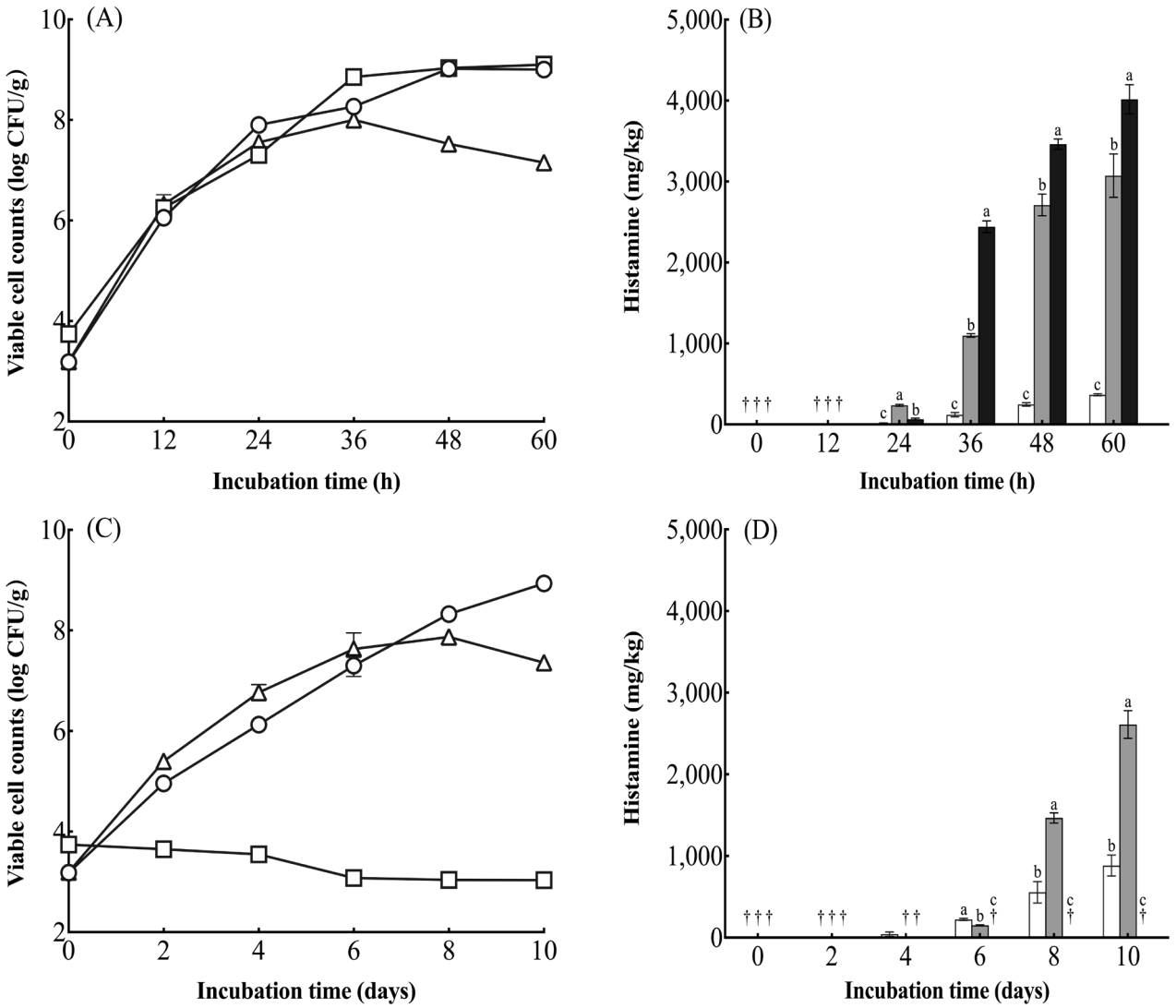

Fig. 2 Wang et al.

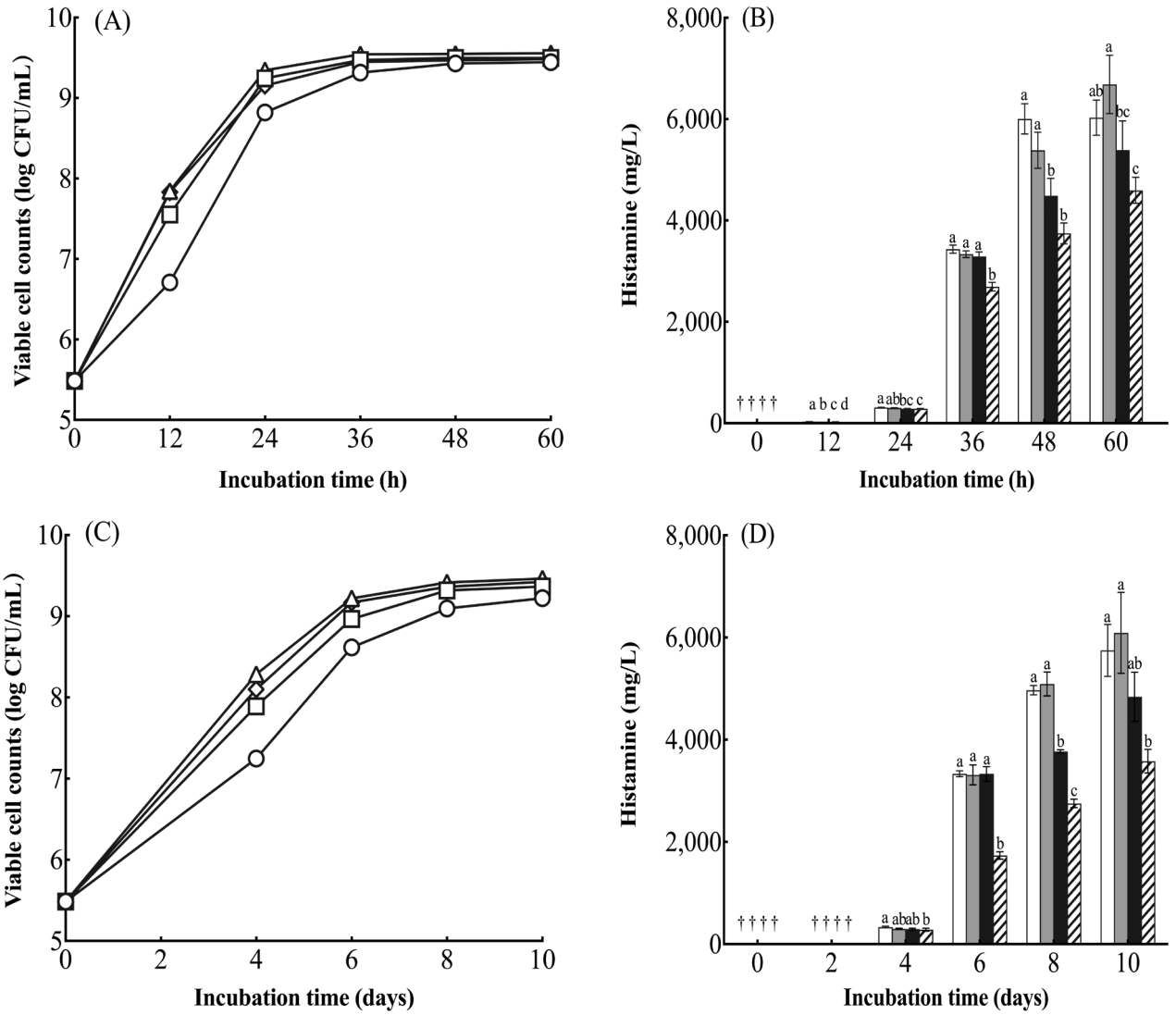

Fig. 3 Wang et al.

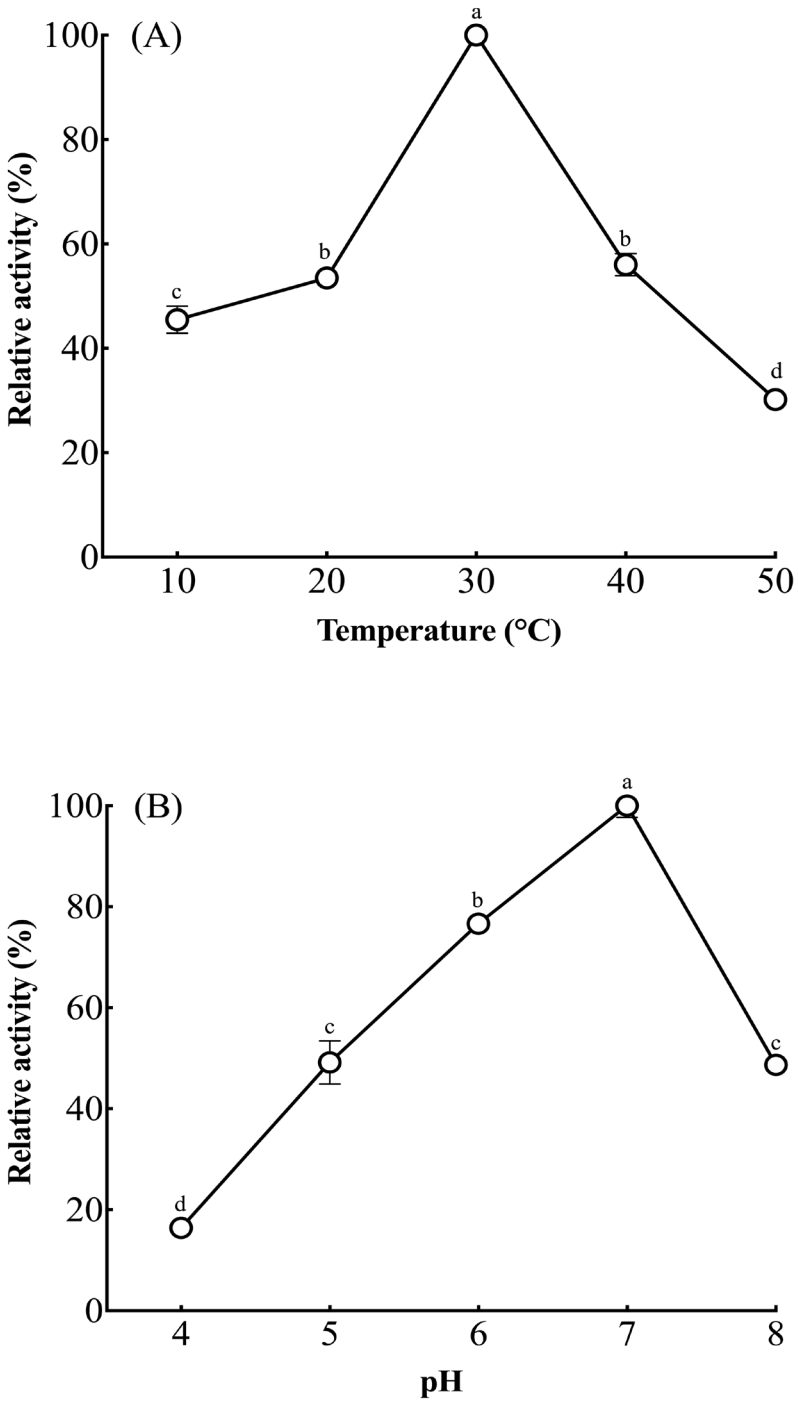

Fig. 4 Wang et al.

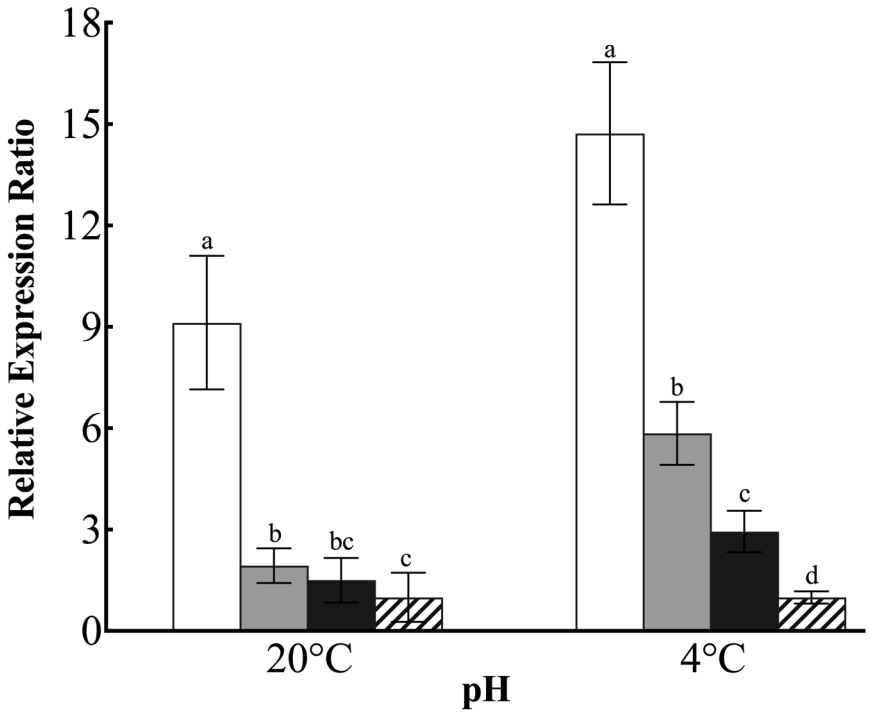

Table. 1 Wang et al.

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