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Isolation of bacteria capable of degrading histamine in fermented mackerel (*Scomberomorus guttatus*)

[1]Men, T.T., [2]Son, N.H., [1]Quynh, T.T., [1]Tai, V.P., [1]De, T.Q., [3]Van, T.T.B. and [3,*]Chau, T.T.

[1]*College of Natural Sciences, Can Tho University, Can Tho city, 94000, Vietnam*
[2]*Vinh Long University of Technology Education, Vinh Long city, 85000, Vietnam*
[3]*Institute of Food and Biotechnology, Can Tho University, Can Tho city, 94000, Vietnam*

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Abstract

Histamine, a biogenic amine, can be formed in foods via decarbonizing reactions of histidine and can cause health problems in consumers. Thus, histamine levels in foods, especially fermented seafood, are strictly controlled during harvesting, preserving, processing, and delivery. In this study, fermented mackerel (*Scomberomorus guttatus*) was collected and used to identify histamine-degrading bacteria. Five strains of bacteria grown in histamine medium were isolated, of which two strains (HF1 and HF2) showed the highest biomass in the 50 ppm histamine-supplied medium. After culturing for 24 hrs, the bacteria showed the highest activities at pH 7.0 and 30°C, and the efficiency of histamine degradation catalyzed by HF1 and HF2 strains was 34% and 31%, respectively. Biochemical characteristics investigation and 16S-rRNA sequencing of the HF1 indicated that the bacterial strain HF1 belonged to *Pseudomonas aeruginosa* and was identified as *Pseudomonas aeruginosa* HF1.

1. Introduction

Histamine, the biogenic amine, widely occurs in several foods that can poison consumers. The compound can be found in several foods, such as wine, cheese, and fish sauce (Stratton *et al*., 1991; Lonvaud-Funel and Joyeux, 1994; Kimura *et al.*, 2001; Hungerford, 2010). Fish and fermented fish contain histamine, which is formed by the decarbonization reactions of histidine, a natural amino acid in fish, under the effect of certain contaminated bacteria in foods (Ohtsu, 2010; Center for Food Safety and Applied Nutrition, U.S. Department of Health and Human Services, FDA, 2022). The substance is heat-resistant (*The Merck Index,* 1976) and cannot be destroyed by cooking. The toxication of histamine can cause allergic reactions with mid to severe symptoms, depending on the quantity of histamine. In some cases, heavily poisonous victims can be dead if they are not cured promptly (Zaman *et al.*, 2010). Thus, methods of reducing histamine to the lowest level have been paid attention to.

The application of histamine-degrading bacteria effectively reduces the quantity of histamine in fermented fish. Some recent studies have isolated bacterial strains capable of degrading histamine in fermented fish products. Some strains of bacteria capable of degrading histamine have also been isolated from fish sauce, such *as Staphylococcus xylosus, S. carnosus, Bacillus amyloliquefaciens, Arthrobacter crystallopoietes, Brevibacterium linens* (Kimura *et al.,* 2001; Hungerford, 2010; Tapingkae *et al.,* 2010; Zaman *et al.,* 2010; Lee *et al.,* 2016). Kung *et al.* (2017) also introduced four strains of *Lactobacillus plantarum* that can degrade histamine from Miso soybean sauce products. However, studies on the ability to degrade histamine of bacteria in the fermented mackerel (*Scomberomorus guttatus*) are still limited. Therefore, this study was carried out to isolate histamine-degrading bacteria from fermented mackerel and to investigate the influence of environmental conditions such as pH and temperature on the growth and development of potential bacterial strains to identify their characteristics.

2. Materials and methods

2.1 Samples of fermented mackerel

The fermented mackerel *(Scomberomorus guttatus*) was made by mixing the slice of mackerel, salt, and some dried rice starch, then incubated for 2 to 6 months. The fermented fish - qualified for Vietnamese National Standards (2010) - was collected at a traditional market in the Mekong Delta, Vietnam (10.026705160083612, 105.77673476093634). The samples were stored at 4°C, transported to the laboratory, and kept at -18°C for

*Corresponding author.
Email: *chautuan@ctu.edu.vn*

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further investigation.

2.2 Isolation of the histamine-degrading bacteria

Approximately 1 g of the homogenized fermented fish sample was added to the 10 mL of mineral medium containing 1 g/L glucose, 2 g/L yeast extract, 5 g/L NaCl, 0.5 g/L K_2HPO_4, pH 7.0±0.2, 1 g/L histamine dihydrochloride. The samples were circularly shaken and incubated at 30°C for 7 days. Then the incubated samples stood for 30 mins. The supernatant was used to isolate the histamine-degrading bacteria in a minimal mineral medium (1 g NaCl, 0.5 g NH_4Cl, 0.49 g K_2HPO_4, 0.375 g KH_2PO_4, 0.935 g $MgCl_2$, 0.015 g $CaCl_2$, 1000 mL H_2O) supplemented with 0.1% histamine dihydrochloride. Subsequently, colonies with differences in shape and size were selected for isolation by culturing on TSA medium (30 g/L tryptone soy broth; 15 g/L agar) for 3 days. Then the bacterial strains were identified by the colony and cell morphology; the gram stain and bacterial cell were observed by the optical microscope (Olympus BX51TF, Japan) at 1000× magnification.

2.3 Growth of isolated bacteria in the histamine-supplied medium

The colony of each bacterial strain was inoculated into the TSB medium (30 g/L Tryptone soy broth) by shaking at 200 rpm, at room temperature for 24 hrs, and the optical density at the wavelength of 600 nm (OD_{600nm}) was corrected to be 0.6. Approximately 10 µL of the treated sample was transferred to the minimum mineral medium supplemented with 50 ppm histamine dihydrochloride. The control sample was inoculated similarly to the above-described condition except for the addition of 50 ppm histamine (control medium). Then, the optical density of the bacteria was observed in the medium after 24 hrs of culturing.

2.4 pH and temperature effects on the growth of isolated bacteria

Potential bacterial strains were tested for growth in the histamine dihydrochloride supplemented medium with the variant pH values of 5, 6, 7, 8, and 9; at the temperature of 25°C, 30°C, 35°C, and 40°C. Samples were circularly shaken at 200 rpm and measured the OD_{600nm}.

2.5 Histamine-degrading capacity of isolated bacteria

After growing in the TSB medium, 1 mL of the bacterial suspension, corrected the OD_{600nm} to 0.6, was adjusted into 100 mL of TSB medium with 50 ppm histamine dihydrochloride. Samples were incubated at 30°C for 24 hrs and centrifuged to collect the supernatant. Then the residual histamine in the sample was analyzed by high-performance liquid chromatography (HPLC) and determined by fluorospectrophotometer from 400 to 600 nm with an excitation of 320 nm (Tahmouzi *et al.*, 2011).

2.6 Biochemical testing and identification of bacteria strains degrading histamine

Morphology, Gram staining, catalase, oxidase reactions, and 16S-rRNA sequencing were performed to identify the histamine-degrading bacteria. The primers of 27F (5-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTG-TTACGACTT-3') (Frank *et al*., 2008) were used to amplify the 16S-rRNA gene. The PCR reactions contained 12.5 µL master mix, 0.5 µL primers, and 5 µL DNA, filled up to 25 µL with water. The reactions were carried out at 95°C (6 mins), 40 cycles - 5°C (30 s), 55°C (30 s), 72°C (30 s), and 72°C (5 mins). Then, products were washed and examined by 1.5% agarose gel electrophoresis, measured the A_{260nm}/ A_{280nm}, and sequenced by the Sanger method (Blazej *et al*., 2006). The products were carried out the PCR, 96°C (1 min), 25 cycles - 96°C (10 s), 50°C (5 s), 60°C (4 mins) - with 10 µl of BigDye™ Terminator Cycle Sequencing Kit (Thermo Fisher Scientific Inc, Waltham, MA, USA), washed with ethanol and sequenced using HiDi Formamide. The products were determined nucleotide sequence by ABI PRISM 3130 Genetic Analyzer (Thermo Fisher Scientific Inc); the sequence was read by FastPCR Software (PrimerDigital Ltd, Helsinki, Finland) and checked for similarity with the gene data bank (Genbank) using the BlastN tool.

2.7 Data analysis

GraphPad Prism software (GraphPad Software LLC, USA) was used to store, process data, calculate and plot graphs. The software and the Minitab 16 software were used for ANOVA statistical analysis using Tukey's test. Data were shown as mean ± standard deviation (SD) of triplicates.

3. Results

3.1 Phenotypic characteristics of the degrading histamine enables bacteria

The results showed that five bacterial strains could grow in the medium supplemented with histamine from the fermented fish samples. The colony shape of the five isolated bacterial strains shows that four strains are round, and another is irregular (Table 1). One strain expresses opaque white, three strains are light, dark, and white yellow, respectively, and the other indicates translucent white, making the change of the medium color. All strains show the entire margin, raised form,

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Table 1. The morphological characteristics of colonies and cells of isolated bacterial strains after 72 hrs of culturing on TSA medium.

Sample	Strain	Colony characteristics						Cell characteristic	
		Form	Color	Elevation	Margin	Surface	Size (mm)	Shape	Gram
Fermented mackerel	HF1	circular	translucent white	raised	entire	smooth	0.2	Short rod	–
	HF2	circular	light yellow	raised	entire	smooth	0.1	cocci	–
	HF3	circular	dark yellow	raised	entire	smooth	0.2	cocci	–
	HF4	circular	White yellow	raised	entire	smooth	0.1	cocci	–
	HF5	irregular	opaque white	raised	entire	smooth	0.3	cocci	–

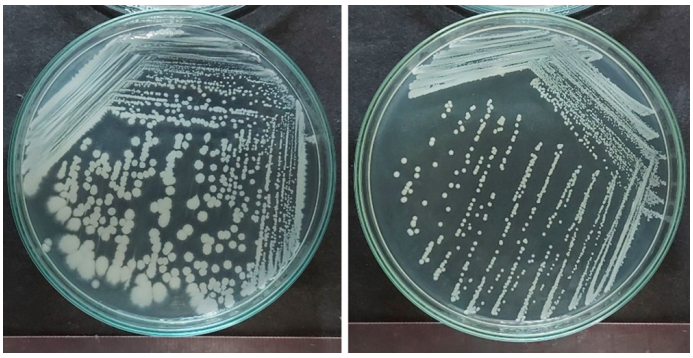

Figure 1. Colony morphology of some representative strains on TSA medium after 72 hrs of culture. The homogenized fermented fish sample was added to the 10 mL mineral medium, incubated, and isolated as described in materials and methods. The colonies with the difference in shape and size were selected for isolation by culturing on the TSA medium for 72 hrs.

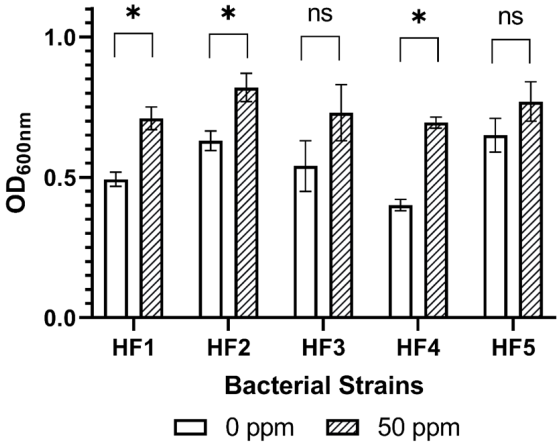

Figure 2. Differences in the growth of bacteria isolated in the medium with and without the addition of 50 ppm histamine dihydrochloride. Data are expressed as mean±SD of triplicate experiments and differences were expressed using t–test and ANOVA. *statistically significantly different ($P \leq 0.05$), ns: not statistically significantly different ($P>0.05$).

and smooth surface. The colony form and shapes of some bacterial strains are presented in Figure 1.

3.2 Growth ability of isolated bacteria in the histamine-supplied medium

After isolating the colonies, the strains were cultured in the TSB medium. The results found that the bacterial cells showed biomass after 24 hrs in the culture medium with and without the addition of 50 ppm histamine dihydrochloride. The results indicated the differences in the optical density of the bacteria in the control and 50 ppm histamine dihydrochloride media.

Figure 2 indicates that the isolated bacterial strains could grow in the 50 ppm histamine dihydrochloride medium after 24 hrs of culture. Among five investigated bacterial strains, the strains (HF1, HF2 and HF4) produced significantly different biomasses compared to incubated in the medium without histamine dihydrochloride. The remaining bacterial strains produced similar the biomass in both the sample and control media. The HF1 and HF2 placed first and second in biomass production capacity, respectively. Those are potential bacterial strains that could be able to degrade histamine and should be used to carry out further experiments.

3.3 Effects of pH and temperature on the growth of isolated bacteria

The experiments were done to find the effects of pH on the HF1 and HP2 bacteria, and the two bacterial strains presented different biomass in variant pH (Figure 3A). At pH 5.0, HF1 and HF2 grew and created biomass in the histamine dihydrochloride supplement after 24 hrs. The highest optical density of HF1 and HF2 was shown at pH 7.0 and reached the value of 0.869 and 0.907, respectively. Besides, the optical density did not differ significantly between the two bacteria.

Figure 3B displays the effect of the temperature of the histamine dihydrochloride medium on bacterial growth and showed that HF1 and HF2 had different biomass at a temperature from 25°C to 40°C. after 24 hrs of culture, HF1 reached the highest biomass when cultured at 30°C. The optical density at the wavelength of 600 nm was 0.916. In contrast, those of HF2 reached the maximal from 30°C to 35°C and from 0.680 and 0.701, respectively. The two bacteria' biomass was significantly different from those cultured at 25°C and 40°C. Thus, the optimum temperature for the growth of both strains was 30°C.

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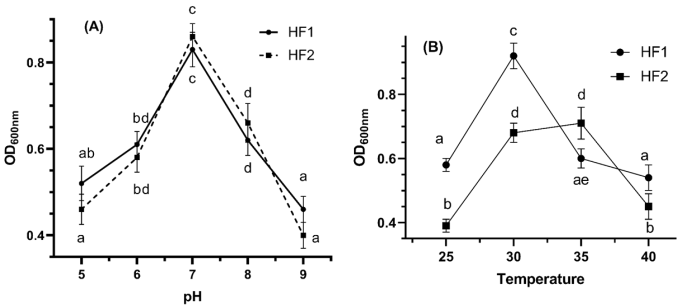

Figure 3. The growth of two bacterial strains, HF1 and HF2, under the effect of variant culture conditions: (A) effect of pH; (B) effect of temperature. Data are expressed as mean±SD of triplicate experiments and differences were expressed using t–test and ANOVA. Data with different notations are statistically significantly different ($P \leq 0.05$).

3.4 Histamine-degrading capacity of isolated bacteria

Based on the findings on the growing ability of bacteria, HF1 and HF2 strains were used to investigate the histamine-degrading ability in the TSB medium supplemented with histamine dihydrochloride. The HPLC and fluorescent methods were used to investigate the ability of HF1 and HF2 to degrade histamine. The results (Figure 4) indicated that the strains were able to resolve after 24 hrs of culture, and the degrading ability of HF1 and HF2, significantly different from the control without the addition of the bacteria, was 34% and 31% of the initial amount of histamine, respectively. Thus, it is concluded that the two strains were potential histamine -biodegraded bacteria.

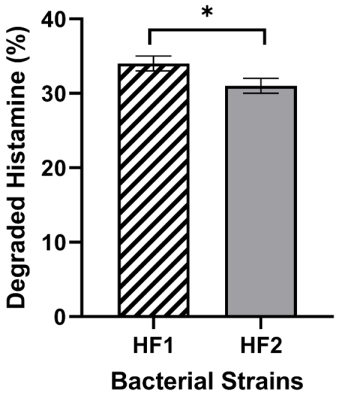

Figure 4. Histamine degradation capacity of HF1 and HF2 strain after 24 hrs of culturing. Data are expressed as mean±SD of triplicate experiments and differences were expressed using t–test and ANOVA. *statistically significant differences ($P \leq 0.05$).

3.5 Biochemical properties and strain of bacteria strains degrading histamine

The HF1 strain grew faster and degraded more histamine than those of the HF2 strain. Thus, the former was selected for 16S-rRNA sequencing. Then the HF1 bacterial strain sequences were compared with those of the NCBI Gene Bank using BlastN. Figure 5 presents that the 16S-rRNA sequence of the HF1 bacterial strain indicated a 99% homology with species of the genus *Pseudomonas*, in which the HF1 had the highest similarity with the bacteria *Pseudomonas aeruginosa* LGMT12 (99.02%). In addition, the observation result of biochemical characteristics of HF1 showed that it could assimilate citrate, hydrolyze gelatin, and produce catalase (Table 2). These characteristics coincided with *Pseudomonas aeruginosa* and were identified as *P. aeruginosa* HF1.

Figure 5. The tree of *Pseudomonas aeruginosa* produced by BlastN pairwise alignments.

Table 2. The biochemical characteristics of HF1 and HF2.

Strain	Characteristic reaction			
	Catalase	Citrate utilizing	Gelatinase	Mobility
HF1	+	+	+	+
HF2	+	+	+	+

4. Discussion

In the bacterial cell shape shown in Table 1, there are four bacterial strains with a spherical shape (80%) and a strain with a short rod shape (20%). Gram staining results showed that all stains are Gram-negative. The isolated bacteria varied in form and shape, meaning the Histamine-degrading bacteria were not diverse in the colony morphology.

Zaman *et al.* (2010) isolated bacterial strains of *Bacillus*, such as *B. amyloliquefaciens, B. subtilis, B. himi,* from fish sauce samples; all these strains were able to degrade histamine and also isolated the histamine-degrading bacterial strain from fish sauce, *S. carnosus*. The bacteria were able to degrade 29.1% of histamine compared to the initial. Kung *et al.* (2017) demonstrated the presence of four strains of *Lactobacillus plantarum* that degraded histamine in Miso soybean products. The bacteria were isolated from salted sardines using histamine dihydrochloride-supplied TSB medium. Lee *et al.* (2015) found eight strains of *Bacillus* spp. isolated from the samples in Taiwan showed the capacity of degrading histamine, in which *Bacillus polymyxa* D05-1 showed the ability to degrade histamine with 100% histamine degradation after 24 hrs of culture. In this

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study, five bacterial strains capable of degrading histamine were isolated from the fermented mackerel. Three bacterial strains of HF1, HF2, and HF4 showed high biomass growth in a TSB medium supplemented with 50 ppm histamine dihydrochloride, (Figure 2) consistent with previous studies on isolating the histamine-degrading bacteria from fermented products.

Previous studies have also shown the effect of pH on the growth of bacteria and histamine decomposition (Namwong *et al.,* 2005) and demonstrated that the optimal pH for histamine degradation of *Exiguobacterium profundum* strain was 7.0. In other studies, the ability of *Virgibacillus campisalis* to degrade histamine increased with pH from 6.0 to 9.0 and reached the optimal value from 7.5 to 8.0. *Bacillus polymyxa* had been exposing the ability to degrade histamine in the pH range from 4.0 to 10. Histamine was resolved with the maximal quantity at pH 7.0 (100%) (Lee *et al.,* 2012, Lee *et al.,* 2015). In this study, the HF1 and HF2 strains produced high biomass at pH 7.0, compatible with previous studies on the effect of pH on the growth of histamine-degrading bacteria (Figure 3A).

Previous studies have also shown the effect of temperature on bacterial growth and histamine breakdown. According to Namwong *et al.* (2005), *E. profundum* isolated from Thai fish sauce could effectively degrade histamine at the optimum temperature of 37°C. Another author (Lee *et al.,* 2012) demonstrated the ability to degrade the histamine by *V. campisalis*. This bacterial strain could grow at 15 to 40° C and optimally at 37°C. In this study, both HF1 and HF2 strains had high biomass growth at 30°C after 24 hrs of culture (Figure 3B), consistent with previous studies on the effect of temperature on the growth of histamine-degrading bacteria. It is the potential bacterial strain for applying thermophilic bacteria in treating histamine from fermented fish products.

Zaman *et al.* (2010) demonstrated that strains of *Bacillus* spp., and *Staphylococcus* spp., isolated from the fish sauce with up to 15% salt concentration, could degrade from 28.9 to 59.9% of the amount of histamine in the medium compared to that of initial. Research by Lee *et al.* (2015) introduced six histamine-degrading bacteria (75%) collected from salted fish samples belonged to *Bacillus,* including *B. cereus, B. polymyxa, B. licheniformis, B. amyloliquefaciens* and *B. subtilis,* among them, the degraded histamine ability of *B. polymyxa* D05-1 was 34% of the histamine content in the product. In this study, two bacteria, HF1 and HF2, were identified as histamine-degrading strains, with the HF1 strain showing the highest efficiency in degrading histamine, reaching 34%.

Histamine is formed via decarbonizing the amino acid histidine reactions by catalyzing L-histidine decarboxylase (Ohtsu, 2010). However, the amine can be destroyed by oxidation, methyl transferring, or dehydration pathways under the effects of enzymes such as diamine oxidase, histamine - N - methyltransferase, histamine oxidase, or histamine dehydrogenase (Biegański *et al.,* 1983; Dapkevicius *et al.,* 2000; Bakke *et al.,* 2005; Maintz and Novak, 2007).

Some authors (de la Torre *et al.,* 2018; Luengo and Olivera, 2020) found that the bacterial strain *Pseudomonas aeruginosa* can catabolize and degrade biogenic amines (BAs; 2-phenylethylamine, tyramine, dopamine, epinephrine, norepinephrine, octopamine, histamine, tryptamine, serotonin, agmatine, cadaverine, putrescine, and some fatty amines). These findings are associated with the result that *P. aeruginosa* HF1 was able to degrade 34% histamine (Figure 4) in the medium consisting of 50 ppm histamine under experimental conditions.

5. Conclusion

This study successfully isolated five bacterial strains that can degrade histamine in fermented mackerel and identified that the HF1 and HF2 showed the highest activity at the optimum conditions, pH 7.0 and 30°C and that their highest efficiency of degrading histamine was 34% and 31% in the 50 ppm histamine addition medium, respectively. Based on the sequencing results and identification of biochemical characteristics, the HF1 strain was identified as *P. aeruginosa* HF1.

Conflict of interest

The authors declare no conflict of interest.

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