

Isolation and Screening of Histamine-Producing Bacteria from the First Six Months of the Cat Hai Fish Sauce Fermentation Process

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Abstract

Histamine is considered to be a hazard in fish sauce, and histamine poisoning usually causes symptoms such as a runny nose, asthma (bronchospasm), urticaria, rash, itching, swelling (eyelids, puffy lips), inflammation, and redness of the conjunctiva. In this study, Cat Hai fish sauce, one of the major traditional fish sauce manufacturers in Vietnam, was used to investigate the variation in histamine content during the fermentation process and to isolate histamine-producing bacteria. Six Dich Chuop samples corresponding to the first sixth months from the beginning of fermentation were collected for these purposes. The results showed that the content of histamine in the six samples corresponding to the first six months from the beginning of fermentation tended to increase during fermentation, reaching the highest rate of 604.85 ppm in the fifth month. A total of 50 isolates were collected from TSA medium and used for screening histamine-producing bacteria on HBI medium. Among these bacteria, four isolates (CH2.4, CH3.3, CH4.4, and CH5.1) were capable of producing histamine, and the highest producing isolate, CH5.1 (from 5th month), was identified as *Tetragenococcus halophilus*. Furthermore, this *Tetragenococcus halophilus* was determined to have the highest histamine production in HBE supplemented with 1% histidine at 50°C, pH 6.0, and 25% NaCl.

Keywords

Cat Hai fish sauce, histamine, histamine-producing bacteria, fermentation process

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Introduction

Fish sauce is a protein solution that mainly consists of amino acids, and is used as a spice for cooking, sauce, or food in the daily meal (Fukami *et al.*, 2004). Vietnam has about 2,800 fish sauce

production facilities, producing more than 215 million liters per year, worth over VND 4,800 billion (Department of Agriculture, Forestry and Fisheries, 2014). However, Vietnam's fish sauce exports account for only about 3-5% of the production (Vietnam Online Quality, 2016). The main cause of the current low export volume is that there are no well-established brands of Vietnamese fish sauce overseas. In addition, another important issue is that traditional Vietnamese fish sauce often encounters technical barriers with some international quality standards. One of the important quality targets is histamine.

The traditional fermented fish sauce production process consists of two major stages, namely hydrolysis (primary fermentation) and odorization (secondary fermentation). In the primary fermentation stage, fish protein is hydrolyzed into amino acids. Fermentation helps to complete the hydrolysis process to form the flavor of the fish sauce. The traditional fish sauce production in each locality has different stages, but it follows a strict rule of thumb and usually lasts from 12 to 18 months.

Despite having a high nutritional value, the presence of biological amines in the fish sauce at high levels, particularly histamine, is a risk to human health (Askar *et al.*, 1993; Tsai *et al.*, 2007; Zaman *et al.*, 2010). Histamine is formed by the separation of the α -carboxyl group from the histidine amino acid. Histidine is one of the non-essential amino acids that the human body does not synthesize itself, and which must be obtained from food. Histidine is often found in foods such as fish, meat, eggs, and dairy products (Wickham, 2011). Histamine poisoning usually causes symptoms such as a runny nose, asthma (bronchospasm), urticaria, rash, itching, swelling (eyelids, puffy lips), inflammation, and redness of the conjunctiva (Mahidol *et al.*, 2003). According to Codex 302-2011, histamine is considered to be a hazard in fish sauce and the contents of this compound must not exceed 400 ppm. However, traditional Vietnamese sauce with histamine content ranging from 700-3000 ppm was reported (Union of Science and Technology in Binh Thuan, 2013). This leads

to a risk of unsafe use of fish sauce due to histamine poisoning.

There are three main methods of reducing histamine levels in fish sauce: physical, chemical, and microbiological methods. However, the physical and chemical methods have the disadvantages of high cost, difficulty in application, and unsafe methods for the users. Therefore, microbiological methods with the advantages such as ease of application, lower price, and safety become feasible methods to control the amount of histamine in fish sauce, but to apply these methods requires more research.

The aim of this research was to determine the changes in histamine content in Dich Chuop Cat Hai fish sauce during the first six months of fermentation and isolate and screen histamine-producing bacteria from its product. This data provide the premise for further research to inhibit histamine-producing bacteria in fish sauce from which to improve the quality and serve the increasing demand of consumers.

Materials and Methods

Materials

Samples collection

Cat Hai fish sauce is produced by the stirring method, and the fermentation time is one year. In this study, we sampled fish sauce in the first 6 months of fermentation to isolate histamine-producing bacteria.

Dich Chuop, fish materials (usually salted fish) decomposed through fermentation to make fish sauce, was taken from the Cat Hai Seafood Processing Service Joint Stock Company - Cat Hai Town - Hai Phong province. The samples were collected from the raw materials during the first month to the sixth month, and sampling occurred every month from the same tank. Dich Chuop samples were stored at 4-6°C in plastic bottles.

Media use

Three types of media were used in this present study as follows: HBI - Medium for screening histamine-producing bacteria (g L^{-1}):

5 g trypton; 5 g yeast extract; 10 g histidine; 0.06 g bromoresol purple, 1 g CaCO₃; 25 g agar; 200 g NaCl; pH 6.47 (Nga, 2016); TSA - Medium (Trypticase Soy Agar Histidine) for isolation of and conservation of the bacteria (g L⁻¹): 15 g tryptone, 5 g soy peptone; 5 g sodium chloride, 12 g agar No. 2; pH 7.3 ± 0.2 (Lab, United Kingdom) with 20% NaCl; and HEB - Medium for culturing and determining the bacteria's ability to produce histamine (g L⁻¹): 5 g tryptone; 2.5 g K₂HPO₄; 10 g L-histidine; 200 g NaCl; pH 6.0 (Nga, 2016).

Methods

The flow diagram of the general experiments is as follows:

Sampling

Samples were taken according to TCVN 5676-90 from different points in the tank (four corners of the tank, on the surface, at the bottom, and in the middle of the tank as shown in Figure 1).

Firstly, 1% of the Dich Chuop volume was taken from different tanks each month of fermentation before being mixed together in a container, which was called the intermediate sample. Finally, 2000 mL of Dich Chuop was taken from the container of intermediate sample to be used for further analysis.

The samples were then stored at 4-6°C for 7 days for the analysis of histamine content and isolation of bacteria which are capable of producing histamine in different stages of fermented fish sauce.

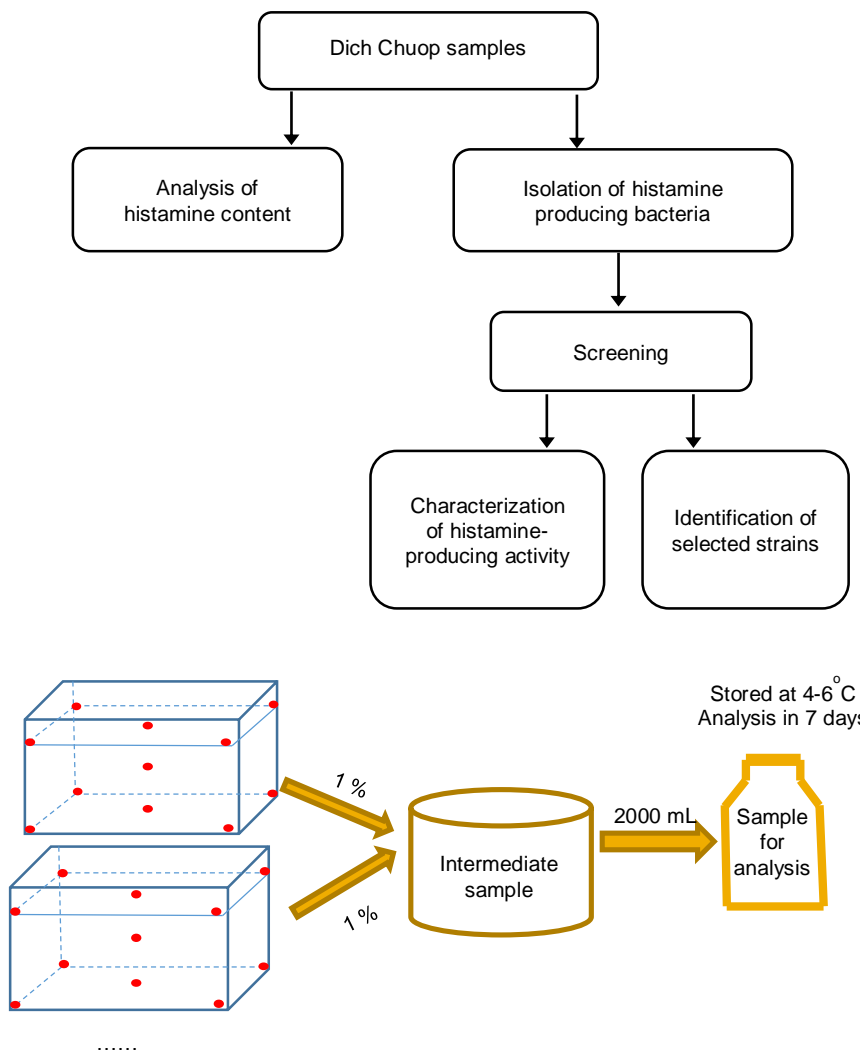


Figure 1. Diagram of sampling methods following TCVN 5676-90

Determination of histamine content by HPLC

The equipment used for the determination of histamine content in the samples were a separation system including an HPLC (Agilent) with detection by fluorescence spectrophotometer, and XDB C18 column (Agilent) with an inside diameter of 4.6 mm and length of 250.0 mm.

The protocol methods followed Yoshida *et al.* (2012) and TCVN 8352:2010 with several modifications. In short, 100 μ L samples were homogenized in 900 μ L Methanol: H₂O (75:25) and then centrifuged for 10 min at 6000 g. After 2 μ L of supernatant was collected, 4 μ L solution of Na₂B₄O₇ (pH 10.0), 4 μ L OPA (ortho-phthalaldehyde), and 10 μ L KH₂PO₄ were added.

The chromatographic conditions were as follows: A phase (KH₂PO₄ 0.01M, pH 4.0): 75 and B phase (Acetonitrile 100%): 25. The flow rate was 1 mL min⁻¹, and detection was at 230 nm and 450 nm. The column temperature was 40°C and the injection load was 5 μ L. In terms of the scope of application, the LOD (Limit of Detection) was 5 ppm and the LOQ (Limit of Quantification) was 15 ppm.

Isolation of bacteria from samples

From the 06 samples taken at different fermentation stages, bacteria isolation was carried out on TSA medium, at pH 7.2, and with a concentration of 20% NaCl. Descriptions of bacterial colonies were based on the color, size, surface structure, and outer edge of colonies.

Screening histamine-producing bacteria

All bacteria from the previous experiment were cultured on HBI medium to determine the histamine producing bacteria. If the colonies grown on HBI medium had a purple color, this indicated that the particular bacteria could decarboxylase amino acids (Hsien *et al.*, 2010).

Identification of the selected strains

Identification of the bacteria followed the methods of Marc *et al.* (2003) and Filipe *et al.* (2008) with several modifications. The strains were selected based on maximum enzyme activity and identified based on the

morphological observations and the comparison of 16S rDNA fragments.

As part of the morphological tests, Gram staining, colony size, shape of bacteria, and bacteria mobility were performed. Sequence analysis of 16S rDNA was as follows. DNA was extracted and purified according to the CTAB method (the current protocol for the isolation of DNA in molecular biology) and purification was determined by spectrophotometrically measurements at the ratio of A260/A280. The values should be higher than 1.8 for further PCR amplification. The 16S rDNA gene fragments were amplified using universal primers, including forward primer 27F (5'-AGAGTTTGATCCTGGCTCAG - 3') and reverse primer 1492R (5'-GGTTACCTTGTTACGACTT - 3'). PCR was performed under the following conditions: initial denaturation at 94°C for 5 min; 30 cycles of denaturation at 94°C for 45 sec, annealing at 55°C for 45 sec, extension at 72°C for 90 sec, and a final extension at 72°C for 5 min. The DNA sequences were then analyzed and aligned using BLAST.

The effects of several factors on the histamine production of the isolated strain

The strain which had the highest histamine producing ability of previous experiment was used in this experiment.

Effect of table salt (NaCl) concentration

The strain was cultivated in HEB liquid medium containing 1% L-histidine (free-base) with NaCl at different concentrations of 0, 15, 20, 25, or 30% and incubated at 37°C in a shaker incubator at 200 rpm for 4 days. The histamine concentration was determined in the supernatant after cell removal by centrifugation of cultured broth at 6000 rpm at 4°C for 15 min.

Effect of pH

The effect of pH was determined by incubating the bacteria into HEB liquid medium containing 1% histidine (free-base) and incubated at 37°C in a shaker incubator at 200 rpm for 4 days at different pHs (4.0, 5.0, 6.0, 7.0, or 8.0). Similarly, the histamine produced was determined in media as described above.

Effect of temperature

The effect of temperature was determined by incubating the bacteria into HEB medium with histidine (free-base) at different temperatures of 30, 40, 50, or 60°C in a shaker incubator at 200 rpm for 4 days. The supernatant was obtained by centrifugation of cultured broth at 6000 rpm at 4°C for 15 min.

Data analyses

Mean values were taken from the measurements of three replications from each treatment. The standard deviations of the means were calculated. Analyses were completed using Microsoft Excel 2013.

Results and Discussion

The variation of histamine content during fish sauce fermentation

As reported by other authors, during the six beginning months of fish sauce fermentation, the histamine content is the most apparent (Jung *et al.*, 2013). Histamine in the samples was analyzed according to the methods mentioned above.

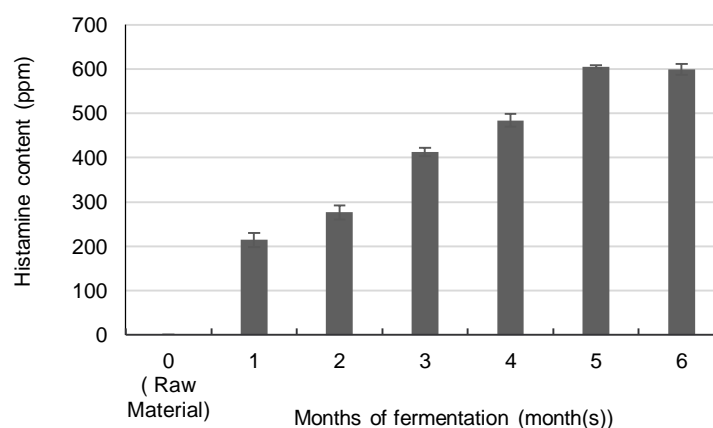
The results show that the raw material samples have histamine levels below the detection level (<5 ppm) (Figure 2). However, the concentration of histamine in Dich Chuop tended to increase gradually over time. Of note, the fifth month of fermentation had a histamine

concentration of 604.85 ppm. From the first month to the fifth month, there was a marked increase in histamine levels, starting at 214.95 ppm in the first month and then reaching the highest level in the fifth month at 604.85 ppm. The histamine content reduced by the sixth month, but this decrease was not statistically different. These results can be explained by the effects of the histidine decarboxylase enzyme found in fish muscle and bacteria available in fish that will degrade histidine to histamine, thereby resulting in increased histamine levels.

The upward trend of histamine content of Dich Chuop Cat Hai fish sauce from the first month to the fifth month during fermentation is similar to that of Nha Trang fish sauce in the same fermentation period. However, histamine in Dich Chuop Nha Trang fish sauce was lower than Dich Chuop Cat Hai fish sauce; specifically, the histamine level of Dich Chuop Nha Trang fish sauce in the fifth fermentation month was only 233 ppm (Nga, 2016). This difference can be explained by the fact that the different raw materials have different histamine contents.

Isolation and screening of histamine-producing bacteria

In the present study, six Dich Chuop Cat Hai samples from the first to the sixth months of the fermentation were used. All colonies of all isolates were described in detail in terms of color,



Note: Vertical bars represent \pm SD, $n = 3$.

Figure 2. Histamine content at different months of the fermentation processes

Table 1. Bacterial isolates from Dich Chuop in the first six months of fermentation

Dich Chuop sample (month)	No. of bacterial isolates	Code of bacterial isolates
1 st	14	CH1.1 to CH1.14
2 nd	8	CH2.1 to CH2.8
3 rd	8	CH3.1 to CH3.8
4 th	8	CH4.1 to CH4.8
5 th	7	CH5.1 to CH5.7
6 th	5	CH6.1 to CH6.5
Total	50	

size, surface structure, and the outer edge of colonies. The numbers and codes of bacterial isolates from each sample are presented in Table 1.

It can be clearly seen from Table 1 that the number of colonies in the first month of fermentation was higher than those in the following months. From the fermented fish sauce samples collected in the first month, 14 bacterial colonies were isolated, whereas from the sixth month samples, only 5 bacterial colonies were isolated. This indicates that the bacteria that are able to grow in high salinity conditions and adapt to the nutrient availability of the fish sauce will survive (Hien, 2017).

Following bacterial isolation, a total of 50 bacteria from the prior experiment were cultured on HBI medium to determine histamine producing bacteria. The colonies that grew on the HBI medium had a purple color, which indicated that the particular bacteria could decarboxylase amino acids. The results showed that among the 50 strains isolated from the 6 Dich Chuop samples on TSA medium, only 4 strains, namely CH2.4, CH3.3, CH4.4, and CH5.1, were able to grow on HBI medium and their colonies turned purple. It can be suspected that the four strains are capable of producing

enzymes that convert histidine to histamine.

The histamine-producing activity of the four isolated strains was determined by inoculating the isolates in HEB supplemented with 1% L-histidine and incubating them at 37°C for 4 days. One milliliter of the culture broth was taken for quantitation of histamine (Nga, 2016).

The histamine concentration at 0 h of culture was 0 ppm. The histamine content after 4 days of culture is shown in Table 2.

The results of the analysis of histamine content in Table 2 show that all of four bacterial strains were capable of producing histamine. Thus, it can be concluded that all of these four strains contributed to the increase in histamine content in the fermented fish sauce. CH5.1 produced the highest histamine levels, about 383.92 ppm after 4 days of culture, followed by CH4.4 (227.22 ppm). These two strains were isolated from the Dich Chuop at the fourth and fifth months of Cat Hai fish sauce fermentation, and these are also the time points when fish sauce had the highest histamine content.

The CH5.1 strain was further identified and investigated for its histamine production under different cultivation conditions. The results are presented in the sections below.

Table 2. Histamine production ability of isolated strains

Code of isolates	Histamine content after 4 culture days (ppm)
CH2.4	188.65 ± 2.30
CH3.3	198.48 ± 2.16
CH4.4	227.22 ± 1.18
CH5.1	383.92 ± 1.15

Result for 16S rDNA sequencing of CH5.1

Homologous sequences for the DNA sequence of the CH5.1 isolate were searched for in the sequence database using the BLAST program. The BLAST searches showed that the CH5.1 isolate was most closely related to *Tetragenococcus halophilus*, originating from China and Thailand, with the query coverage of 97% and the max nucleotide identities 100% (Table 3). The resulting sequences that most closely matched the CH5.1 isolate were isolated in Thailand.

Tetragenococcus halophilus are Gram-positive, rod-shaped, anaerobic, and salty. The study by Nga (2016) reported that a *Tetragenococcus halophilus* isolate had the highest histamine production in Nha Trang fish sauce during the fifth month.

In a study on the kinetics and frequency of bacteria involved in the fermentation process for the production of Cat Hai fish sauce, it was reported that the genus *Tetragenococcus* had a frequency of 88.8% during the fermentation process of Cat Hai fish sauce (Hien, 2017). This

indicates that this strain has adapted to the high salt concentration conditions of fish sauce.

Characterization of the histamine-producing activity of CH5.1

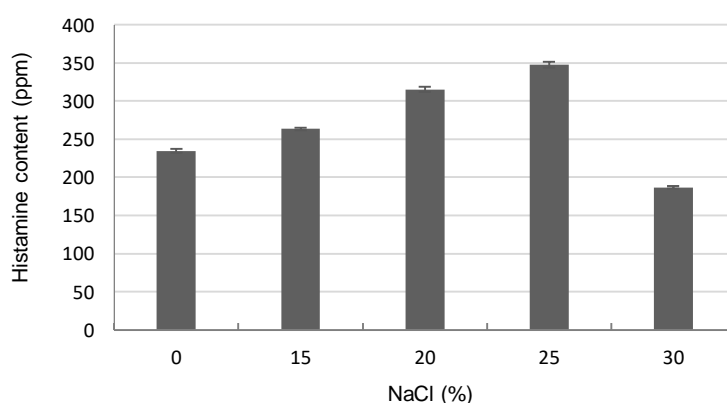
Effect of salt table concentration on histamine-producing activity

Tetragenococcus halophilus CH5.1 was cultured in HEB medium supplemented with 1% histidine with 0, 15, 20, 25, and 30% salt concentrations. The results of the analysis of histamine content after 4 days of culture are illustrated in Figure 3.

It can be seen that the salt concentration influences the histamine production ability of *Tetragenococcus halophilus* CH5.1. When the medium had a 0% salt concentration, the histamine content determined after 4 days of culture was 234.62 ppm. An increase of the salt concentration to 15% increased the histamine production ability, specifically, at 15% NaCl, after 4 days of culture the histamine content was determined to be 263.33 ppm, and the histamine

Table 3. BLAST search using complete DNA sequence of the bacteria

No	GenBank bacteria	Host, country	Accession	Query cover (%)	Identity (%)
1	<i>Tetragenococcus halophilus</i> , strain HGA-2	China	MG988273.1	97	100
2	<i>Tetragenococcus halophilus</i> , strain M3M5	Thailand	KU132381.1	97	100
3	<i>Tetragenococcus halophilus</i> , strain M1M5	Thailand	KU132380.1	97	100
4	<i>Tetragenococcus halophilus</i> , strain SP37-2	Thailand	AB665248.1	97	100
5	<i>Tetragenococcus halophilus</i> , strain KS87-1	Thailand	AB665248.1	97	100



Note: Vertical bars represent \pm SD, $n = 3$.

Figure 3. Effect of salt concentration on the histamine-producing activity of *T. halophilus*

content continued to increase to 347.76 ppm when cultured in the medium with a salt level of 25%. Increasing the salt concentration will affect the metabolism of bacteria capable of producing the enzyme decarboxylase. Some species of lactic acid bacteria isolated from fish sauce such as *Tetragenococcus muriaticus* have the ability to generate histamine during growth and development, and activity of histidine decarboxylase can be maintained at a 20% salt concentration (Kimura *et al.*, 2001). The histamine-producing ability of *Tetragenococcus halophilus* CH5.1 decreased as the culture medium increased to a salt concentration of 30% (only 186.85 ppm). This may be because at high salt concentrations, the bacteria growth is restricted, thus the amount of histamine produced is lower than when cultured under lower salt concentration conditions.

In the study by Jesebel *et al.* (2012) about the influence of salt concentration on histamine formation in fermented Tuna Viscera (Dayok), it was found that high salt concentrations >25% retards microbial histidine decarboxylase activity. This phenomenon can be attributed to reduced microbial cell activity due to the presence of high sodium chloride concentrations causing withdrawal of water and other soluble contents from the cell through osmosis and thus retarding or inhibiting their growth.

Effect of temperature on histamine-producing activity

Temperature plays an important role in enzyme production. The fermentation process of fish sauce takes place in both winter and

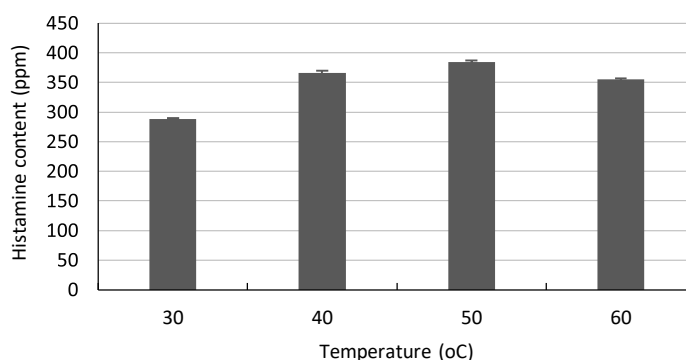
summer, so there are times when the outdoor temperature increases up to 60°C. To investigate the histamine production ability of bacteria in temperatures ranging from 30 to 60°C, cultures of *Tetragenococcus halophilus* CH5.1 were grown in different temperatures.

The histamine production ability of *Tetragenococcus halophilus* CH5.1 increased as the temperature of culture increased from 30°C to 50°C, and the histamine content was the highest when being cultured at 50°C, reaching 384.17 ppm (Figure 4). In the study by Isabel *et al.* (2008) on the effects of different factors on histidine decarboxylase activity from *Pediococcus parvulus*, it was reported that the temperature suitable for this strain producing the enzyme was 40°C, which is lower than the temperature of *Tetragenococcus halophilus* CH5.1 strain. As the culture temperature increased from 50°C to 60°C, the histamine content declined. This can be explained by the fact that high temperatures tend to inhibit bacterial growth as well as enzyme activity, thereby leading to the lower amount of histidine converted to histamine.

Effect of pH on histamine-producing activity

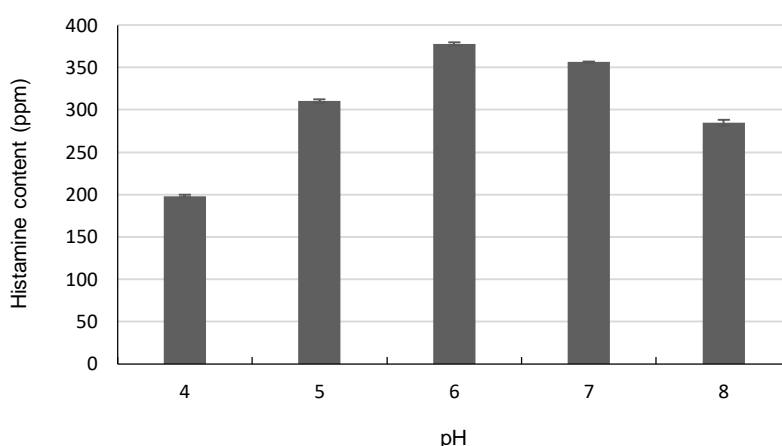
To determine the effect of pH on the histamine production activity of the bacterial strain *Tetragenococcus halophilus* CH5.1, the bacterial strain was cultured in HBE medium at pH 4.0, 5.0, 6.0, 7.0, and 8.0.

It is evident that the pH affects the enzymatic activity of *Tetragenococcus halophilus* CH5.1 (Figure 5). When the pH of the medium increased



Note: Vertical bars represent \pm SD, $n = 3$.

Figure 4. Effect of temperature on the histamine-producing activity of *T. halophilus*



Note: Vertical bars represent \pm SD, $n = 3$.

Figure 5. Effect of pH on the histamine-producing activity of *T. halophilus*

from 4.0 to 6.0, the histamine production increased. From pH 6.0 to 8.0, the ability of the *Tetragenococcus halophilus* CH5.1 strain to metabolize histidine to histamine decreased. Therefore, it can be monitored that pH 6.0 is the appropriate pH for this bacterium to produce histamine.

In the study by Masayo *et al.* (1984), it was shown that the pH suitable for some strains of bacteria, such as psychrophilic and halophilic histamine-forming bacteria that produce histamine, ranges from 5.0 to 6.0.

We can conclude that the conditions suitable for *Tetragenococcus halophilus* CH5.1 to produce histamine in HEB supplemented by 1% L-histidine was at 50°C, pH 6.0, and 25% of NaCl.

Conclusions

Histamine levels tended to increase, reaching the highest rate of 604.85 ppm in the fifth month. Among the 50 strains isolated from six Dich Chuop samples on TSA medium, four strains (CH2.4, CH3.3, CH4.4, and CH5.1) of histamine-producing bacteria were screened. The CH5.1 strain isolate from the fifth month was found to be capable of producing the highest histamine and was identified as *Tetragenococcus halophilus*. Further research of this isolate determined that it had the highest histamine production in HBE supplemented

with 1% histidine at 50°C, pH 6.0, and 25% NaCl. Consequently, these results could be used as a premise for further studies to inhibit histamine-producing bacteria by measures such as covering the canvas to change the fermentation temperature or adding water to change the salt concentration and pH, thus creating conditions less suitable for histamine-producing bacteria.

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