Fisheries Journal, 16 (1), 490-503 (2026)
http://doi.org/10.29303/jp.v14i3.2142

ISOLATION AND IDENTIFICATION OF HISTAMINE-PRODUCING PSYCROTROPHIC BACTERIA FROM BIG EYE TUNA

Isolasi dan Identifikasi Bakteri Psikrotrofik Pembentuk Histamin dari Tuna Mata Besar

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(Received March 2nd 2026; Accepted March 6th 2026)

ABSTRACT

This study aimed to isolate psychrotrophic histamine-forming bacteria (BPH) from bigeye tuna using Niven and EMB (eosine methylene blue) pre-screening media, and to identify the obtained BPH based on their biochemical and molecular characteristics. BPH were isolated from bigeye tuna that had been incubated at 16 oC for 5 days. Positive BPH isolates from Niven and EMB media were then grown in TSBH (trypticase soya broth histidine) medium at 5, 16, and 30 oC to test their activity in forming histamine using TLC (thin layer chromatography). Selected BPH were then identified biochemically and molecularly. BPH with high histamine-forming ability were obtained from Niven medium as many as 16 isolates (39.02%) and from EMB medium as many as 3 isolates (5.35%). The highest histamine-forming activity was obtained at 30 oC and then biochemical testing was carried out for all isolates at that temperature. Presumptive biochemical characterization of 19 isolates obtained 6 isolates that have different biochemical characteristics, namely oxidase, motility, gelatin hydrolysis ability, and the ability to grow in 10% NaCl. The isolates came from Niven medium with codes TMB-N4, TMB-N9, TMB-N25, and TMB-N26 and from EMB medium with codes TMB-E38 and TMB-E40. The results of molecular testing for 6 isolates obtained the genus Morganella with a similarity percentage of >99% and biochemical characterization that leads to the species Morganella morganii subsp. sibonii. The acquisition of Morganella morganii subsp. sibonii bacteria from both EMB and Niven medium indicates that these bacteria are important organisms in the formation of histamine in fish that experience temperature abuse conditions during handling.

Keywords: Isolation, Niven, Eosine Methylene Blue, Psychrotrophic Histamine-Forming Bacteria, Morganella morganii subsp. Sibonii

ABSTRAK

Penelitian ini bertujuan untuk mengisolasi bakteri pembentuk histamin (BPH) psikrotrof dari tuna mata besar dengan menggunakan medium pra-penapisan Niven dan EMB (eosine

methylene blue), serta mengidentifikasi BPH yang didapat berdasarkan karakteristik biokimia dan molekuler. BPH diisolasi dari tuna mata besar yang telah dinkubasi pada suhu 16 ^{o}C selama 5 hari. Isolat BPH positif dari medium Niven dan EMB kemudian ditumbuhkan dalam medium TSBH (trypticase soya broth histidine) pada suhu 5, 16, dan 30 ^{o}C untuk diuji aktivitasya dalam membentuk histamin menggunakan KLT (kromatografi lapis tipis). BPH terpilih selanjutnya diidentifikasi secara biokimia dan molekuler. BPH dengan kemampuan pembentukan histamin yang tinggi diperoleh dari medium Niven sebanyak 16 isolat (39,02%) dan dari medium EMB sebanyak 3 isolat (5,35%). Aktivitas pembentukan histamin tertinggi diperoleh pada suhu 30 ^{o}C dan selanjutnya dilakukan pengujian biokimia untuk seluruh isolat pada suhu tersebut. Karakterisasi presumtif biokimia dari 19 isolat diperoleh sebanyak 6 isolat yang memiliki perbedaan karakteristik biokimia, yaitu oksidase, motilitas, kemampuan menghidrolisis gelatin, dan kemampuan tumbuh dalam NaCl 10%. Isolat tersebut berasal dari medium Niven dengan kode TMB-N4, TMB-N9, TMB-N25, dan TMB-N26 serta dari medium EMB dengan kode TMB-E38 dan TMB-E40. Hasil pengujian molekuler untuk 6 isolat diperoleh Genus *Morganella* dengan persentase kemiripan >99% serta karakterisasi biokimia yang mengarah pada spesies *Morganella morganii subsp. sibonii*. Perolehan bakteri *Morganella morganii* subsp. *sibonii* baik dari medium EMB dan Niven mengindikasikan bahwa bakteri tersebut merupakan organisme penting dalam pembentukan histamin pada ikan yang mengalami kondisi penyalahgunaan suhu selama penangan.

Kata kunci : Isolasi, Niven, Eosine Methylene Blue, Bakteri Pembentuk Histamin Psikrotrofik, *Morganella morganii subsp. sibonii*

INTRODUCTION

Tuna is an Indonesian export commodity to various countries and is a fish with important economic value. Indonesia has exported 1.3 million tons of skipjack tuna (20.06% of global production) to various countries, such as the United States, Japan, and Korea between 2020 and 2022 (Hartanto *et al*., 2021). Tuna from Indonesia as an export product still faces several obstacles, including rejection due to product damage. As many as 64% of the total 146 rejection cases that occurred throughout 2010 were caused by the presence of pathogenic bacteria and toxins produced, such as histamine, at concentrations above 50 ppm; 26% were due to unhygienic conditions; 6% were due to chemical residues; and 4% were due to misbranding (Kushendarto *et al*., 2018). The presence of histamine in fishery products can cause scombroid fish poisoning (SFP). SFP can be characterized by the appearance of symptoms of vasoactive and psychoactive health disorders, including skin (primarily flushing), digestive, hemodynamic (hypotension), and neurological symptoms (Kung *et al*., 2017).

Histamine is formed through a series of histidine decarboxylation reactions by the enzyme L-histidine decarboxylase (L-HDC). Bacteria containing the enzyme L-HDC are the primary cause of histamine formation in scombroid fish (Yamaki *et al*., 2020). Several species have been isolated from tuna, including Morganella morganii, Klebsiella pneumoniae, and Hafnia alvei, which produce histamine at levels above 1000 ppm (Lee *et al*., 2016). In addition, several types of psychrotrophic histamine-forming bacteria (HBs) have also been found in tuna, including Photobacterium kishitanii, P. phosphoreum, and M. psychrotolerans, which can produce histamine at 2°C (Kung *et al*., 2017). These bacteria are the primary cause of histamine in fishery products stored at low temperatures, especially chilled products. Based on the aforementioned issues, an effective and efficient strategy is needed to suppress the growth of HBs.

Isolation of HBs faces many challenges, one of which is false positives on selective media. Only approximately 2% of all bacterial colonies grown on Niven's medium are capable of producing histamine (Hongpattarakere *et al*., 2016). To overcome this, modifications to the isolation process are necessary. One such approach is the use of selective media specifically targeted to specific bacterial groups before using HBs. The media that have been used for the modification of BPH isolation techniques are VRBGA (violet red bile glucose agar) for Enterobacteriaceae, TCBS (thiosulfate citrate bile salt) for Vibrionaceae, and PIA (pseudomonas isolation agar) for Pseudomonaceae (Zgomba Maksimovic *et al*., 2018). In addition, there are several media that can also be used, namely S110 for Staphylococci, MRS (Man-Rogossa Sharpe Agar) for lactic acid bacteria and EMB (eosin methylene blue agar) for Enterobacteriaceae (S. H. Kim *et al*., 2000). Therefore, in this study, isolation was carried out using EMB and Niven selective media to increase the number of histamine-forming psychrotrophic bacteria so that false positives can be minimized.

RESEARCH METHODS

Tools and Materials

Bigeye tuna (Thunnus obesus) fish samples were prepared using Niven medium (NIVEN agar with a composition of 2% bacteriological agar (Oxoid), 0.1% L-histidine (Merk), 0.002% phenol red (Scharlau), 0.1% tryptone (Oxoid), 0.2% yeast extract (Oxoid), 0.5% NaCl (Merk), and 0.1% CaCO3), TSBH (TSB + 1% L-histidine), Butterfield's phosphate buffer (BPB) (Merk), tryptose soya broth (TSB) (Oxoid), HCl (Merk), NaOH (Merk), distilled water, spirits, TLC Plate in silica gel 90 F254 (20x20 cm), TLC eluent [Methanol: Ammonia (20:1) v/v], ninhydrin spray (300 mg ninhydrin in 97 mL n-butanol and 3 mL glacial acetic acid) (Brand), and 70% ethanol. Geneaid Presto™ Mini Gdna Kit protocol, 1x TAE buffer, agarose gel (genetic science), marker, fluorosafe, universal 16S rRNA primers (27F and 1492R), Morganella morganii ATCC 25830 bacteria, HDC specific primers, hairdryer, microcentrifuge, and UV-laminary, electrophoresis tool, biorad thermal cycler.

Research Stages

The research stages were carried out with fish sample preparation, isolation on selective media, screening 1, screening 2, testing the levels and activity of histamine formation using the TLC (thin layer chromatography) method and processed using ImageJ software (Margareta *et al*., 2020). Biochemical characterization was carried out based on Bergey's manual and molecular identification of bacteria was carried out using the Sanger method (Takahashi *et al*., 2003). Statistical analysis was carried out descriptively based on the results of screening, biochemical tests, molecular identification and phylogenetic trees.

Sample Preparation

Sampling was conducted using fish collected at the Sadeng fishing port. Bigeye tuna samples were randomly taken from plastic tubs, taking samples from the top, bottom, middle, right, and left sides. Six fish were collected, with an average weight of 172 grams and a length of approximately 18 cm. The samples were transported using Styrofoam containers filled with ice cubes and fish in a 3:1 ratio. The fish were then placed in sterile HDPE plastic bags and incubated for approximately 5 days.

Isolation and Screening

The fish that had been incubated for 5 days, each fish was then taken from the meat near the gills, stomach, dorsal, and tail (Hongpattarakere *et al*., 2016). The meat was weighed until it reached a weight of 300g, then mortared until it became a dough. 25 grams of fish meat

was then taken and mixed with 225 mL of BPB (butterfield's phosphate buffered) (pH 7.2), homogenized until evenly distributed. 1 mL of the homogenate was then taken and put into 9 mL of BPB for dilution. Dilution was carried out sequentially until the 7th dilution (10-7) was obtained. 100 µL of the 10-2 – 10-7 dilution solution was taken and each was put into a selective medium using the spread plate method. The selective medium used was Niven agar and EMB agar (S.-H. Kim *et al*., 2001). The medium is then incubated for ± 5 days at a temperature of 16 oC or until colonies appear.

Testing of histamine levels and activity

Histamine levels and activity were tested using modified Thin Layer Chromatography (TLC) (Margareta *et al*., 2020). Initially, 1,000 µL of TSBH medium was taken and placed into a microtube, then centrifuged at 12,000 rpm for 15 minutes. A 0.5 µL supernatant was taken and spotted onto a TLC plate. The plate was placed in a chamber containing a 20:1 eluent of methanol and ammonia. The mobile phase ascent process took 40-60 minutes. The plate was then dried with a hair dryer and sprayed with ninhydrin (300 mg ninhydrin in 97 mL n-butanol pro analysis and 3 mL glacial acetic acid). The plate was dried again with a hair dryer until a slightly purplish red spot appeared. Histamine concentration was analyzed using the densitometry method, which is by calculating the area of the spot that appeared with the help of ImageJ software.

Molecular identification

Molecular identification was performed based on the method (Takahashi *et al*., 2003). The primers used were universal primers 27F (5'-AGA GTT TGA TCC TGG CTC AG- 3') and 1492R (5'GGT TAC CTT GTT ACG ACT T- 3') for 16S rRNA (Hwang *et al*., 2019) and hdc-f (5'-TCH ATY ARY AAC TGY GGT GAC TGG RG-3') and hdc-r (5'-CCC ACA KCA TBA RWG GDG TRT GRC C-3') (Yamaki *et al*., 2020). Universal primers were used for molecular identification and HDC primers to determine the presence of the HDC gene in the isolate. The amplified gene was then electrophoresed according to the method (Labella *et al*., 2018).

Biochemical Characterization

Biochemical testing was carried out based on the Bergey's Manual of Determinative Bacteriology reference, and the species determination was based on (O'Hara *et al*., 2000).

Data Analysis

Analysis of isolation and screening data, biochemical characterization and molecular identification was carried out descriptively.

RESULT

Isolation and screening

The isolation process stages and results are shown in Table 1 for EMB medium and Table 2 for Niven medium. A total of 19 BPH isolates were obtained during the isolation and screening phase, each coded Nv 1, Nv 2, Nv 3, Nv 4, Nv 5, Nv 7a, Nv 7b, Nv 8a, Nv 9, Nv 10, Nv 12, Nv 23, Nv 25, Nv 26, Nv 27, and Nv 28 for isolates from Niven pre-screening medium; and EM 38, EM 39, and EM 40 for isolates from EMB pre-screening medium.

Table 1. Number of suspected BPH colonies from the screening phase using EMB medium

Medium	Total number of colonies	Type	Quantity per type	Screening 1 (Niven)	Screening 2 (TLC Test)
EMB (pre-screening)	460*	EI	2	0	0
		EII	4	0	0
		EIII	19	18	0
		EIV	23	23	0
		EV	3	3	3
		EVI	3	3	0
		EVII	2	2	0
	Total		56	49	3

Description: * the number of colonies that grew at dilution levels of 10-4, 10-5, 10-6, and 10-7 which were less than or equal to 250. Each dilution was spread in duplicate with the number of colonies for each dilution being 178 and 214; 27 and 35; 4 and 1; 1 and 0.

Table 2. Results of colony sampling and screening from Niven medium

Medium	Total number of colonies	Type	Quantity per type	Screening 1 (Niven)	Screening 2 (TLC Test)
Niven (pre-screening)	446*	NI	7	7	5
		NII	6	6	4
		NIII	7	7	7
		NIV	11	11	0
		NV	10	10	0
	total		41	41	16

Description:* the number of colonies that grew at dilution levels of 10-4, 10-5, 10-6, and 10-7 which were less than or equal to 250. Each dilution was spread in duplicate with the number of colonies for each dilution being 218 and 197; 18 and 0; 10 and 0; 0 and 3.

Characterization of BPH Isolates with Biochemical Testing

The purpose of the biochemical testing conducted at this stage is to obtain distinguishing characteristics based on the basic biochemical tests of the first stage. Tests include Gram, catalase, oxidase, motility, aerobic and anaerobic, glucose fermentation and gas formation, oxidative-fermentative (OF), gelatinase, and the ability to grow on NaCl, as well as histamine production at 5°C. The distinguishing characteristics obtained are the basis for selecting isolates followed by 16S rRNA sequencing for molecular identification.

Table 3. Grouping of BPH isolates from bigeye tuna samples based on biochemical test results

Testing	Kelompok																					
	B1			B2					B3	B4	B5		B6									
	Nv 10	Nv 12	**EM 40**	**Nv 4**	Nv 5	Nv 7b	Nv 1	Nv 27	**EM 39**	**Nv 26**	Nv 23	**Nv 9**	Nv 8a	Nv 7a	Nv 2	Nv 3						
Gram	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-						

Oxidase	+	+	+	-	-	-	-	-	-	-	-	-	+	+	+	+	
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Motility	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	
Aerobic	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Anaerobic	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
OF	F	F	**F**	**F**	F	F	F	F	**F**	**F**	F	**F**	F	F	F	F	
Gelatinase	+	+	+	-	-	-	-	-	+	-	-	-	-	-	-	-	
NaCl 10%	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	
Histamin (ppm)*	≤25	≤25	**59**	**43**	≤25	≤25	≤25	≤25	**45**	**82**	≤25	**58**	46	57	≤25	≤25	

Note: Bold letters represent isolates selected for further 16S rRNA sequencing. * Histamine concentration at 5 oC for 15 days of incubation.

Molecular identification of 16s rRNA and phylogenetic tree

16S rRNA identification was performed to determine the species based on the similarity of isolates from basic biochemical testing to available references. Isolates EM 40 (B1), Nv 4 (B2), EM 39 (B3), Nv 26 (B4), Nv 9 (B5), and Nv 25 (B6) underwent 16S rRNA molecular identification. Sequencing results showed that all isolates were >99% similar to Morganella morganii subsp. sibonii, as shown in Table 4.

Table 4. 16S rRNA Molecular Identification

No	Sample	Type	Percentage
1	Nv 4	Morganella morganii subsp. sibonii	99,63%
2	Nv 9	Morganella morganii subsp. sibonii	99,26%
3	Nv 25	Morganella morganii subsp. sibonii	99,71%
4	Nv 26	Morganella morganii subsp. sibonii	99,56%
5	EM 39	Morganella morganii subsp. sibonii	99,71%
6	EM 40	Morganella morganii subsp. sibonii	99,71%

Description: BLAST sequencing in percentage of bacterial similarity. Genes were read in the 1500 bp region (16S rRNA).

The value of the closeness of kinship between isolates can also be determined by phylogenetic tree analysis. Isolates resulting from the phylogenetic tree analysis are shown in Figure 1. Based on these results, all isolates have a taxonomically close relationship with Morganella morganii subsp. sibonii with a closeness value of 89 and 70 between isolates in one clade with a distance of 0.002. The higher the closeness value in one clade, the more likely the bacteria are to be the same organism (Labella *et al*., 2018). Therefore, the isolates from the study are suspected to be in the genus Morganella.

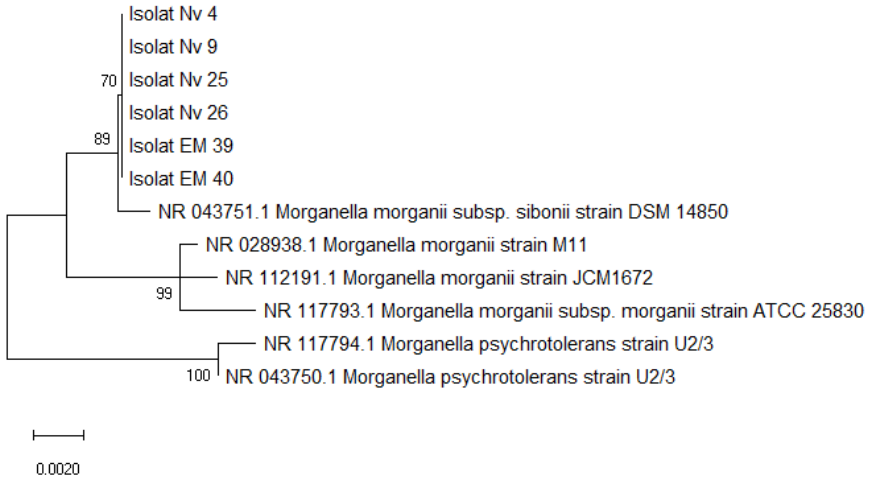

Figure 1. Phylogenetic tree of Morganella morganii subsp. Sibonii

Description: The tree was obtained using the neighbor-joining method with the MEGA X application. The values in the clade were taken with 1000 bootstrap resampling.

Electrophoresis results of HDC gene (histidine decarboxylase)

Confirmation of the presence of the histidine decarboxylase (HDC) expression gene and calculation of histamine formation activity of the isolates Morganella morganii TMB-N4, M. morganii TMB-N9, M. morganii TMB-N25, M. morganii TMB-26, M. morganii TMB-E39, and M. morganii TMB-E40 were carried out at this stage. All tested isolates had the HDC coding gene. This was indicated by the visualization of an electrophoretic band at a size of approximately 700 bp (Figure 2). The HDC expression gene is the gene responsible for the formation of the HDC enzyme. This gene was read at a wavelength of 709 bp (Takahashi *et al*., 2003).

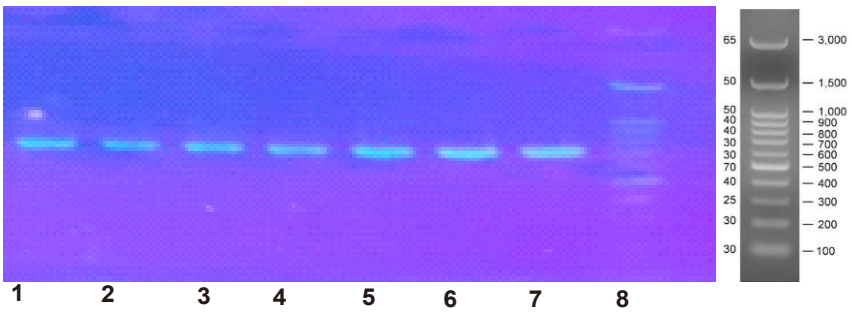

Figure 2. HDC gene electrophoresis

Description: 1. Morganella morganii isolate ATCC 25830, 2-7 Morganella morganii isolates TMB-N4, TMB-N9, TMB-N25, TMB-N26, TMB-E39, and TMB-E40, 8 are Geneaid 100 bp DNA ladder III markers. Visualization is read at a size of 700 bp using UV-Vis 302 nm.

Semi-quantitative histamine measurements yielded histamine production at temperatures of 5, 16, and 30°C, with concentrations of approximately >40, 400, and 900 ppm, respectively (Table 5). Among all histamine-forming bacteria, Morganella has consistently been reported as a productive histamine producer in histidine decarboxylase broth (Ozogul, 2004) and in both TSBH and TFIB (Margareta *et al*., 2020). The results showed that Morganella morganii TMB-N4, M. morganii TMB-N9, M. morganii TMB-N25, M. morganii TMB-26, M. morganii TMB-E39, and M. morganii TMB-E40 were capable of producing histamine at temperatures of 5, 16, and 30°C, with 30°C showing the highest concentration.

Table 5. Histamine quantification and presence of the HDC gene

No	Sample	hdc	Histamine [a] concentration (ppm)		
			5	16	30
1	TMB-N4	+	43	478	1116
2	TMB-N9	+	58	534	971
3	TMB-N25	+	72	569	1162
4	TMB-N26	+	82	484	1049
5	TMB-E39	+	45	464	966
6	TMB-E40	+	59	584	906

Description: a is the histamine concentration at each incubation temperature.

Biochemical Characterization Results

The purpose of biochemical testing at this stage was to confirm the suspected subspecies of Morganella morganii subsp. sibonii based on 16S rRNA sequencing results. The biochemical characterization results of Morganella morganii are shown in Table 6. Molecular identification results for all isolates point to the genus Morganella.

Table 6. Biochemical Characteristics of Isolates Morganella morganii TMB-N4, M. morganii TMB-N9, M. morganii TMB-N25, M. morganii TMB-26, M. morganii TMB-E39, and M. morganii TMB-E40

Biochemical characteristics	Isolate												
	1	2	3	4	5	6	7	TMB-N4	TMB-N9	TMB-N25	TMB-N26	TMB-E39	TMB-E40
Gram	-	-	-	-	.	-	-	-	-	-	-	-	-
Shape	R	R	R	R	.	R	R	R	R	R	R	R	R
Oxidase	-	-	-	-	.	-	-	-	-	+	-	-	+
Catalase	+	+	.	+	.	+	.	+	+	+	+	+	+
Indole	+	+	+	+	+	+	+/-*	+	+	+	+	+	+
Motility	+	+/-*	+/-*	+	.	+/-*	+/-*	+	-	+	+	+	+
MR	+	+	+	+	.	+	+	+	+	+	+	+	+
VP	-	-	-	-	.	-	-	-	-	-	-	-	-
Citric	-	-	-	-	-	-	-	-	-	-	-	-	-
H_2S Production	-	-	-	-	.	-	-	-	-	-	-	-	-
Urease	+	+	+	+	+	+	+	+	+	+	+	+	+
Phenylalanine deaminase	+	+	+	+	.	+	+	.	.	.	.	.	.
Ornithine dekarboksilase	+	+/-*	+/-*	+	+	-	+/-*	+	+	+	+	+	+
Lisin dekarboksilase	-	+/-*	+/-*	+	+/-*	+	+/-*	+	+	+	+	+	+
Arginin dihidrolase	-	-	.	-	.	-	.	.	.	.	.	.	.
Motility	+	+/-*	-	+	.	+	+	+	+	+	+	+	+
Gelatin	-	-	-	-	.	-	-	-	-	-	-	+	+

Biochemical characteristics	Isolate												
	1	2	3	4	5	6	7	TMB-N4	TMB-N9	TMB-N25	TMB-N26	TMB-E39	TMB-E40
Gram	-	-	-	-	.	-	-	-	-	-	-	-	-
Glucose	+	+	+	+	.	+	+	+	+	+	+	+	+
Trehalose	**.**	**-**	**-**	**-**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**+**
Mannitol, Lactose, Sucrose, Maltose	-	-	-	-	.	-	-	-	-	-	-	-	-
Oxidation-fermentative	F	.	.	F	.	.	.	F	F	F	F	F	F
Gas from glucose	.	+	+	+	.	+	+	+	+	+	+	+	+

Description: * is a variable between biogroups; - (1% to 15% positive); (.) no test; F fermentative; R (rod/stem shape); + (strain 85% to 100% positive). Bold letters indicate key tests to differentiate Morganella morganii subsp. morganii and Morganella morganii subsp. sibonii. 1. Morganella morganii (S. H. Kim *et al*., 2000); 2. Morganella morganii subsp. morganii (Jensen *et al*., 1992); 3. Morganella morganii subsp. morganii (O'Hara *et al*., 2000); 4. Morganella morganii (Cowan *et al*., 1993); 5. Morganella morganii subsp. sibonii (Emborg *et al*., 2006); 6. Morganella morganii subsp. sibonii (Jensen *et al*., 1992); 7. Morganella morganii subsp. sibonii (O'Hara *et al*., 2000);

DISCUSSION

The yield of 5.35% positive BPH isolates from EMB was lower than that of (S. H. Kim *et al*., 2000), which stated that 90% of colonies obtained from EMB incubated at 35°C were true BPH. The lower number of isolates in this study is likely due to the use of a lower incubation temperature of 16°C. According to (S. H. Kim *et al*., 2001), various types of BPH obtained from EMB medium are mesophilic bacteria, including Klebsiella sp., Citrobacter sp., Serratia sp., Enterobacter sp., and Pantoea sp. Therefore, at higher temperatures, most BPH growth in EMB medium is inhibited, affecting TLC results as a secondary screening method for determining true BPH.

Enterobacter, which are commonly found at lower temperatures, produce biogenic amines in addition to histamine. This is thought to have caused false positives in screening stage 1, which resulted in histamine not appearing as a spot on TLC in screening stage 2. False positives are errors in reading color changes in selective media due to an increase in pH caused by the presence of other biogenic amines besides histamine. Enterobacteriaceae found at low temperatures are producers of the biogenic amines putrescine and cadaverine. These bacteria include Kluyvera in-termedia, Yersinia kristensenii, Y. rodhei, Serratia grimesii, S. ficaria, Providencia vermicola, and Obesumbacterium proteus, which were incubated in VRBGA medium at refrigerated temperatures for 28 days (Mavromatis & Quantick, 2002). The BPH isolated in screening stage 1 in this study is thought to only produce low concentrations of histamine, making it undetectable on the TLC plate.

The yield of BPH from Niven medium was higher than that from EMB medium, at 39.02%. This is thought to be because Niven medium is capable of growing all types of BPH (Otsuka *et al*., 2022). Similar results were reported in a study (Rodtong *et al*., 2005) where Niven medium incubated at 35°C was able to obtain 40% positive BPH from the total number of suspected BPH colonies as a pre-screening medium. Similarly, (Kuda *et al*., 2012) who

isolated BPH from scombroid and non-scombroid fish using Niven medium obtained 57.3% positive BPH at an incubation temperature of 15°C. The selectivity of Niven medium was not affected by incubation temperature, although in this study a lower temperature was used. This indicates that Niven medium is a selective medium suitable for BPH isolation at lower temperatures, so that more BPH in this study were obtained from Niven medium incubated at 16°C.

The isolates obtained from EMB and Niven medium were isolated at 16°C. It is possible that the BPH in this study are psychrotrophic. The 5-day isolation at 16°C in this study was sufficient time for the growth of histamine-producing psychrotrophic bacteria. Psychrotrophic bacteria have a lifespan between 5°C and 30°C. The results of this study indicate that EMB medium is less suitable for isolating psychrotrophic BPH, as only a small number of BPH were obtained. EMB medium is generally used to select mesophilic bacteria, both lactose and non-lactose fermenters (S. H. Kim *et al*., 2001). Therefore, in this study, approximately 7 times more isolates were obtained from Niven medium than from EMB. Semi-quantitative histamine measurements revealed histamine formation at temperatures of 5, 16, and 30°C, with concentrations of approximately >40, 400, and 900 ppm, respectively (Table 5). Among all histamine-forming bacteria, Morganella has consistently been reported as a productive histamine producer in histidine decarboxylase broth (TSBH and TFIB) (Margareta *et al*., 2020). The presence of the histidine decarboxylase gene also plays a role in histamine formation. Histidine decarboxylase is an enzyme that specifically decarboxylates histidine to histamine. Without this enzyme, the concentration of histamine formed in food is not very high (Anderegg *et al*., 2020). It is possible that this species entered fish through post-harvest contamination, as it is rarely found in fresh fish and is isolated only from damaged fish (S. H. Kim *et al*., 2001). Several studies have shown that the genus Morganella is a histamine producer. Emborg *et al*. (2006) discovered the bacterium Morganella psychrotolerans capable of producing histamine at refrigerated temperatures. Furthermore, S. H. Kim *et al*., 2000, isolated 374 histamine-producing bacteria during the spoilage of tuna (Thunnus thynnus), and 47 of these bacteria belonged to the genus Morganella.

The results showed that Morganella morganii TMB-N4, M. morganii TMB-N9, M. morganii TMB-N25, M. morganii TMB-26, M. morganii TMB-E39, and M. morganii TMB-E40 were capable of producing histamine at 5, 16, and 30°C, with 30°C showing the highest concentration. Based on the study data, all isolates were capable of producing >40 ppm histamine at 5°C within 15 days, and the number increased with increasing incubation temperature. This finding provides important information regarding M. morganii isolates that can produce histamine at concentrations approaching 50 ppm at 5 oC for 15 days of incubation. Isolates Morganella morganii TMB-N4, M. morganii TMB-N9, M. morganii TMB-N25, M. morganii TMB-N26, M. morganii TMB-E39, and M. morganii TMB-E40 are suspected to be psychrotrophic bacteria because of their ability to form histamine at 5 oC. According to (Devivilla *et al*., 2019) the definition of psychrotrophic bacteria is bacteria that are able to reproduce and survive at temperatures of 4 oC to 42 oC, with an optimal temperature of 22 oC to 28 oC. This is supported by (Hongpattarakere *et al*., 2016) who stated that psychrotrophic bacteria can grow in liquid media and cloud it within 14 to 21 days at 5°C. Therefore, the isolates from this study are mesophilic bacteria that have the potential to produce histamine at low temperatures when stored for a long time. The results of this study also indicate that the genus Morganella is a productive histamine-forming bacteria, especially when temperature fluctuations occur during handling (Han *et al*., 2024).

All isolates were able to produce acid from the fermentation of trehalose and glucose, but were unable to utilize trehalose, mannitol, maltose, lactose, and sucrose. All six isolates were able to hydrolyze urea, decarboxylate lysine and ornithine, and did not utilize citrate.

They were MR positive and acetoin production negative (VP negative). Based on these data, the isolates belonged to the genus Morganella (Nevado *et al*., 2023). The genus Morganella was first discovered and isolated from a patient suffering from summer diarrhea and described by Morgan, who later named it Morgan's bacillus. This bacterium is characterized by not fermenting lactose, sucrose, salicin, sorbitol, maltose, inositol, trehalose, rhamnose, sorbitol, arabinose, adolnitol, dextrin, xylose, and raffinose. Some carbon sources that this bacterium can ferment include glycerol, galactose, and levulose. Other characteristics of this species, according to O'Hara *et al*., 2000, include indole production, gelatin dissolution, some non-notyl fermentation, and some H2S formation. Jensen *et al*., 1992, described two subspecies: Morganella morganii subsp. morganii and Morganella morganii subsp. sibonii, based on their ability to ferment trehalose. All six isolates found in this study were capable of fermenting trehalose. This ability is the key to distinguishing between Morganella morganii subsp. morganii and Morganella morganii subsp. sibonii.

BPH not only plays a role in producing histamine as a secondary metabolite, but also acts as a spoilage bacterium due to its ability to produce several other biogenic amine compounds. The presence of high levels of biogenic amine compounds in a fishery product is an indicator of fish damage (Visciano *et al*., 2012). Suppression of BPH growth is necessary to maintain fish freshness so that the export value for fresh tuna products (chilled tuna) remains high. Based on the results of this study, Morganella morganii subsp. sibonii is able to produce histamine at a temperature of 5 oC within 15 days of incubation. A rapid handling process and maintaining the established cold chain system, namely at a temperature of 4 oC, is necessary to suppress BPH growth (Hatipoglu *et al*., 2023) because histamine-forming bacteria appear in fishery products due to abused temperatures during the handling and processing process.

CONCLUSION

A total of 41 suspected BPH isolates from Niven medium and 49 suspected BPH isolates from EMB medium were obtained from the isolation stage. BPH with high histamine-forming ability was obtained from Niven medium as many as 16 isolates and from EMB medium as many as 3 isolates. The results of characterization and identification of BPH, both from Niven and EMB medium, were all suspected to be the genus Morganella and the results of molecular identification for 6 isolates were suspected to be the species Morganella morganii subsp. sibonii.

ACKNOWLEDGEMENT

The author would like to express his gratitude to the Ministry of Education, Culture, Research, and Technology through funding from the Scientific Research Program under the Independent Research Grant scheme for Lecturers. Akhmad Awaludin Agustyar as the provider of pure culture of Morganella morganii ATCC 25830.

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