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Rapid Detection of HDC Gene in *Enterobacter aerogenes* from Fish Products Using *in Silico* PCR for Food Safety and Allergy Risk Assessment

Deteksi Cepat Gen HDC pada Enterobacter aerogenes dari Produk Perikanan Menggunakan in Silico PCR untuk Penilaian Keamanan Pangan dan Risiko Alergi

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ABSTRACT

Histamine poisoning is a significant food safety concern associated with fish and fishery products. Enterobacter aerogenes is one of the histamine-producing bacteria capable of converting L-histidine into histamine through the histidine decarboxylase (HDC) enzyme. The accumulation of histamine in fish and fishery products can lead to allergic reactions and foodborne illnesses, highlighting the need for rapid detection methods. This study aimed to design and optimize primers targeting the HDC gene in E. aerogenes and validate their specificity using in silico PCR. The HDC gene sequence was retrieved from the NCBI GenBank database, and primers were designed using Primer3Plus software. The in silico PCR analysis was performed to assess the specificity of the primers against the genome of E. aerogenes and other histamine-producing bacteria species. The optimized Primer Pair 1 successfully amplified a 230 bp target region, showing high specificity for E. aerogenes in product size and annealing position with no cross-reactivity to other histamine-producing bacteria species. These findings demonstrate the potential of in silico PCR as a rapid and cost-effective screening tool for detecting histamine-producing bacteria in fish and fishery products. However, further in vitro validation is required to confirm the applicability of these primers in real-world food safety and allergy risk assessments.

ABSTRAK

Keracunan histamin merupakan masalah keamanan pangan yang signifikan yang terkait dengan konsumsi ikan dan produk perikanan. *Enterobacter aerogenes* adalah salah satu bakteri penghasil histamin yang mampu mengubah L-histidin menjadi histamin melalui enzim histidin dekarboksilase (HDC). Akumulasi histamin dalam ikan dan produk perikanan dapat menyebabkan reaksi alergi serta penyakit bawaan makanan, sehingga diperlukan metode deteksi yang cepat. Studi ini bertujuan untuk merancang dan mengoptimalkan primer yang menargetkan gen HDC pada *E. aerogenes* serta memvalidasi spesifisitasnya menggunakan *in silico* PCR. Urutan gen HDC diperoleh dari database NCBI GenBank, dan perancangan primer dilakukan menggunakan perangkat lunak Primer3Plus. Analisis *in silico* PCR dilakukan untuk mengevaluasi spesifisitas primer terhadap genom *E. aerogenes* dan spesies bakteri penghasil histamin lainnya. Pasangan primer yang telah dioptimalkan (Primer Pair 1) berhasil mengamplifikasi wilayah target sepanjang 230 bp, menunjukkan spesifisitas tinggi terhadap *E. aerogenes* dalam ukuran produk dan posisi annealing tanpa adanya reaktivitas silang dengan spesies bakteri penghasil histamin lainnya. Hasil ini menunjukkan bahwa *in silico* PCR berpotensi menjadi alat skrining yang cepat dan hemat biaya untuk mendeteksi bakteri penghasil histamin dalam ikan dan produk perikanan. Namun, validasi lebih lanjut melalui uji in vitro diperlukan untuk memastikan aplikabilitas primer ini dalam penilaian keamanan pangan dan risiko alergi secara nyata.

INTRODUCTION

Fish and its products are one of the most widely consumed food across the globe. They are considered a staple food in the diets of millions of people. The existing fish processing techniques and processes have provided various types of products, both for personal consumption and for commercial distribution (Tidwell and Allan, 2001; Delgado et al., 2003). However, fish and their derivative products are often susceptible to contamination by microorganisms. The situation was even more difficult by the fact that fish and fishery products themselves were very easily perished (Ahmed, 2020). Cases of food safety that have received serious attention in fish products are the high incidence of contamination by pathogenic bacteria that capable of producing toxins. Improper production, distribution, and storage phases can trigger the formation of various toxins which lead to serious health consequences for consumer (Novoslavskij et al., 2016; Sheng and Wang, 2020; Comi and Iacumin; 2025).

Food safety within the seafood industry is a key issue. Among severe toxins related to fish consumption, histamine, a biogenic amine substance, is one of the most concerning toxins all over the world. Histamine contamination remains one of the top causes related to fish consumption foodborne illness. Histamine was responsibly known for a broad series of adverse effects, commonly referred to as scombroid poisoning (Visciano et al., 2014; Feng et al., 2016; Hungerford, 2021). Histamine poisoning is not only a significant public health concern, but also an important economic activity problem, including the food business. This poisoning can involve fresh fish that, by their appearance, appear fresh and are consumed shortly after capture. Consumers may not realize that they have consumed fish or processed fish products that contain histamine until they experience symptoms of poisoning (Economou et al., 2017).

Histamine is produced through the bacterial decarboxylation of histidine, an amino acid highly concentrated in fish species like tuna, mackerel, and sardines. Generally, bacteria involved in this reaction belong to the genera of *Enterobacter*, *Klebsiella, Morganella*, *Photobacterium*, *Proteus*, and *Raoultella*. Those genera are known for their capability to produce histamine at a higher level (<1000 mg/L) under ideal condition (Björnsdóttir-Butler et al., 2010; Enache *et al*., 2013; García-Tapia et al., 2013; Bjornsdottir-Butler et al., 2015; Hu et al., 2015; Guo et al., 2019; Oktariani et al., 2022). Among these, the important one is *Enterobacter aerogenes* since it can grow well in variable environments, especially under conditions of poor storage or handling of fish products. This pathogenic bacterium contains the Histidine Decarboxylase (HDC; EC 4.1.1.22) gene, which encodes the enzyme responsible for converting L-histidine into histamine. The enzyme remains stable even if the bacteria are inactive, which can be due to cell damage or frozen storage conditions. During fish decomposition, these bacteria will develop with the resultant formation of this toxic histamine that upon ingestion may provoke symptoms ranging from rashes and headaches to more serious anaphylactic reactions (Surya et al., 2019; Hassan et al., 2020).

The conventional methods for histamine-producing bacteria detection, such as microbiological culturing and biochemical analysis, have significant limitations despite their efficacy. Most of those methods are extremely time-consuming, require special laboratory equipment, and may not be practical for rapid testing or high volume of samples. Other commonly used methods, such as numerous chromatographic methods, ELIZA, and biosensors, focus more on detecting the concentration of histamine in the sample, rather than on the histamine-producing bacteria (Nevado et al., 2023). This paper focuses on developing urges for quicker and more reliable methods to detect histamine-producing bacteria before they reach the consumer. One of the promising solutions to answer this challenge include the use of molecular techniques, such as Polymerase Chain Reaction (PCR), a technique that allows one to rapidly amplify and then detect the evidence through specific genetic markers. More specifically, the HDC gene provides a suitable biomarker for identifying bacteria capable of producing histamine using the PCR method. However, in vitro PCR is faster than the traditional methods, but still requires biological samples, reagents, and equipment. Therefore, its application becomes limited when the screening has to be done rapidly on a large scale. Rapid methods are very much essential in preventing poisoning due to histamine and avoiding the access of such products to the market (Bjornsdottir-Butler et al., 2011; Wongsariya et al., 2016). Given the very high spoilage rates of fish products and their possible buildup of histamine even under refrigeration, it becomes urgent to have effective and rapid diagnostic tools for ensuring food safety.

The molecular detection methods, including PCR, have held promise for faster and more accurate identification of histamine-producing pathogens. However, even with conventional PCR, samples and reagents are still needed physically, which means it can hardly be scaled up for routine food testing. In this respect, an *in silico* PCR was executed to detect the HDC gene in *E. aerogenes* as a virtual and highly specific screening tool that could accelerate the process of food safety assessment. *In silico* PCR is a computational approach used to predict PCR amplification outcomes based on a given set of primers (probes) targeting DNA sequences from a sequenced genome or transcriptome. This technique utilizes the latest genome data to theoretically simulate the amplification of specific DNA regions. The software provides detailed information on the starting position of each PCR product (amplicon), its expected length, and a virtual representation of its electrophoretic mobility. The researchers are able to virtually amplify DNA sequences from digital genomic data retrieved from genomic databases such as NCBI (Bikandi et al., 2004; Ethica et al., 2020a). Given the speed and cost-effectiveness of pathogen detection methodologies in early-stage screenings without the need for actual physical samples, *in silico* PCR is expected to enhance histamine-producing bacteria detection in fish and fishery products, supporting proactive food safety and allergy risk management. These benefits give a wider overview of any possible contamination that happens in the sample. Therefore, *in silico* PCR may present a very good potential to improve the routine protocols of food safety by offering a scalable method with extreme sensitivity.

This study aims to design and optimize the PCR primers that will have a specific target on the HDC gene in *E. aerogenes* using bioinformatics tools and then be tested through *in silico* PCR simulations for efficacy and specificity to amplify the HDC gene. Moreover, the findings are expected to contribute for developing rapid molecular diagnostics in food safety by demonstrating the feasibility and advantages of *in silico* PCR for the rapid detection of foodborne pathogens. These findings will subsequently provide indications as to how the use of virtual PCR methods may be integrated into routine food safety monitoring in order to protect consumers against allergic reactions and improve public health in general.

METHODS

Data Collection

The study required an extensive search for bacterial genomes based on fish products from public databases. The research focused on *E. aerogenes*, a well-known histamine producer usually observed in seafood. The HDC gene in *E. aerogenes* was selected due to its known involvement in histamine production. These targeted genomic sequences were retrieved from the GenBank sequence database by the National Center for Biotechnology Information (NCBI), including its protein sequence data (Yu and Zhang, 2011).

Primer Design

Primer design was performed from a selected complete open reading frame (ORF) of the HDC gene in *E. aerogenes*. Several recommended primer pairs were generated based on the targeted gene sequence using the web-based software Primer3Plus at *https://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi*. Those primer pairs were sorted and focused on those flanking conserved regions of the targeted gene to get the most optimal primer pair as a genomic biomarker. The optimized primer pair was then applied to run the PCR procedure (Bikandi et al., 2004; Hung and Weng, 2016; Ethica et al., 2020b).

In Silico PCR

The optimized primer pair was tested using *in silico* PCR at *http://insilico.ehu.eus/PCR/*. The high specificity to the targeted gene and the least likely to cause off-target amplifications were highly desired (Gupta et al., 2017). The virtual PCR was run using a genome database of the main genera that involved in histamine production in fish products, namely *Enterobacter*, *Klebsiella*, *Morganella*, *Photobacterium*, *Proteus*, and *Raoultella*. All genomes of species from the six genera stored in the *in*

silico PCR program database were used to test the specificity of the optimized primer pair.

Data Analysis and Visualization

The nucleotide and protein sequence of the complete HDC gene in *E. aerogenes* was selected in FASTA format. The protein sequence was then visualized using SWISS-MODEL at *https://swissmodel.expasy.org/* (Biasini et al., 2014; Waterhouse et al., 2018). The primer design results were analyzed to determine whether the constructed primer passed all the parameters of a reliable primer. The following parameters were used to optimize the design of the primer pair, such as physical characteristics, primer performance to avoid primer dimerization, and lack of secondary structure formation. The physical characteristics mainly refer to primer length (18-25 nucleotides), melting temperature (Tm=58-62°C), and GC content (40-60%) provided by Primer3Plus. The possibility of primer dimer and secondary structure formation was confirmed using OligoEvaluator at *https://www.oligoevaluator.com/* (Kariyanna et al., 2020; Watahiki et al., 2020). The specificity of the optimized primer pair was verified by running *in silico* PCR using the total genome of *E. aerogenes* in the database. The amplicon must be specific to the target gene and not to other genes or species. The specificity of the optimized primer pair to the target gene is indicated by the presence of only one PCR product band with the exact size as when the primer was designed. In contrast, the genomic sequences of other histamine production-associated bacterial species from the six genera were also retrieved for use as controls to strengthen the specificity of the optimized primer pair. Verification for the primer position in the HDC gene sequence was performed using Sequence Manipulation Suite (SMS 2): Primer Map Online Analysis Tools at *https://www.bioinformatics.org/sms2/primer_map.html* (Betsy and Siva, 2023). Amplicon sequence compatibility was confirmed using VectorBuilder's Sequence Alignment tool at *https://en.vectorbuilder.com/tool/sequence-alignment.html* (Oliveira-Andrade et al., 2024).

RESULT AND DISCUSSION

The Feature of HDC in *Enterobacter aerogenes*

Accumulation of histamine in fish and its products has made histamine food poisoning, also well known as scombroid poisoning. The evidence shows that scombroid poisoning is a big public health hazard. Histamine poisoning outbreaks have been reported worldwide, with symptoms ranging from harmless headaches and skin rashes to more serious and acute allergic reactions which include respiratory distress. The major challenge in managing histamine contamination is the inadequacy of conventional detection methods, which are mostly time-consuming, labor-intensive, and require specialized equipment that may not be readily available in every setup.

This type of foodborne illness is caused by the bacterial involvement of certain species such as *E. aerogenes* that carry the gene for HDC, which catalyzes the conversion of L-histidine to histamine. The gene later expresses a product of HDC enzyme (HDC; EC 4.1.1.22), an enzymatic protein under accession number AKQ19164.1 of the protein database (Table 1), shown in 3D structure (Figure 1). The 3D structure shows how the amino acid chain comprising the HDC enzyme folds to form its functional group. The HDC enzyme removes the α-carboxylate group of the substrate of L-histidine into histamine, active more in low pH (4-5,5) and temperature of 20-37ºC (Milicevic et al., 2014; Sahu et al., 2015).

Table 1. Protein sequence of HDC in *E. aerogenes* (GenBank: AKQ19164.1)

Database	Accession Number	Sequence (in FASTA Format)	Size
Protein	AKQ19164.1	MSLSIADQNKLDAFWSYCVKNRYFNIGYPESADFD YTMLERFLRFSINNCGDWGEYCNYLLNSFDFEKEV MEYFSGIFKIPFAESWGYVTNGGTESNMFGCYLGR ELFPEGTLYYSKDTHYSVAKIVKLLRIKSQLVESQ PDGEMDYDDLINKIRTSGERHPIIFANIGTTVRGA VDNIAEIQKRIAALGIPREDYYLHADAALSGMILP FVEDPQPFTFADGIDSIGVSGHKMIGSPIPCGIVV AKKANVDRISVEIDYISAHDKTISGSRNGHTPLMM WAAVRSHTDAEWHRRIGHSLNMAKYAVDRFKAAGI DALCHKNSITVVFPKPSEWVWKKHCLATSGNVAHL ITTAHHLDSSRIDALIDDVIADLAQRAA	378 aa

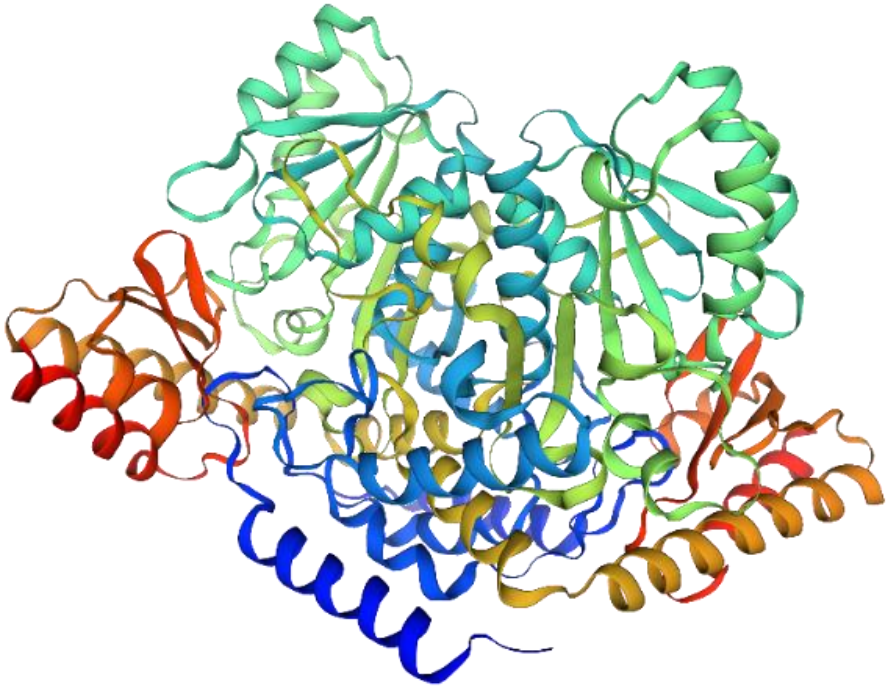


Figure 1. The HDC enzyme in *E. aerogenes* shown in 3D structure

Optimization of Primer Pair Design

The HDC gene in *E. aerogenes* was retrieved from the GenBank sequence database under accession number KP728799.1 of the nucleotide database. It encodes the complete sequence of the *E. aerogenes* strain DI-311 histidine decarboxylase gene (Table 2). The HDC gene sequence was used to construct all primer pairs optimized for *in silico* PCR. The nucleotide sequence under accession number KP728799.1 was used as a DNA template for designing the PCR primers. After obtaining the primer design, one pair of primers was then selected from several generated primer pairs that best met the optimization parameters.

Table 2. Gene sequence of HDC in *E. aerogenes* strain DI-311 (GenBank: KP728799.1)

Database	Accession Number	Sequence (in FASTA Format)	Size
Nucleotide	KP728799.1	ATGTCTTTATCTATTGCCGATCAAAATAAACTCGA TGCATTTTGGTCATACTGTGTAAAAAATCGCTACT TTAATATTGGCTACCCTGAATCTGCTGATTTTGAT TACACCATGCTGGAGCGTTTTCTGCGTTTCTCAAT TAATAACTGTGGTGACTGGGGGGAGTACTGTAACT ATTTACTCAACTCTTTCGACTTTGAAAAAGAAGTC ATGGAGTATTTCTCCGGCATATTCAAAATCCCCTT TGCGGAAAGCTGGGGCTATGTCACCAACGGCGGCA CAGAAAGTAATATGTTTGGTTGCTATCTGGGAAGA GAACTGTTCCCGGAAGGCACGCTCTACTATTCAAA AGATACTCACTATTCCGTCGCCAAAATCGTCAAAC TGCTGCGTATCAAATCGCAACTGGTGGAATCTCAG CCAGACGGAGAAATGGATTATGACGATTTGATCAA TAAAATCAGGACCTCAGGCGAACGCCATCCCATCA TTTTCGCCAATATTGGCACAACGGTACGCGGCGCT GTTGATAATATCGCTGAGATACAGAAACGTATCGC TGCGCTGGGGATCCCACGTGAAGATTATTATCTAC ACGCGGATGCCGCGCTAAGCGGCATGATCTTGCCT TTCGTGGAGGATCCACAACCGTTTACCTTCGCGGA TGGTATCGATTCGATTGGCGTTTCCGGACACAAAA TGATCGGCTCTCCGATTCCATGCGGCATTGTGGTA GCCAAGAAAGCCAACGTCGACCGTATCAGCGTAGA GATCGACTACATCTCCGCCCACGATAAAACGATTT	1137 bp

The optimization of primer pairs targeting the HDC gene in *E. aerogenes* was a critical step in ensuring the specificity and efficiency of the *in silico* PCR. Several primers were obtained from generating primers using Primer3Plus are listed in Table 3. All pairs of primers that have been generated show that the physical characteristics (primer length, melting temperature, and GC content) have met the specified parameter criteria. OligoEvaluator was used to verify the possibility of primer dimer and secondary structure formation. None of the primers showed any potential for forming primer dimers, while Primer Pair 1 showed the lowest tendency for secondary structure formation among other primer pairs. Thus, Primer Pair 1 was selected as the primer pair to be further tested using *in silico* PCR. *In silico* specificity of the optimized Primer Pair 1 resulted in one clear amplicon of the expected size (230 bp) against the genomic sequences of *E. aerogenes* and no other species.

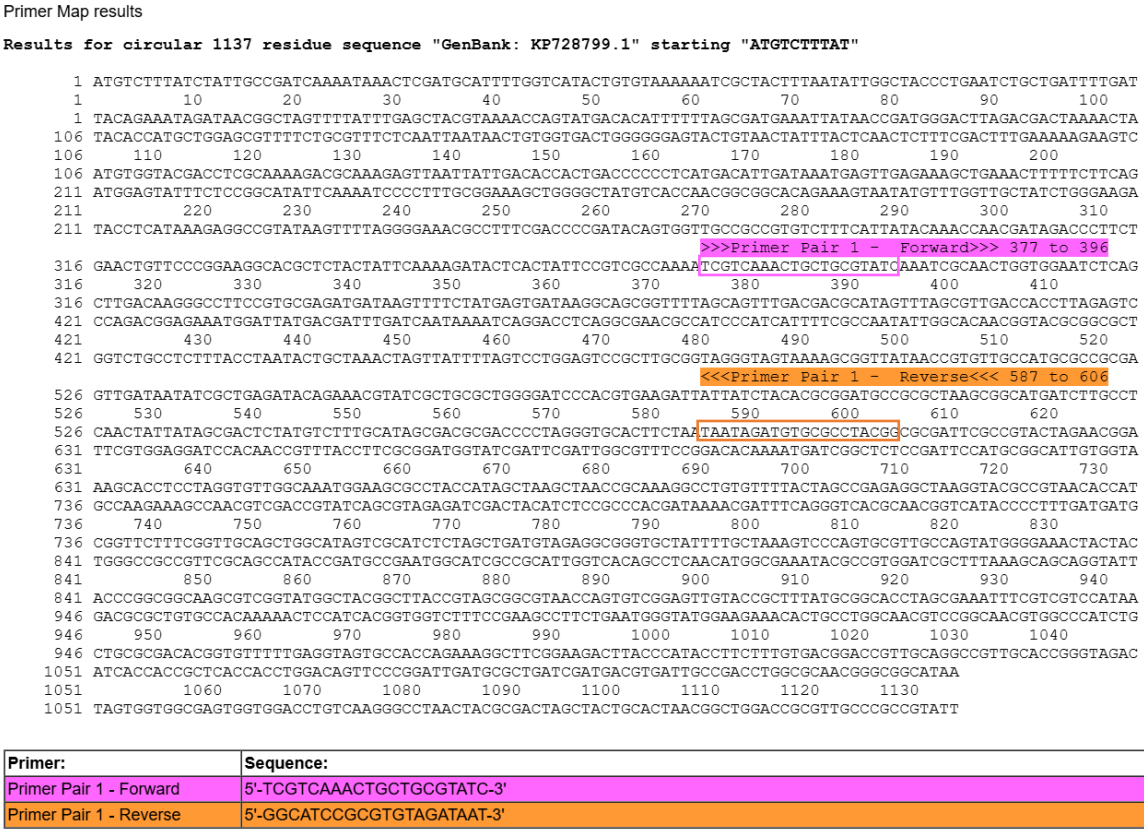
Table 3. The primer candidates from the HDC gene in *E. aerogenes*

Primer	Amplicon Size	Sequence	Feature
Pair 1	230 bp	Forward (start=377) TCGTCAAACTGCTGCGTATC	Length=20 bp Tm=60.0°C GC=50.0% Primer Dimer=no Sec. Structure=none
		Reverse (start=606) GGCATCCGCGTGTAGATAAT	Length=20 bp Tm=60.0°C GC=50.0% Primer Dimer=no Sec. Structure=very weak

Pair 2	150 bp	Forward (start=415) TCTCAGCCAGACGGAGAAAT	Length=20 bp Tm=60.0°C GC=50.0% Primer Dimer=no Sec. Structure=moderate
		Reverse (start=564) CGCAGCGATACGTTTCTGTA	Length=20 bp Tm=60.0°C GC=50.0% Primer Dimer=no Sec. Structure=weak
Pair 3	151 bp	Forward (start=414) ATCTCAGCCAGACGGAGAAA	Length=20 bp Tm=60.0°C GC=50.0% Primer Dimer=no Sec. Structure=moderate
		Reverse (start=564) CGCAGCGATACGTTTCTGTA	Length=20 bp Tm=60.0°C GC=50.0% Primer Dimer=no Sec. Structure=weak
Pair 4	168 bp	Forward (start=397) AAATCGCAACTGGTGGAATC	Length=20 bp Tm=59.9°C GC=45.0% Primer Dimer=no Sec. Structure=none
		Reverse (start=564) CGCAGCGATACGTTTCTGTA	Length=20 bp Tm=60.0°C GC=50.0% Primer Dimer=no Sec. Structure=weak
Pair 5	192 bp	Forward (start= 415) TCTCAGCCAGACGGAGAAAT	Length=20 bp Tm=60.0°C GC=50.0% Primer Dimer=no Sec. Structure=moderate
		Reverse (start= 606) GGCATCCGCGTGTAGATAAT	Length=20 bp Tm=60.0°C GC=50.0% Primer Dimer=no Sec. Structure=strong

The correctness of the primers' position on the HDC gene sequence of *E. aerogenes* (GenBank: KP728799.1) was verified to ensure that Primer Pair 1 will attach to the designated position of the targeted gene. Specific primer design can indeed be done for a limited number of organisms. However, neither of these primer design tools and procedures assures accurate nucleotide matches between primer and target sequence. Therefore, accuracy needs to be ensured by rechecking not only the size of the product but also the position of the primer attachment to the target DNA (Ye et al., 2012). The Primer Map Online Analysis results showed that the predicted attachment location of Primer Pair 1 during the annealing of PCR was completely compatible with the target DNA sequence (Figure 2). The position of Primer Pair 1 was started at the base sequence number 377 to 606 of the target gene, exactly as the primer design results in the prior step of this study. Thus, the *in silico* PCR procedure

using Primer Pair 1 can be carried out with such verification results.



Primer:	Sequence:
Primer Pair 1 - Forward	5'-TCGTCAAACTGCTGCGTATC-3'
Primer Pair 1 - Reverse	5'-GGCATCCGCGTGTAGATAAT-3'

Figure 2. The result of compatibility and primer position in the HDC gene sequence

In Silico PCR Performance

Primer pair specificity was further confirmed by *in silico* PCR on genome sequences of bacteria associated with the production of histamine in fish products. *In silico* PCR program was developed for PCR simulation against up-to-date sequenced prokaryotic genomes. The list of genomes and associated data are updated shortly after their availability at NCBI. Once the bacterial genome is selected, the user must define the primers and set the maximum amplicon length. The primer sequence can include degenerate nucleotides (N), and the maximum allowable amplicon length is 10,000 bp. Additionally, plasmids can be included into the experiment (Bikandi et al., 2004). Advancements in sequencing technology and decreasing costs have led to the sequencing and annotation of numerous genomes in public databases. This abundance of genomic data enables the application of *in silico* PCR. By simulating PCR reactions, *in silico* PCR aids in selecting newly designed primers and identifying potential issues before primer synthesis or laboratory-based experiments (Yu and Zhang, 2011).

Virtual amplification was performed using the total genome of various bacteria from six main genera producing HDC enzymes, namely *Enterobacter*, *Klebsiella*, *Morganella*, *Proteus*, *Photobacterium*, and *Raoultella*, which are recorded in the *in silico* PCR program database (Table 4). A total of 29 strains from the six genera in the *in silico* PCR database were used to test the specificity of the optimized primer pairs. Two strains were from species previously used to construct the primer pairs, namely *E. aerogenes* EA1509E and *E. aerogenes* KCTC 2190. The rest were strains from other species (code numbers 3-12 for different species from the genus *Enterobacter*, code numbers 13-24 for strains from the genus *Klebsiella*, code number 25 for strains from the genus *Morganella*, code numbers 26 for strains from the genus *Photobacterium*, code numbers 27-28 for strains from the genus *Proteus*, and code numbers 29 for strains from the genus *Raoultella*).

Table 4. The list of bacteria producing HDC enzymes used in the *in silico* PCR program

Code Number	Selected Strains
1	*Enterobacter aerogenes* EA1509E
2	*Enterobacter aerogenes* KCTC 2190
3	*Enterobacter asburiae* LF7a
4	*Enterobacter cloacae* EcWSU1
5	*Enterobacter cloacae* SCF1
6	*Enterobacter cloacae* subsp. cloacae ATCC 13047
7	*Enterobacter cloacae* subsp. cloacae ENHKU01
8	*Enterobacter cloacae* subsp. cloacae NCTC 9394 draft genome
9	*Enterobacter cloacae* subsp. dissolvens SDM
10	*Enterobacter sakazakii* ATCC BAA-894
11	*Enterobacter sp.* 638
12	*Enterobacter sp.* R4-368
13	*Klebsiella oxytoca* E718
14	*Klebsiella oxytoca* KCTC 1686
15	*Klebsiella pneumoniae* 342
16	*Klebsiella pneumoniae* CG43
17	*Klebsiella pneumoniae* JM45
18	*Klebsiella pneumoniae* KCTC 2242
19	*Klebsiella pneumoniae* NTUH-K2044
20	*Klebsiella pneumoniae* subsp. pneumoniae 1084
21	*Klebsiella pneumoniae* subsp. pneumoniae HS11286
22	*Klebsiella pneumoniae* subsp. pneumoniae MGH 78578
23	*Klebsiella pneumoniae* subsp. rhinoscleromatis strain SB3432
24	*Klebsiella variicola* At-22
25	*Morganella morganii* subsp. morganii KT
26	*Photobacterium profundum* SS9
27	*Proteus mirabilis* BB2000
28	*Proteus mirabilis* HI4320
29	*Raoultella ornithinolytica* B6

Note: The genomic sequences of highlighted species are used as DNA templates of primer design for *in silico* PCR amplification

In silico PCR amplification was then generated using Primer Pair 1. The results of virtual amplification using *in silico* PCR are presented in Figure 3-4. Based on the presence of amplicons on virtual gel electrophoresis, strains with code numbers 1-2 produced amplicons in the form of single DNA bands with the planned size, which is 230 bp. Primer Pair 1 successfully amplified the target HDC gene only in *E. aerogenes* while showing no results with genomes of other *Enterobacter* species (Figure 3). The results confirm the high specificity of Primer Pair 1 in targeting the HDC gene of *E. aerogenes* without cross-reacting with other *Enterobacter* species. On the other hand, strains with code numbers 3-29 did not show any PCR products (Figure 4a-e). This finding highlights the robustness of the primer design in distinguishing *E. aerogenes* from other closely related bacteria, reinforcing its potential application in rapid molecular detection methods.

Selected strains

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1 - Enterobacter aerogenes EA1509E
2 - Enterobacter aerogenes KCTC 2190
3 - Enterobacter asburiae LF7a
4 - Enterobacter cloacae EcWSU1
5 - Enterobacter cloacae SCF1
6 - Enterobacter cloacae subsp. cloacae ATCC 13047
7 - Enterobacter cloacae subsp. cloacae ENHKU01
8 - Enterobacter cloacae subsp. cloacae NCTC 9394 draft genome
9 - Enterobacter cloacae subsp. dissolvens SDM
10 - Enterobacter sakazakii ATCC BAA-894
11 - Enterobacter sp. 638
12 - Enterobacter sp. R4-368
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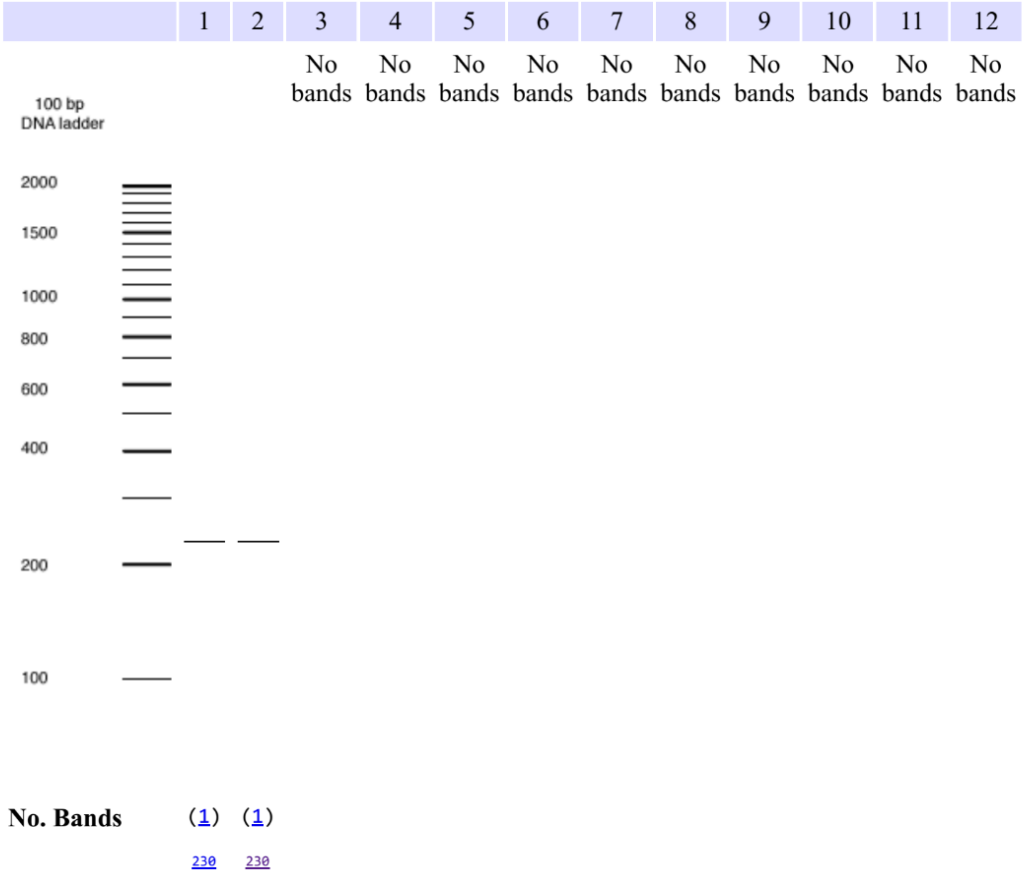


Figure 3. *In silico* PCR result using Primer Pair 1 and 12 strains of the genus *Enterobacter*

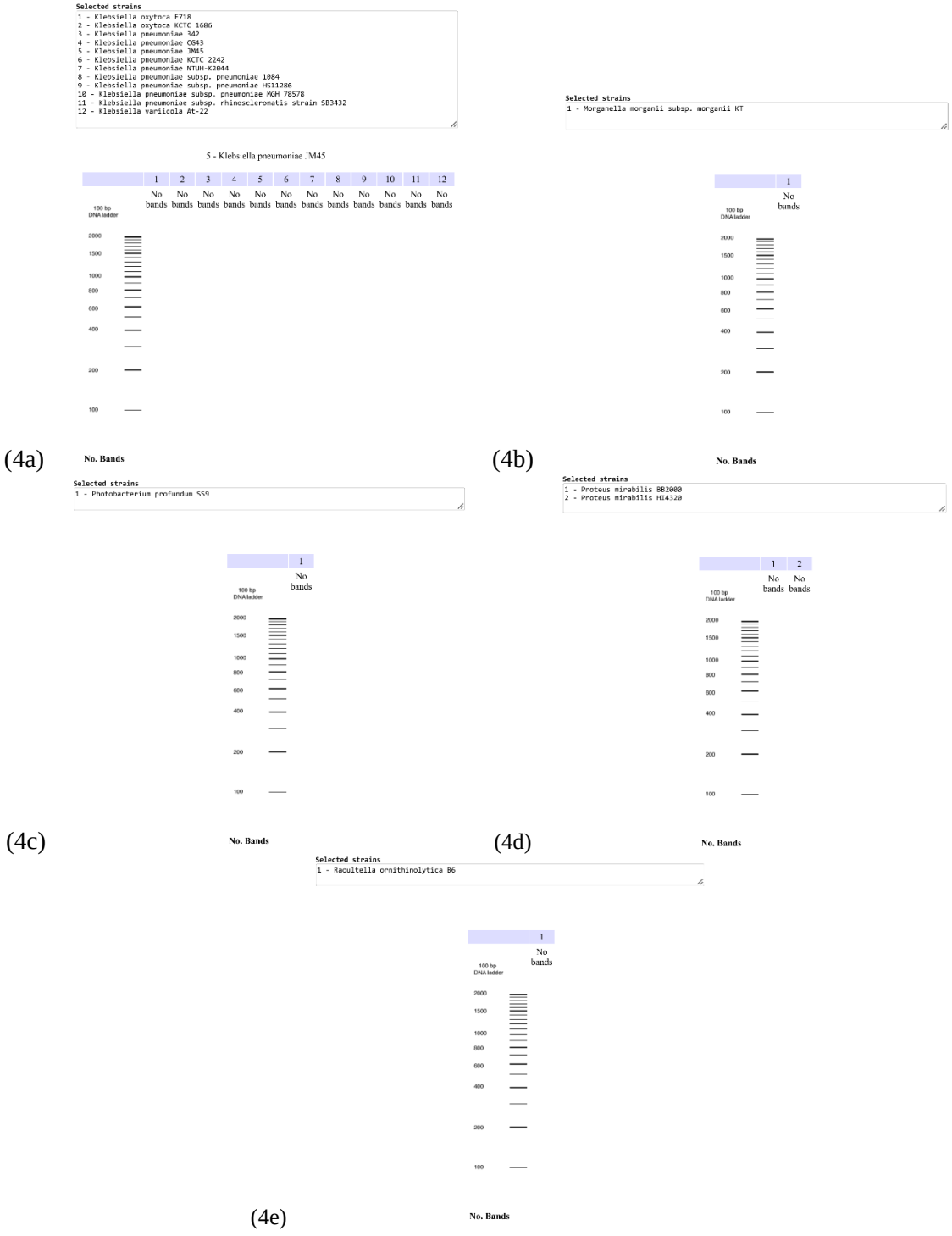
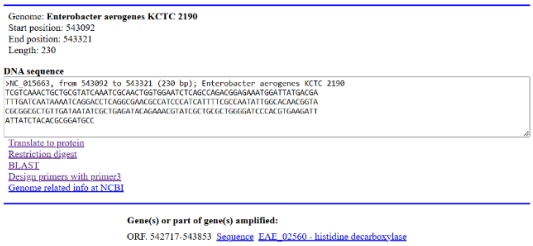


Figure 5. *In silico* PCR result using Primer Pair 1 and 17 strains from five other selected genera: (a) 12 strains of the genus *Klebsiella*; (b) 1 strain of the genus *Morganella*; (c) 1 strain of the genus *Photobacterium*; (d) 2 strains of the genus *Proteus*; and (e) 1 strain of the genus *Raoultella*

PCR Products Verification

After the *in silico* PCR was performed, the correctness of the amplicon products, as viewed from virtual gel electrophoresis, needed verification according to the compatibility of the amplified DNA fragments, both in terms of product size and identity/similarity alignment percentage. The validation of *in silico* PCR performance and results was concluded by evaluating their alignment with target sequences, identifying their location and orientation, and assessing binding efficiency (Kalendar et al., 2024). *E. aerogenes* EA1509E and *E. aerogenes* KCTC 2190 were strains that produced the desired *in silico* PCR product using Primer Pair 1. The amplicon sequences of both strains are virtually collected from the *in silico* PCR program (Figure 5a-b). There are PCR products with the same product size (230 bp) produced from 2 different *E. aerogenes* strains. However, the position of the HDC gene in both strains is in a different sequence from each total genome recorded in the *in silico* PCR database, namely *E. aerogenes* EA1509E at base sequence 4839702-4839931 in template strand of DNA and *E. aerogenes* KCTC 2190 at base sequence 543092-543321 in coding strand of DNA.

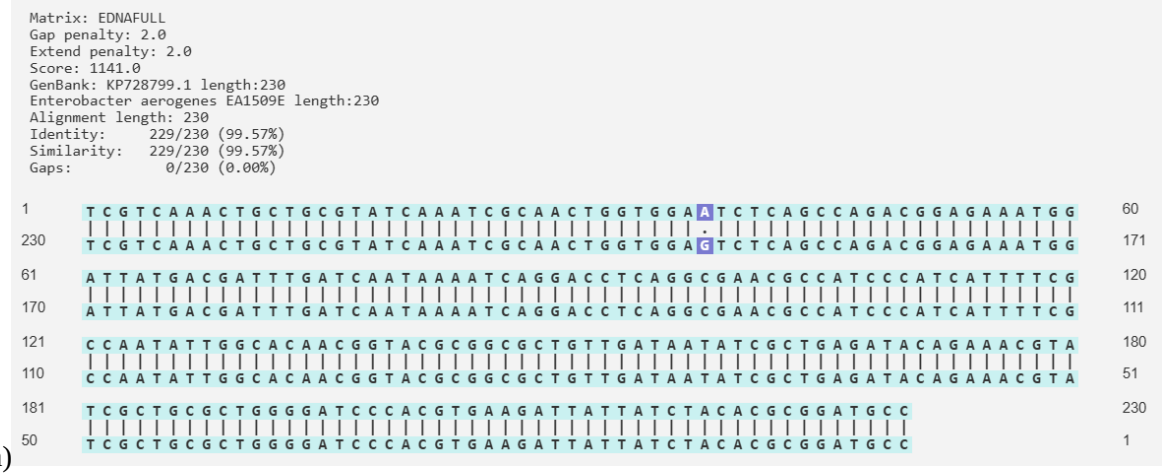
(5a)



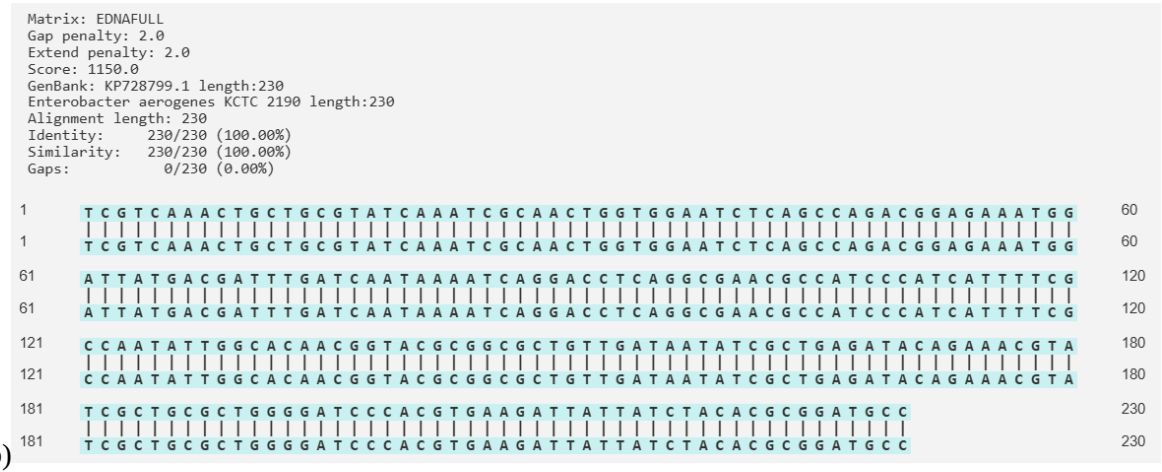
(5b)

Figure 5. The amplicon sequences of the HDC gene performed by *in silico* PCR using Primer Pair 1: (a) *E. aerogenes* EA1509E; (b) *E. aerogenes* KCTC 2190

Amplicon sequences generated from 2 strains of *E. aerogenes* also have compatibility in terms of identity/similarity alignment percentage. The results of VectorBuilder's Sequence Alignment analysis have shown very high compatibilities in the amplicon of both *E. aerogenes* strains (Figure 6a-b). The identity/similarity alignment percentage in *E. aerogenes* EA1509E was recorded at 99.57% with a difference of only 1 base in sequence 38 (one base A in the target sequence was replaced by G), while in *E. aerogenes* KCTC 2190 the percentage reached 100.00%. Based on the overall results of the analysis, Primer Pair 1 can be said to be suitable for use as a biomarker for *in silico* PCR of the HDC gene in *E. aerogenes*.



(6a)



(6b)

Figure 6. The amplicon sequence compatibility performed by VectorBuilder's Sequence Alignment: (a) *E. aerogenes* EA1509E; (b) *E. aerogenes* KCTC 2190

Advantages and Limitation of *In Silico* PCR

In silico PCR presents multiple advantages over traditional bacterial detection methods. While conventional microbiological and biochemical assays require intensive culturing steps and histamine quantification, *in silico* PCR provides a rapid, inexpensive alternative method that does not rely on physical samples or laboratory reagents. This approach enables high-throughput screening of bacterial genomes, making it particularly useful for large-scale food safety assessments. Additionally, it allows researchers to test and optimize primer specificity before conducting in vitro or in vivo experiments, thereby reducing unnecessary trial-and-error in the laboratory. Computational optimization and testing of primers at a genomic scale enables an even higher level of precision and reliability in bacterial identification, ensuring that only the most reliable primers are selected for further application (Bikandi et al., 2004; Ethica et al., 2020a). As a result, *in silico* PCR can serve as a preliminary step in molecular food safety diagnostics, complementing existing detection methods and accelerating the process of pathogen identification (Mohd-Afendy et al., 2013; Ethica et al., 2020b). Further optimization ensured the primer pairs were capable and efficient in identifying the HDC gene correctly, while their speed and applicability for diagnostics in food safety showed potential. The successful design of such PCR primers illustrates the effectiveness of bioinformatics techniques in the advancement of molecular detection methodologies toward a more reliable high-throughput screening of histamine-producing bacteria in fish and fishery products.

Despite its several advantages, *in silico* PCR has certain limitations that must be addressed. The accuracy of this method is highly dependent on the availability of complete and well-annotated bacterial genome sequences in the databases. Any incompleteness or inaccuracy in genomic data might affect the reliability of primer design and specificity testing (Bikandi et al., 2004). However, *in silico* PCR provides only strong theoretical predictions and does not always reliable for potential variations in real-world conditions, such as the efficiency of DNA extraction, primer performance under different PCR conditions, or the presence of inhibitory substances in food samples (San Millán et al., 2013). The specificity of *in silico* PCR results depends on the bioinformatics algorithms used. If the algorithm does not fully consider genomic variations, primer off-target binding, or unintended amplicons, it may lead to false-positive or false-negative predictions (Bikandi et al., 2004; San Millán et al., 2013). Further in vitro PCR validation is essential to confirm the practical applicability of the designed primers.

Implication of Findings and Future Considerations

The findings of this study confirm that the HDC gene in *E. aerogenes* allows for rapid and precise detection using *in silico* PCR, demonstrating the potential of this method as an effective

preliminary screening tool in food safety applications. The ability to identify histamine-producing bacteria at the genetic level provides a proactive approach to preventing histamine accumulation in fish and fishery products, which is crucial for minimizing the risk of foodborne illnesses (Hungerford, 2010; Kanki et al., 2004). By integrating *in silico* PCR into food safety monitoring systems, regulatory bodies and food industries can improve their ability to detect hazardous bacteria and prevent outbreaks of histamine poisoning, thereby enhancing consumer protection and public health (Lehane and Olley, 2000; Visciano et al., 2014). Furthermore, early detection of histamine-producing bacteria enables some intervention strategies, such as improved storage conditions, stricter hygiene measures, and the removal of contaminated products from distribution channels, ultimately reducing the incidence of histamine poisoning in consumers. Future studies should focus on experimentally testing the selected primers in real fish and fishery product samples to assess their sensitivity, specificity, and robustness under standard laboratory conditions. Integration of this *in silico* PCR method into laboratory-based approaches will lead to a more comprehensive and improved approach for histamine-producing bacteria detection, enhanced food safety protocols, and reduced the risk of histamine poisoning incidents worldwide.

CONCLUSION

This study successfully demonstrated the potential of *in silico* PCR as a rapid and reliable method for detecting the Histidine Decarboxylase (HDC) gene in *E. aerogenes*, a key histamine-producing bacterium in fish and fishery products. The optimized Primer Pair 1 was designed with the amplicon size of 230 bp, performed a high specificity, and was successfully validated through virtual PCR simulations, confirming its effectiveness in targeting the HDC gene. The application of *in silico* PCR provides a valuable preliminary screening tool for food safety assessments, offering advantages such as cost-effectiveness, speed, and the ability to analyze multiple bacterial genomes without actual physical samples being required.

Despite its benefits, *in silico* PCR depends on the availability of complete genome sequences and requires further validation through in vitro experiments to confirm its practical value of the results obtained in real food samples. Future research should focus on experimental PCR testing using seafood-derived bacterial isolates to assess primer efficiency under standard laboratory conditions. Integrating *in silico* PCR with traditional molecular detection methods can significantly enhance food safety monitoring, allowing for early identification of histamine-producing bacteria and reducing the risk of histamine-related foodborne illnesses. By leveraging bioinformatics tools for molecular diagnostics, this study contributes to the advancement of rapid food safety detection techniques, ultimately supporting public health efforts in preventing histamine poisoning in seafood consumers.

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