Inhibitory effect of bacteriocin - producing lactic acid bacteria against histamine - producing bacteria isolated from fish

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Abstract

The objective of this present work was to identify the histamine - producing bacteria isolated from fish samples collected from Chidambaram market. The bacteriocin - producing lactic acid bacteria (LAB) isolated from sea water from Vellar estuary was evaluated for the antimicrobial effects of the crude bacteriocin on the growth and histamine accumulation of histamine - producing bacteria. In the present study ten histamine producing bacteria were isolated and identified as Proteus morganii (2), Klebsiella Pneumoniae (3), Citrobacter freundii (2), Enterobacter aerogens (2) and Escherichia coli (1), Among the ten histamine producing bacterial strains, three histamine producing bacteria such as Proteus morganii (H1), Klebsiella pneumonia (H2) and Enterobacter aerogens (H3) produce hdc A gene (370bp) which is responsible for histamine production. The four lactic acid bacterial strains were identified as Lactobacillus casei (L1), Lactobacillus brevis (L2), Lactobacillus fermentum (L3) and Lactobacillus plantarum (L4) found to produce an antibacterial compound with inhibitory activity against the tested histamine - producing bacteria.

The crude bacteriocin of Lactobacillus fermentum(L3) observed highest inhibition against the growth of Proteus morganii (12.5mm). The crude bacteriocin of Lactobacillus casei (L1) showed lowest activity (4.5mm) against Enterobacter aerogens. These antimicrobials therefore play a role in inhibiting the production of histamine in fermented fish products and in preventing seafood-related foodborne disease caused by histamine producing bacteria.

Keywords: Bacteriocin, Histamine, Lactic acid bacteria.

Introduction

Fish and seafood are among the most imperative sources of protein in many parts of the world than other foods; but fish is a highly fresh food that spoils shortly after death if not properly preserved[12]. Feeding rotten fish leads to food poisoning outbreaks such as scombroid poisoning. Scombroid poisoning is also known as histamine poisoning, but because it is a mild disease, it is typically not fully documented in most countries[1].

It is termed as a poisoning associated with certain forms of dark meat consumption of Scombroidae and Scombrosocidae families, which normally contain very large quantities of histidine free in their muscle tissues[11].Histidine is transformed by the microbial decarboxylase enzyme histidine to histamine. One such food poisoning is the consumption of spoiled fresh, frozen fish and tinned fish products which contain remarkably high levels of histamine resulting in histamine fish poisoning[4]. Freshly caught fish have levels of histamine under 2ppm. Fishes that have histamine levels in excess of 20ppm cause aggressive symptoms in humans. According to the Food and Drug Administration (FDA), histamine levels between 20 and 50 ppm showed degradation in the fish. The FDA "action level" for histamine in raw, frozen or canned tuna is 50ppm[5,6].

Fortunately, some lactic acid bacteria (LABs) have been studied in recent years to degrade biogenic amine by producing amine oxidase enzymes or antimicrobials[7]. LAB typically designated as generally recognized as safe (GRAS) status in foods may also have a bio-preservative effect against other microorganisms[17].

As a result of competition for nutrients and/or the development of antagonistic compounds such as organic acids, diacetyl, acetoin, hydrogen peroxide, antibiotics and bacteriocins[17], bacteriocins are defined as ribosomally synthesized and extracelluarly released peptides or protein molecules produced by specific bacteria during the primary phase of growth, though antibiotics are usually secondary metabolites[20]. Brillet et al[3] studied that inhibition of *Listeria monocytogenes* in cold smoked salmon by application of bacteriocin produced by *Carnobacterium divergens* V41 can be used as bio-preservative.

The use of bacteriocins as a bio-preservative in seafoods to increase the shelf-life also inhibits pathogens and avoids food spoilage[3]. The growth range and histamine accumulation of *Streptococcus thermophilus* PRI60 decreased by bacteriocin forming *Lactococci* strains[18]. The previously mentioned studies indicate that bacteriocin applications in food industries can extend food shelf-life, inhibit the growth of foodborne pathogens during food processing, prevent harmful bacteria from forming toxic substances, minimize economic losses due to food spoilage and reduce the use of chemical preservatives[7].

The objective of this study was isolation and identification of the histamine producing bacteria, the genetic basis for histamine production and analysis by polymerase chain reaction, isolation of bacteriocin-producing LAB from sea water and evaluating the inhibitory effects of the crude bacteriocin on the growth and histamine accumulation of histamine-producing bacteria.

Material and Methods

Isolation of histamine producer from seafoods: Fish samples were obtained from the fish market in Chidambaram and they were serially diluted. 0.1ml from 10^{-4}, 10^{-5}, 10^{-6} dilutions were spread plated over the surface of nutrient agar medium. The colonies were observed.

Identification of histamine producer strain: Isolated colonies were plated on the mineral medium incorporated with filter sterilized histidine of 1g/100 ml and kept at room temperature 28± for 24h. The colonies were observed using zone of clearance.

Genetic basis for histamine production

PCR analysis (Location of gene responsible): The procedure for histidine-decarboxylase gene (*hdcA*) amplification and detection consists of 1. Template DNA preparation, 2. DNA Amplification (PCR) and 3. DNA detection or Product conformation by AGE – Agarose Gel Electrophoresis.

Template DNA Preparation

Boiling Lysis method: This method was used to prepare template DNA from test bacterial sample, negative control bacterial and positive control bacteria.

Histamine producer strain was incubated overnight at 37ºC on nutrient agar plates. Thereafter, a colony from the plate was suspended in 100µl of sterile distilled water in a 1.5 ml micro-centrifuge tube and boiled for 10 min. The lysate was chilled on ice and then spun at 6000rpm for 5min in a micro-centrifuge to pellet the debris. 2µl of the supernatant was directly used as template in the PCR reactions. The same method was followed for the preparation of Template DNA from Test bacterial samples.

PCR Reaction conditions: Amplification of the gene was performed in a total volume of 50 µl, containing 100µM (each) of dATP, dCTP, dGTP and dTTP, 100pM of each primer, 10X PCR buffer, 1.5 mM $MgCl_2$, 1.5 U *Taq* DNA polymerase and 0.025% BSA. The above reaction recipe was prepared as 2X Master Mix and used for further reactions.

Primers: Primer sets are: JV16HC/JV17HC (JV16HC: 5'-A GATGGT ATTGTT TCT TAT G-3', JV17HC: 3'-AGACCAT ACACC ATA ACC TT-5'). Primers were synthesized from Bio Serve India Pvt. Ltd., Hyderabad, India. Each primer was used at a concentration of 100pM per reaction

PCR Reaction Recipe

Master Mix	25µl
Template DNA	2µl
Forward Primer	2µl
Reverse Primer	2µl
Water	19µl
Total Volume	50µl

The PCR cycling conditions were: Initial denaturation at 94ºC for 2 minute, denaturation of primer at 94ºC for 1 minute, primer annealing at 60ºC for 1 minute and extension at 72ºC for 2 minute for a total of 35 cycles followed by a 7 minute final extension period. Amplification was performed in a Lark Cycler L125+ thermocycler.

Agarose gel electrophoresis: The PCR products were run on 2% agarose in 1X TAE buffer. The gel was stained with ethidium bromide .10µL of PCR products were loaded into sample wells and 90V was used for 30minutes. The gel was visualized and photographed under transilluminator.

Isolation of *Lactobacillus sp*. from seawater: Marine samples were taken from Vellar estuary and they were serially diluted. 0.1ml from 10^{-4}, 10^{-5}, 10^{-6} dilutions were spread plated over the surface of the MRS agar plates. The plates were incubated at 37˚C for three days. The colonies were observed.

Preparation of culture supernatants: The bacteriocin producing strains was grown in MRS broth (pH 5.5) at 37 °C for 24-30 hrs. The isolated LAB cultures were centrifuged at 10000 rpm for 5 min. and then the supernatant was adjusted to pH 6.5-7.0 with 1 N NaOH[2].

Extraction of crude bacteriocin: The cell free supernatant from Lactic acid bacterial culture was treated with solid ammonium sulphate to 40, 50 and 60 per cent saturation. The mixture was stirred for 2 h at 4 °C and centrifuged at 20000xg for 1 h (4 °C). The precipitate was resuspended in 5 ml of sodium phosphate buffer (50 mM; pH 7.0), then the crude extract was stored at 20 °C.

Inhibition of histamine producer by *Lactobacillus sp.*: 250 ml of nutrient agar was prepared, autoclaved and poured on the plates. It was swabbed with histamine producer colonies. The wells were punctured on the plates and 0.1 ml of crude bacteriocins of *Lactobacillus* bacterial strain was added and kept for incubation at 37^0C for three days.

Results and Discussion

Isolation and identification of histamine producing bacterial strains: In the present study ten histamine producing bacteria from fish samples were collected from Chidambaram market. *Proteus morganii* (2), *Klebsiella Pneumoniae* (3), *Citrobacter freundii* (2), *Enterobacter aerogens* (2) and *Escherichia coli* (1) were identified based on biochemical characterization, the results are given in the table 1 and 2. Among the ten histamine producing bacterial

strains, three histamine producing bacteria such as *Proteus morganii* (H1), *Klebsiella pneumonia* (H2) and *Enterobacter aerogens* (H3) produce hdc A gene(370bp) which is responsible for histamine production successfully amplified by Polymerase chain reaction (Figure 1).

Likewise, the Enterobacteriaceae family is mainly responsible for decomposing the Scombroid fish. Among different enteric bacteria, *Proteus vulgaris Morganella morgini, Enterobacter cloacae, Klebsiella pneumoniae, Hafnia alvei, Proteus mirabilis, Enterobacter aerogens, Serratia planticola, Serratia liquefaciens* and *Citrobacter freundii* have been identified in previous studies as histamine formers in fish[19].

The results are in agreement with the histamine-producing bacteria and bacteriocinogenic lactic acid bacteria (LAB) isolated from Myeolchi-jeot according to sequence analysis of the 16S rRNA gene to determine the inhibitory effects of the bacteriocin on the growth and histamine accumulation of histamine-forming bacteria and to assess the physico-chemical properties of the bacteriocin. Based on 16S rRNA gene sequences, histamine-forming bacteria were identified as *Bacillus licheniformis* MCH01, *Serratia marcescens* MCH02, *Staphylococcus xylosus* MCH03, *Aeromonas hydrophila* MCH04 and *Morganella morganii* MCH05 [16].

Isolation and identification of bacteriocin producing lactic acid bacterial strains: In the present study, lactic acid bacterial isolate was isolated from marine water sample. Microscopic identification of the isolate could determine the rod shaped cells, gram positive, catalase negative, non-motile rods and oxidase negative which showed the usual basic characteristics of Lactobacilli.

Table 1
Identification of Histamine producers

Tests	H1	H2	H3	H4	H5
Gram staining	-	-	-	-	-
Oxidase	-	-	-	-	-
Indole production	-	-	-	-	+
Methyl red	+	-	-	+	+
Voges Proskauer	+	+	+	-	-
Citrate(Simmons)	+	+	+	+	-
H_2O_2 production	+	-	-	+	-
Urea hydrolysis	+	+	-	+	-
Phenylalanine deaminase	+	-	-	-	-
Lysine decarboxylase	-	+	+	-	+
Arginine di hydrolase	-	-	-	+	-
Ornithine decarboxylase	+	-	+	-	-
Motility	+	-	+	+	-
D-Glucose (Acid)	+	+	+	+	+
D-Glucose (Gas)	+	+	+	+	-
Acid production: D-Adonitol	-	+	+	_	-
L-Arabinose	-	+	+	+	+
Cellobiose	-	+	+	+	-
Glycerol	+	+	+	+	+
Lactose	-	+	+	+	-
Maltose	-	+	+	+	+
D-Mannitol	-	+	+	+	+
D-Mannose	-	+	+	+	-
Raffinose	-	+	+	+	+
Sucrose	-	+	+	+	-
D-Xylose	-	+	+	+	+
Nitrate reduction	+	+	+	+	+
Pigment	-	-	-	-	-
Catalase production	+	+	+	+	+
Identified as	*Proteus morganii*	*Klebsiella pneumonia*	*Enterobacter aerogens*	*Citrobacter freundii*	*E.coli*

+ Positive; -negative

Table 2
Identification of Histamine producers

Tests	H6	H7	H8	H9	H10
Gram staining	-	-	-	-	-
Oxidase	-	-	-	-	-
Indole production	-	-	-	-	-
Methyl red	+	-	-	+	-
Voges proskauer	+	+	+	-	+
Citrate (Simmons)	+	+	+	+	+
H_2O_2 production	+	-	-	+	-
Urea hydrolysis	+	+	-	+	+
Phenylalanine deaminase	+	-	-	-	-
Lysine decarboxylase	-	+	+	-	+
Arginine di hydrolase	-	-	-	+	-
Ornithine decarboxylase	+	-	+	-	-
Motility	+	-	+	+	-
D-Glucose (Acid)	+	+	+	+	+
D-Glucose (Gas)	+	+	+	+	+
Acid production: D-Adonitol	-	+	+	_	+
L-Arabinose	-	+	+	+	+
Cellobiose	-	+	+	+	+
Glycerol	+	+	+	+	+
Lactose	-	+	+	+	+
Maltose	-	+	+	+	+
D-Mannitol	-	+	+	+	+
D-Mannose	-	+	+	+	+
Raffinose	-	+	+	+	+
Sucrose	-	+	+	+	+
D-Xylose	-	+	+	+	+
Nitrate reduction	+	+	+	+	+
Pigment	-	-	-	-	-
Catalase production	+	+	+	+	+
Identified as	*Proteus morganii*	*Klebsiella pneumonia*	*Enterobacter aerogens*	*Citrobacter freundii*	*Klebsiella pneumonia*

+ Positive; -negative

Based on the biochemical characterization and carbohydrate utilization, pattern of bacterial isolates was identified as *Lactobacillus casei* (L1), *Lactobacillus brevis* (L2), *Lactobacillus fermentum* (L3) and *Lactobacillus plantarum* (L4). The results are presented in table 3. Similar characters for lactic acid bacteria were described earlier by Kandler and Weiss[9].

In previous studies the lactic acid bacteria were isolated and characterized from fish and prawn[15]. From marine environment, *Lactobacillus lactis* strain was isolated and the bacteriocin showed broad range of inhibitory activity against some important food borne bacterial pathogens[14].

Inhibitory activity of crude bacteriocin of lactic acid bacteria against the histamine producing Bacteria: As shown in the table 4, the four lactic acid bacterial strains isolated from sea water were found to produce an inhibitory activity against histamine producing bacteria such as *Proteus morganii* (H1), *Klebsiella pneumonia* (H2) and *Enterobacter aerogens* (H3). The crude bacteriocin of *Lactoobacillus fermentum* (L3) inhibited the growth *of Proteus morganii* (12.5mm) and *Klebsiella pneumoniae* (11.7mm).

The crude bacteriocin of *Lactoobacillus plantarum* (L4) inhibited the growth of *Klebsiella pneumonia* (10.7mm). The crude bacteriocin of *Lactobacillus brevis* (L2) inhibited the growth of *Klebsiella pneumonia (10.1mm).* The crude bacteriocin of *Lactobacillus casei* (L1) showed lowest activity (4.5mm) against *Enterobacter aerogens.*

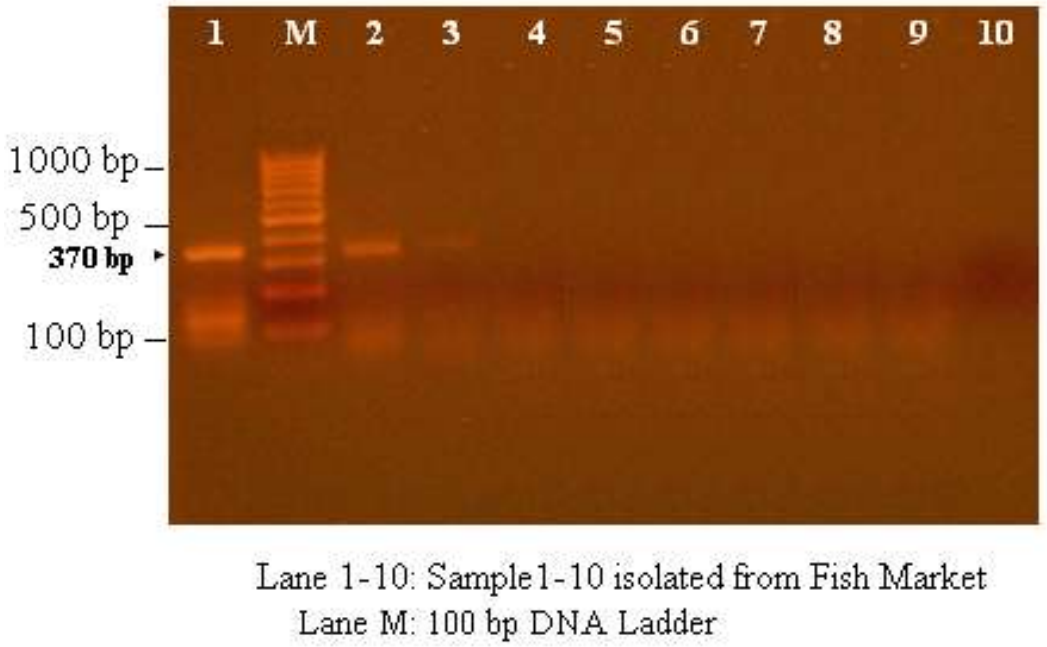
Table 3
Morphological and biochemical characteristics of lactic acid bacterial strains

Morphological Characters	**L1**	**L2**	**L3**	**L4**
Gram reaction	+	+	+	+
Spores	-	-	-	-
Shape	Rods	Rods	Rods	Rods
Size	0.8 µm x 2 µm	1.0 µm x 3 µm	0.7 µm x 2 µm	0.5µm x 0.8 µm
Motility	-	-	-	-
Biochemical characters				
Catalase test	-	-	-	-
Oxidase test	-	-	-	-
NH3from arginine	-	+	+	-
Gas production from glucose	-	+	+	-
Carbohydrates				
Arabinose	-	+	D	D
Cellobiose	+	-	D	+
Esculin	+	D	-	+
Fructose	+	+	+	+
Galactose	+	D	+	+
Glucose	+	+	+	+
Lactose	D	D	+	+
Maltose	+	+	+	+
Mannitol	+	-	-	+
Mannose	+	-	+	+
Melezitose	+	-	-	D
Melibiose	-	D	+	+
Raffinose	-	-	+	+
Rhamnose	-	+	-	-
Ribose	+	-	+	+
Salicin	+	-	-	+
Sorbitol	+	-	-	+
Sucrose	+	D	+	+
Trehalose	+	-	D	+
Xylose	-	D	D	D
Identified as	*Lactobacillus casei*	*Lactobacillus brevis*	*Lactobacillus fermentum*	*Lactobacillus plantarum*

+ Positive; -negative; D-delayed reaction

Table 4
Inhibitory activity of crude bacteriocin of lactic acid bacteria against the histamine producing Bacteria

Lactobacillus spp.	**Histamine producing strains Zone of inhibition (mm)**		
	***Proteus morganii* (H1)**	***Klebsiella pneumonia* (H2)**	***Enterobacter aerogenes* (H3)**
Lactobacillus casei (L1)	7.5	5.3	4.5
Lactobacillus brevis(L2)	9.2	10.1	8.10
Lactobacillus fermentum (L3)	12.5	11.7	8.4
Lactobacillus plantarum (L4)	9.0	10.7	7.0



Proteus morganii, L2 - *Klebsiella Pneumoniae*, L 3- *Enterobacter aerogens* L4-*Citrobacter freundii* , L5-*Escherichia coli*,L6- *Proteus morganii*,L7- *Klebsiella Pneumoniae*,L8- *Enterobacter aerogens*,L9- *Citrobacter freundii*,L10- *Klebsiella Pneumoniae.*

Figure 1: Ethidium Bromide Stained 1.2% Agarose Gel showing 370 bp amplified product (Lane 1-3) of hdc A gene of Histamine producing strains isolated from Fish market samples by using PCR.

Similarly, the earlier studies demonstrated that growth of histamine producing bacteria *L. buchneri* St2A and its histamine production in cheese samples was controlled by the bacteriocin producing *Enterococci* and *L. lactis* used as starter cultures for cheese production. Our findings are in line with the findings from previous studies[8]. The histamine producing strain *S. thermophilus P*RI60 was inhibited by nisin Z producing *L. lactis subsp. lactis* VR84. However, *L. Subsp lactis. Lactis* EG46-produced lacticin 481 did not demonstrate a lethal action against PRI60 strain but was able to reduce its growth range and accumulation of histamine [19]. In another study the histamine-forming bacteria such *as Pseudomonas sp., Proteus morganii and Micrococcus sp* were inhibited by the bacteriocin producing lactic acid bacteria *L. casei* [13].

S. xylosus No. 0538 obtained from salted and fermented anchovy (Myeolchi-jeot) not only possessed a greater ability to degrade histamine but also a measurable ability to degrade tyramine However, this strain was also found to produce the inhibitory substance(s) identical to bacteriocin and to have the highest antimicrobial activity against *B. licheniformis* strains identified as producing amines. *S. xylosus* no. 0538 demonstrated substantially greater ability to degrade histamine, degrading histamine to approximately 62–68 per cent of its initial concentration within 24 hours.[10]

Conclusion

In conclusion, the bacteriocin producing lactic acid bacteria such as (*Lactobacillus casei* (L1), *Lactobacillus brevis* (L2), *Lactobacillus fermentum* (L3) and *Lactobacillus plantarum* (L4)) reduce the growth of histamine producers increasing the shelf life and also maintain the hygienic quality of fish and seafood products, so it can be used as food bio-preservative for sea food products.

In particular, these inhibitory substances can play a role in controlling histamine production in the fermented fish products and preventing seafood-related food-borne disease caused by bacterially-generated histamine.

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