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ISOLATION AND MOLECULAR CHARACTERISATION OF HISTAMINE PRODUCING BACTERIA ISOLATED FROM FISH

S. BHUVANESWARI[1], G. SUBASHINI[1], K. CHITRA DEVI[1], S. UMAMAHESWARI[2]

1. Assistant Professor, Department of Microbiology, Shrimati Indira Gandhi College, Trichy-2, India.
2. Department of Microbiology, Shrimati Indira Gandhi College, Trichy-2, India

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Abstract: Histamine poisoning is one of the most common chemically induced sea foodborne llnesses reported in the United States Today. U. S. Food and Drug Administration (FDA) promulgated industry guidelines in 1994 to establish procedures for safe processing and importing of fish and fishery products on the basis of the hazards analysis critical control point (HACCP) approach .The most recent HACCP guidelines for control of histamine production recommend specific time and temperature limits for potentially hazardous fish on the basis of species, size, and water temperature at harvest (24). The FDA recommends that fish be placed in a cooling medium or be cooled to a specific temperature within a prescribed period of time. Primary processors bear the burden of proof that proper cooling techniques have been used from harvest to receipt of fish and are expected to implement the necessary cooling guidelines that are intended to achieve a core temperature of 4.4°C or less and maintain this temperature throughout handling, processing and distribution. The present study aimed to isolate the histamine producing bacteria isolated from Scombroid fish. The isolate bacteria was further characterised by using various specific Media (Macconkey, TCBS, Cetrimide and Mannitol salt agar Medium) .The 16s rDNA study was conducted using specific primers and PCR amplification was performed .The gene sequence study was performed and identified as *Pseudomonas aeruginosa*. Further the Histamine producing bacteria was characterised by using UV-Visible, FTIR, and TLC studied.

Keywords: Histamine, *Pseudomonas aeruginosa,* Biogenic amines, 16s rDNA.

Corresponding Author: MS. S. BHUVANESWARI

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INTRODUCTION

Histamine poisoning is the most common foodborne problems caused by Biogenic amines. Histamine is a biogenic amine produced during microbial decomposition of scombroid fish such as mackerel as well as non scombroid fish such as sardines. Histamine fish poisoning (HFP) is described as a food borne chemical intoxication primarily caused by intake of fish muscle containing on elevated amount of histamine. Intake of high level of histamine leads to life threatening food intoxication, food intolerance and allergic reaction. Thus, histamine is a major seafood safety concern (Tao *et al*., 2011). This Histamine intoxication causes cardiovascular, gastrointestinal, and neurological symptoms, such as skin rashes, urticarial, oedema, local inflammation, nausea, vomiting, diarrhoea, cramping, hypotension, headache, palpitation, and oral burning. The symptoms usually resolve within 24 hrs**.** During the food intake process in the human gut, low amounts of histamine are metabolised to a physiologically less active degradation product. However, upon intake of a great amount of histamine with food, the detoxification system is unable to eliminate histamine sufficiently**.** Biogenic amines are produced by microorganisms that are capable of decarboxylating the carboxylic group of amino acids.(Joshi *et al.,*2011). The most commonly reported biogenic amines occurring in food and beverages are histamine, beta-phenylethylamine, tyramine, tryptamine, putrescine, cadaverine, spermine and spermadine. Among these, histamine poisoning is frequently reported linked to consumption of protein rich food such as fish and fish products.

Histamine can cause food poisoning, several quantitative methods have been developed. Early detection of histamine-producing bacteria is very important in the food industry. Microbiological screening method have been developed based on the use of a differential medium containing a pH indicator**.** The most frequently occurring microorganisms associated with fish histamine poisoning are *Enterobactericeae, Morganella morganii, Klebsiella pneumonia,* and *Hafnia.* However, a variety of bacteria capable of producing histamine has been identified in fish.(Hungerford 2010).The US Food and Drug Administration (FDA) have established a defect action level of 50 ppm for fish and fish product

MATERIALS AND METHODS

The fish *(Cyprinus carpio)* sample was collected from Echanary, in Coimbatore district. The fish muscle tissue obtained from each of three locations (head, belly, tail) by swab Method and the swab was immediately placed on sterile 0.1% of peptone water. After sample collection were used for microbiological analysis.

IDENTIFICATION OF BACTERIAL ISOLATES

Fish samples were serially diluted in sterile 0.1% of peptone water and the samples were streaked on the Macconkey agar plate and incubated at 37ºC for 24 hours.Then Gram staining and Motility test was done. Various biochemical tests were carried out according to standard methods such as IMVIC Test,Triple sugar iron test,Citrate utilisation test . (Binaya *et al*.,2013)

CULTURING IN PRODUCTION MEDIUM

The bacterial isolates were cultured in specific medium for histamine production containing Tryptone - 0.5%,Yeast extract -2.0%,L-histidine -0.5%,Nacl -1.5%,Distilled water-100ml.

QUANTIFICATION OF HISTAMINE

Quantification of histamine was carried out using calorimetric method. The absorbance of the color produced was measured immediately after 5 minutes at 496nm. The concentration of histamine in sample was measured at 496nm. The histamine concentration in sample was estimated using the following formula;

$$\text{Histamine (mg/100g)} = \frac{A \times 2 \times 25 \times 100}{5 \times 1000}$$

$$= A \text{ mg /100g}$$

Paper chromatography was done in a beaker containing solution for 5-10 min in order to saturated with solvent vapour. After 10 min yellow-voilet color indicate the presence of histamine. The Rf (Retardation factor) values was measured then The TLC plate was prepared.Then 50µl of sample was loaded on the TLC plate and allow to run in the solvent system consisting of methanol, chloroform, glacial acetic acid, formic acid, distilled water (5:2:1:1:1)mixture. TLC plate was kept over the beaker containing solution for 5-10 min in order it to get saturated with solvent vapour. After 10 min yellow-brown color indicate the presence of histamine. The Rf (Retardation factor) values was measured by the following formula,

$$Rf = \frac{\text{Distance travelled by solute}}{\text{Distance travelled by solvent}}$$

ISOLATION OF DNA (Rajan and Selvi Christy, 2010)

16_Sr DNA ANALYSIS

Total genomic DNA was extracted from 5 ml overnight NB culture of the purified isolates. PCR was performed in a light cycler Eppendorf PCR machine. A1300 bp fragment was obtained by PCR amplification of the16S rDNA gene (Alvarez, 2010) using the primers:

F: 5-AGAGTTTGATCMTGGCTCAG-3

R: 5-CGGGCGGTGTGTACAAGG-3

FISH PRIMER

F1- primer-Furofins Genomics (45-6011-3/4)

F2- primer-Furofins Genomics (45-6011-1/4)

The PCR mixture was composed of 100 ng of genomic DNA, 30 p mol of each primer, 200 lM of dNTPs, 1U of Taq polymerase and 10 µl of 10X PCR reaction buffer, and the reaction volume was adjusted to 100 µl in 0.5 ml Eppendorf tube. The PCR amplification conditions were performed by an initial denaturation step at 94 ^{o}C for 10 min followed by 30 denaturation cycle set 94 ^{o}C for 1 min, annealing at 60 ^{o}C for 1 min and an extension at 72 ^{o}C for 1 min followed by a final extension step at 72 ^{o}C for10 min. Amplicons of 16S rDNA were purified using PCR purification kit (Applied Biosystem 96 well Multi well PCR) PCR Purification Kit, Thermo Scientific). Each of these purified products was sequenced by the chain terminator method (Bioneer Company, Korea), using the two corresponding PCR primers separately. The resulted DNA sequences were phylogenetically analyzed using the BLAST search program. Multiple sequence alignment and molecular phylogeny were performed using MEGA 5.0 software .DNA sequencing was performed by and 16S rDNA sequences were BLAST searched against Gene Bank database (http://www.ncbi.nlm.nih.gov/). Phylogenetic tree was constructed using Treeview software (Page, 1996).Finally Histamine was estimated by using Nanodrop spectrophotometric method compared with standard.

RESULTS

Histamine producing bacteria were isolated from fish sample.Colony morphology of bacterial species were studied by Gram staining. Gram positive and Gram negative bacteria were determined.There was two bacterial species was identified and screened by various biochemical tests.In this investigation *Klebsiella pneumonia* and *Pseudomonas aeruginosa* were isolated (Table 1). The histamine content was estimated by using nano drop spectroscopic method and compared with standard using Nanodrop spectrophotometric method. (Table 2)

TABLE: 1 Morphological and bio chemical characteristics of an organisms

TESTS	**BACTERIAL ISOLATES**	
	K.pneumoniae	***P.aeruginosa***
Culture characteristics	Grey, shiny and mucoid colonies	Pale yellow colonies
Gram staining	-	-
Shape	Rods	Rods
Motility	Non Motile	Motile
Indole	–	–
MR	–	–
VP	+	–
TSI	A/A	AK
Citrate	+	+

'AK' – Alkaline 'AA' - Acid

Table: 2 Estimation of Histamine

S.No	**S1**	**S2**	**Standard**
1	0.048	0.032	0.059
2	0.089	0.041	0.094
3	0.125	0.112	0.126
4	0.247	0.341	0.247
5	0.624	0.456	0.645
6	0.246	0.124	0.254

Figure 1: DNA isolation

1 2

1. DNA of *Pseudomonas aeruginosa*

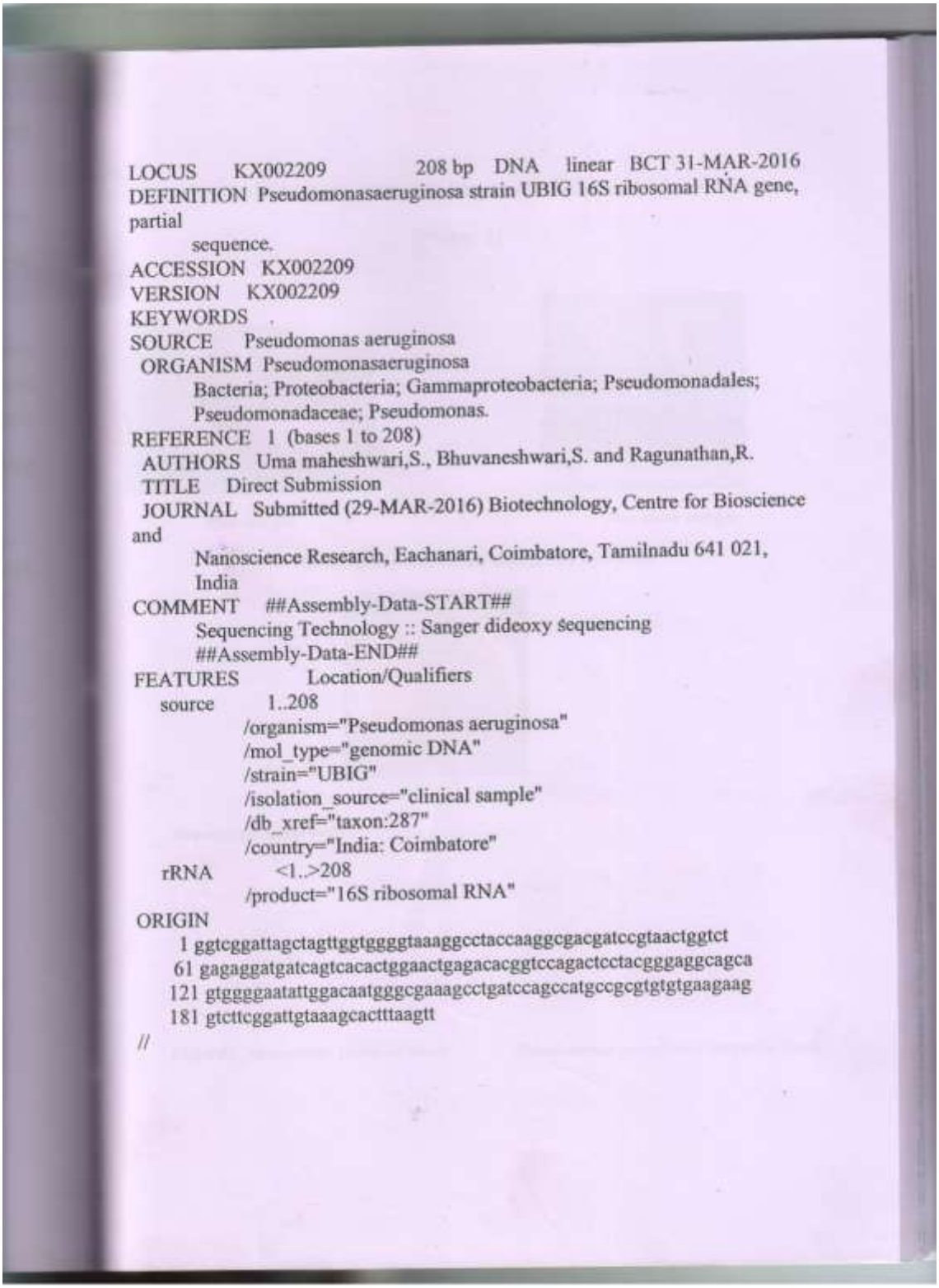
2. DNA of *Klebsiella pneumoniae*

In Paper chromatography the Rf value was calculated *Klebsiella* 0.69 and *Pseudomonas* as 0.75. which is indicates the presence of histamine in the production medium.The TLC showed that histamine was present in both *Klebsiella sp* and *Pseudomonas sp* due to the formation of yellow to brown color band. The Rf value was measured and calculated as 0.32 required for *Klebsiella sp* and 0.37 required for *Pseudomonas sp*. which indicates the presence of histamine in the production medium **.**The DNA molecular weight of the DNA was found to be 856 and 984 Kbs respectively for S_1 and S_2 (Figure 1).

PCR- 16_{sr} RNA

The isolated DNA was further amplified using 16_{sr} RNA universal bacterial primer. The PCR amplified product molecular weight was found to 746 Kbs respectively. PCR product of fish histamine was found to be 848 kbs respectively

PLATE 1: Sequencing of 16srDNA of the isolate.



LOCUS KX002209 208 bp DNA linear BCT 31-MAR-2016
DEFINITION Pseudomonasaeruginosa strain UBIG 16S ribosomal RNA gene, partial
sequence.
ACCESSION KX002209
VERSION KX002209
KEYWORDS .
SOURCE Pseudomonas aeruginosa
ORGANISM Pseudomonasaeruginosa
Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales;
Pseudomonadaceae; Pseudomonas.
REFERENCE 1 (bases 1 to 208)
AUTHORS Uma maheshwari,S., Bhuvaneshwari,S. and Ragunathan,R.
TITLE Direct Submission
JOURNAL Submitted (29-MAR-2016) Biotechnology, Centre for Bioscience and
Nanoscience Research, Eachanari, Coimbatore, Tamilnadu 641 021,
India
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Sequencing Technology :: Sanger dideoxy sequencing
##Assembly-Data-END##
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/isolation_source="clinical sample"
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/country="India: Coimbatore"
rRNA <1..>208
/product="16S ribosomal RNA"
ORIGIN
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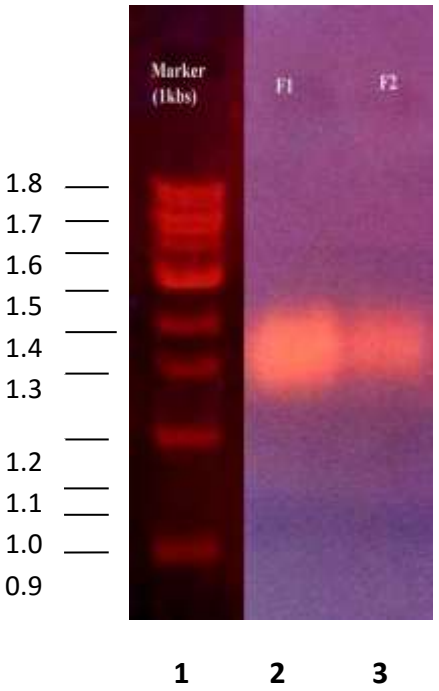
61 gagaggatgatcagtcacactggaactgagacacggtccagactcctacgggaggcagca
121 gtggggaatattggacaatgggcgaaagcctgatccagccatgccgcgtgtgtgaagaag
181 gtcttcggattgtaaagcactttaagtt
//

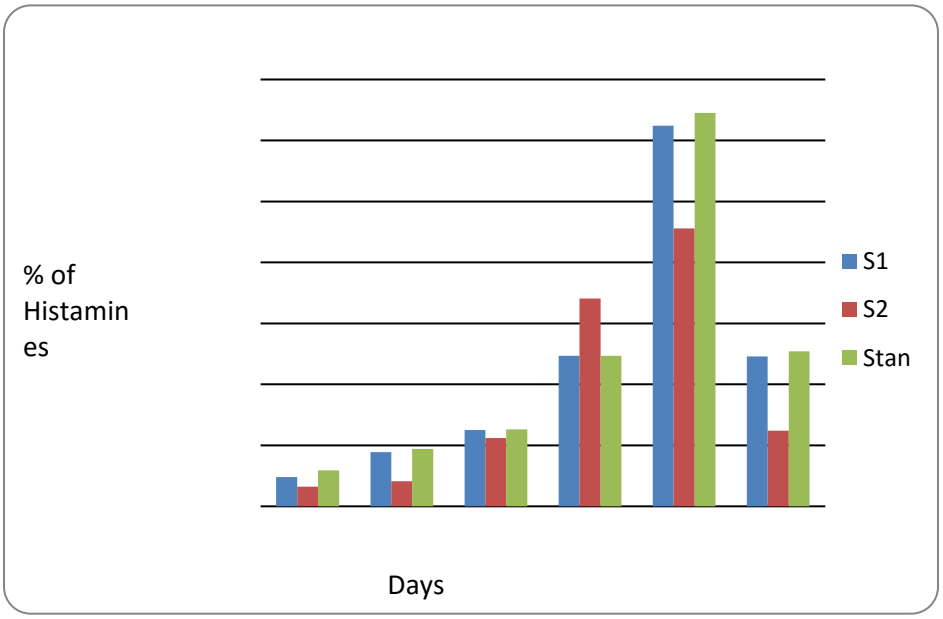
FIGURE 2: PCR amplification

Histamine Producing gene (Fish primer)



1. **Standard marker**
2. ***Pseudomonas aeruginosa***
3. ***Klebsiella pneumoniae***

Figure 3: Estimation of histamine by Nanodrop Spectroscopic method



DISCUSSION

Bacteria was isolated from the skin surface of the fish may be swabbed over over the surface of Nutrient rich medium (Spanggaard *et al*., 2000).

In our study the bacteria was isolate from by skin surface of the fish by swabbed method and serial dilution was done by 1% of peptone broth and then it was streaked on the nutrient medium after the incubation period the further studies was carried out. Histamine is produced by wide range of bacteria but major histamine producing bacteria in fish are Gram negative bacteria such as *Morganella morganii, Photobacterium damselea, Enterobacter aerogenes, Hafnia alvei, Citrobacter freundii, and Escherichia coli* (Ienistea *et al*., 1971).

In our study, we observed two Gram negative histamine producing bacteria were isolated such as P*seudomonas aeruginosa, Klebsiella pneumonia.* The number of methods were used for the histamine testing. In contrast to many of the other seafood toxins, the high action levels established for histamine in fish allow for its detection by a variety of different approaches ranging from simple and inexpensive Thin Layer Chromatography (TLC), paper chromatography, more powerful Liquid Chromatography- mass Spectrometry (LC-MS). High performance liquid chromatography (HPLC) with detection scheme based on precoloumn derivatization. (Brillantes and Samosarn, 2001).

In this study, histamine compound is separated by (TLC), paper chromatography was performed and its confirmed by color changes. Violet color is refered for paper chromatography and brown color refered for the TLC.

Early detection of histamine producing bacteria is very important in the food industry. Microbiological screening methods have been developed based on the use of a differential medium containing a pH indicator. However, DNA based procedures, which focus on the nucleic acid composition of the bacterial genome, are subjected to less time consuming than phenotyphic characterization. Polymerase chain reaction offers a rapid and specific means of detecting and identifying histamine producing bacteria (Bover-Cid and Holzapfel, 1999).

In this study the bacterial DNA was isolated and the PCR gene amplification was carried out using specific primers. The PCR product was subjected to the gene sequencing. The bacterial gene sequence was submitted to NCBI and identified as *Pseudomonas aeruginosa.* The NCBI accession number was KX002209.

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