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Research Article

Occurrence of Histamine Forming Bacteria and Histamine in Dried Fish

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Abstract: Natural spoilage of protein rich foods leads to the production of histamine, which is associated with histamine poisoning throughout the world. Fish and fish products are at times associated with major concern due to the presence of histamine, which at toxic level generates histamine or scombroid poisoning known to be contaminated with high levels of histamine leading to histamine poisoning. Histamine consumption is also considered to be of health concerns at chronic levels due to effects on the physiological functions. Dried fish are known to be one of the most popular fish products consumed in the Indian subcontinent, often prepared under unhygienic conditions. In this present study, fifteen dried fish sold in local retail markets were purchased and screened to determine the occurrence of histamine and histamine-forming bacteria. The levels of pH, salt, moisture content and Aerobic Plate Count (APC) were determined in all samples and ranged from 5.46 to 7.07, 0.5 to 1.19%, 33 to 77.05% and 10.5 to 11.4 log CFU/g respectively. Two strains of *Enterobactercancerogenous* PUFSTFMDf01 and *Enterobacter* sp., PUFSTFMDf02 were identified as histamine forming bacteria in dried fish samples. All fifteen dried fish samples contained an average histamine content of 10.03 mg/100 g which is greater than the guideline value (5 mg/100 g) suggested by USFDA.

Keywords: Dried fish, *Enterobacter*, food safety, food spoilage, histamine, histamine forming bacteria

INTRODUCTION

Drying food is the one of the ancient known food preservation method and is often used even today for the preservation of various foods including fish and Fish is one of the highly perishable food products and is often preserved through such traditional methods such as smoking, salting and drying Processing of sea food by salting and drying involves several steps including back-cutting, degutting, salting and sun-drying. Dried fish has a shelf life of several months to years if stored efficiently. Fish is one of the products with high protein content and the meat is fast degraded by spoilage microorganisms leading to production of undesirable products such as histamine on mild spoilage, production of undesirable changes in the meat and further making the product undesirable for consumption. Salted fish products are known to contain histamine forming bacteria, which on proliferation under favorable conditions may contribute to the increase of toxic amines, leading to histamine poisoning (Jeyasekaran and Jeyashakila, 2003). Histamine at elevated levels generates food poisoning or food allergic condition. Histamine intoxication is possibly the best known food-borne illness associated with eating fish, with a range of symptoms including rash, nausea, urticaria, diarrhea, vomiting, tingling, flushing and itching of the skin. The severity of the symptoms can vary with the amount ingested and the individual's sensitivity to it (Lehane and Olley, 2000). Scombroid fish such as mackerel, tuna, saury and bonito that contain high levels of free histidine in their muscle are often implicated in scombroid poisoning incidents (Taylor, 1986). The dried product acquires a hard consistency with low water activity and high salt content (5-25%). However, large amounts of histamine have often been detected in commercial fishery products of India, including salt-dried products, which are not subjected to thermal treatment, could be the cause of some histamine outbreaks (Chakrabarti, 1991, 1993). Histamine-forming bacteria were found to be high in salted Indian Ilisha, Lethrinids, Tiger perch and Seer fish sold in India (Jeyasekaran and Jeyashakila, 2003). Aim of the proposed study is to screen the histamine forming bacteria and histamine in the market samples of dried fish collected locally.

MATERIALS AND METHODS

Sample collection: A total of 15 dried fish samples were collected from local market. All the samples were collected in sterile containers, transferred to laboratory and subjected to microbiological analysis.

Microbiological analysis of histamine forming bacteria: A portion of the dried fish samples were

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serially diluted with a sterile phosphate buffer saline and 0.1 mL aliquots of the diluents were inoculated into Aerobic Plate Count (APC) agar. The plates were incubated at 35°C for 48 h and the colonies were counted. The bacterial counts in the samples were expressed as Colony Forming Units (CFU/g). Representative isolates were selected and streaked on Nutrient agar plates. Isolates were purified by sequential streaking on MRS agar plates and incubated at 37°C for 48 h. A 0.1 mL aliquot of the diluted sample was spread on Histamine-forming Bacterium Isolation (HBI) agar fortified with L-histidine (Niven *et al*., 1981). The modified Niven's agar plates were incubated at 35°C for 48-72 h purple color colonies shown positive. The positive colonies were picked and streaked onto Trypticase Soy Agar (TSA) to obtain pure cultures.

pH, salt, moisture and nitrogen content determination: Dried fish samples (10 g) were homogenized in sterile blenders with 10 mL of distilled water to make thick slurry. The pH of this slurry was then measured using pH meter. The salt content in each sample was determined according to the AOAC (1995) procedures by homogenizing 2 g of dried fish sample with 18 mL of distilled water. The homogenate was titrated with 0.1 M $AgNO_3$ using 10% w/v K_2CrO_4 solution as an indicator. The moisture content was conducted by drying 3 g of a test sample at 102.0±2.0°C under atmospheric pressure for 2 h. The nitrogen content of the sample was determined by the standard kjedhal procedure of the AOAC (1995).

Identification of histamine forming bacteria: Histamine positive isolates were identified on the basis of colony morphology, gram stain, endospore stain, oxidase and catalase presumptive test. The histamine forming isolates were further confirmed by amplifying and sequencing 1400 bp of the 16_S rDNA. Amplification was performed using the universal primers UNI-L (5-AGAGTTTGATCATGGCTCAG-3) and UNI-R (5-GTGTGACGGGCGGTGTGTAC-3) (Kuhnert *et al*., 1996, 2000). Bacterial cells were cultured overnight in 2 mL of Trypticase Soy Broth at 35°C and then 1.5 mL of grown bacterial cells were harvested by centrifugation at 13,000-16000 rpm for 2 min at room temperature. The cell pellet was processed for template DNA extraction using spin column kit (Himedia, Mumbai). PCR amplification was performed in 50 µL reaction mixture containing 10 mMTris-HCl (pH 8.3), 50 mMKCl, 1.5 mM $MgCl_2$, 20 pmol of each primer, a 0.2 mM concentration each of the four dNTP's, 2.5 U/100 µL of Taq DNA polymerase (Sigma-Aldrich, USA) and template DNA (10 ng). Amplifications were carried out for 35 cycles (94°C for 30 sec, 55°C for 30 sec and 72°C for 60 sec) in Eppendorff Gradient PCR with an initial denaturation (94°C for 4 min) and a final extension (72°C for 7 min) (Kuhnert *et al*., 1996, 2000). Amplified PCR products were detected by electrophoresis on a 1.5% agarose gel staining with ethidium bromide. Amplicons were purified, PCR purification Kit. The sequences were analyzed with the BLAST (NCBI) for the identification of histamine-forming bacteria.

Histamine analysis:

Standard preparation: Histamine dihydrochloride was dissolved in 50 mL of 0.1 M HCl and used as the standard stock solutions.

Sample preparation: Five grams of dried fish sample was ground and transferred to 50 mL centrifuge tubes. The sample was homogenized for 3 min with 20 mL of 6% Trichloroacetic Acid (TCA), centrifuged (10,000 g, 10 min, 4°C) and filtered through Whatman No. 2 filter paper. TCA was added to the filtrates to bring a final volume of 50 mL. Standard histamine solutions and 2 mL aliquots of the food sample extracts were derivatized with benzoyl chloride according to Hwang *et al*. (1997) method. The benzoyl derivatives were dissolved in 1 mL of methanol and 20 µL aliquots were injected for HPLC analysis.

HPLC conditions: The contents of the histamine in the test samples were determined according to Hwang *et al*. (1997) method with slight modification. The detection of histamine was performed using Prominence Ultra-Fast Liquid Chromatographic (UFLC) system (Shimadzu, Japan). Model LC20AD with PDA Detector (set at 233 nm) C18 column (250×4.6 mm) was used for separation. The gradient elution program began with 50:50 (v/v) methanol: water at a flow rate of 0.8 mL/min for 0.5 min, followed by a linear increase to 85:15 methanol: water during the next 6.5 min. the methanol: water mix held constant at 85:15 for 5 min and then decrease to 50:50 (0.8 mL/min) during the next 2 min. Histamine standard was analyzed with test samples to check the chromatographic consistency. The samples were injected twice. Peak heights of the histamine standard were used to prepare the standard curves for determination of histamine concentration in the test sample.

Statistical analysis: Pearson correlation was carried out to determine relationships between pH, salt content, APC, protein, nitrogen and histamine contents in the 15 dried fish samples. All statistical analyses were performed using the Statistical Package for Social Sciences, SPSS Version 19.0 for windows (SPSS Inc., Chicago, IL, USA).

RESULTS AND DISCUSSION

Table 1 shows the microbiological analysis of the dried fish samples. The total count of bacteria on MRS

Table 1: Microbial profile of dried fish samples

Source	Aerobic Plate Count (APC) log cfu/g	Total count on MRS agar log cfu/g	Total count on Niven's agar log cfu/g
Ribbon fish	10.90±0.55	07.26±0.50	06.93±0.45
Tilapia	11.20±0.37	07.40±0.45	06.56±0.11
Sardine	11.06±0.57	06.96±0.58	06.06±0.51
Anchovy	10.50±0.10	06.80±0.86	05.80±0.30
Indian mackerel	11.30±0.15	07.10±0.53	06.40±0.53

Table 2: pH, salt content and histamine content in dried fish products

Source	pH	Salt content (%)	Histamine level (mg/100 g)
Ribbon fish	7.07±0.80	0.75±0.07	06.00±01.45
Tilapia	6.60±0.20	1.19±0.12	06.85±03.20
Sardine	6.91±0.40	0.59±0.14	05.26±01.68
Anchovy	7.66±0.04	0.76±0.02	12.70±11.20
Indian mackerel	5.46±0.21	0.99±0.16	19.30±12.28

Table 3: Organisms identified

Strain No.	Organism	Gene bank accession no.
PUFSTFMDf01	*Enterobactercancerogenous*	KC834378
PUFSTFMDf02	*Enterobacter* sp.	KC834379

and Niven's agar media were 6.8-7.4 log cfu/g and 5.8-6.93 log cfu/g, respectively. The levels of APC in dried fish samples range from 10.5 to 11.3 log cfu/g. This can be noted that the majority of micro-flora in the dried food belongs to lactic acid bacterial group and are potential histamine producers. The pH, salt content and histamine forming bacterial count found in dried fish sample are presented in Table 2. According to pervious report, histamine production increased as the pH raised from 5.3-6.4. Sodium chloride plays an important role in microbial growth and therefore influences the activity of their amino acids decarboxylase (Zaman *et al*., 2009). The bacteria associated with fish spoilage were increasingly stressed when salt content in fish increased to above 1% (Wheaton and Lawson, 1985). In the present study, a total of 30 presumptive colonies were isolated from the Niven Medium plates and screened for histamine production and 18 isolates were able to produce histamine. Based on biochemical and morphological characterization, all strains were identified as *Enterobacter* sp. and *Bacillus* sp. Among them 2 of the confirmed histamine producing isolates were further identified as *Enterobacter* sp., based on 16s rDNA, *Enterobacter* sp., *Enterobacteraerogenus*, *Selariodesleptolepis* (Table 3). Previous studies reported that *Staphylococcus* sp. Yatsunami and Echigo (1991, 1992), *S. epidermidis* and *S. capitis* (Hernandez-Herrero *et al*., 1999), *Enterobacteraerogenous* (Huang *et al*., 2010), *Pantoea* sp. and *E. cloacae*, from salted mackerel, *Bacillus* sp., from fermented fish (Tsai *et al*., 2005, 2006) and *Bacillus megaterium* (Lin *et al*., 2012) were identified as histamine forming bacteria in salted fish and fish products.

The average content of histamine in tested samples was greater than 5 mg/100 g. The average histamine content in dried fish samples were 6, 6.85, 5.26, 12.7 and 19.3 mg/100 g, respectively. Huang *et al*. (2010) reported that dried fish product of *Selariodesleptolepis* had the highest histamine content of 6.31-47.90 mg/100 g. Kung *et al*. (2008) studied that, among the sixteen mullet roe product one sample had 8.18 mg/100 g histamine content. Myeolchi-jeot, a salted and fermented anchovy contained histamine greater than 5 mg/100 g was reported by Mah *et al*. (2002) in. However, fermented fish product contained high histamine contents. For example, Tsai *et al*. (2006) studied that high contents of histamine (26.3-39.4 mg/100 g) were detected in imported fermented fish products in Taiwan. Kuda *et al*. (2007) demonstrated that salted and fermented mackerel and sardine products showed high content of histamine from 12.6 to 30.5 mg/100 g. Previous study reported that most dried milkfish products sold at retail markets in Taiwan contained higher level of histamine greater than 5 mg/100 g guideline value suggested by the USFDA (Hsu *et al*., 2009). For fish, based on data collected from numerous outbreaks the US Food and Drug Administration (FDA) established a histamine hazard action level of 50 mg/100 g (Taylor, 1986). It has also been reported that 20 mg/100 g of histamine may be sufficient to cause the symptoms of scombroid poisoning (CDC, 2000). Present study is the first report on histamine content in the dried fish from Puducherry coast and the concentrations were found to be significantly higher than the (USFDA, 2001) guidelines.

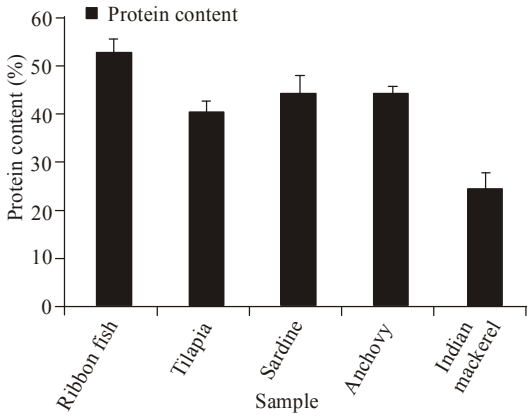

Fig. 1: Protein content in dried fish (percent dry weight)

Pearson correlation was carried out to study the relationship among the pH, salt contents, moisture content, protein and nitrogen content of the tested samples. Figure 1 represents the percentage of protein and nitrogen content in dried fish sample. The protein

content in the dried fish sample ranged 26.8-53.4%. In general, a positive correlation existed among protein and nitrogen content and a negative correlation existed between pH, salt content and histamine content in the 15 tested samples.

CONCLUSION

This study is the first report on histamine and histamine producing bacteria in dry fish in India. Dry fish is a part of the meal in the coastal regions in various Asian countries including India. Results in this study shows that potential histamine producers make a majority of the micro-flora of dry fish. Level of histamine in dry fish sample analyzed was relatively low indicating the low risk. The samples analyzed in this study were high quality market samples and also the number is limited. However, in case of dry fish which are prepared under unhygienic manufacturing conditions and low quality, there may be risk of potential poisoning. More samples from wider geographical locations and various quality conditions needs to be analyzed to properly understand the risk associated with the dry fish with respect to histamine.

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