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Isolation of Histamine Forming Bacteria and Quantification of Histamine from Fermented Mango Pickle

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Pickle is considered as an integral part of Indian meal, especially in southern India. There are several varieties of pickles made out of different vegetables across India and mango pickle is predominant among them. The present study validates histamine level and its associated bacterial population in mango pickles. The preliminary microbial analysis on Niven's agar revealed the population of histamine-producing bacteria in mango pickle. Further confirmation of histamine producing bacteria with PCR using HDC specific primers showed two bacterial isolates had HDC gene and they were identified by 16S rRNA sequencing as *Enterobacter cloacae* PUFSTP86 and *E. cloacae* PUFSTP92. Physicochemical analysis and nutritional properties of mango pickle showed pH (5.3 to 6.4), moisture (67.17 to 74.76 %), ash (85.20 to 88.72 %), nitrogen content (3.60 to 7.46 %) and water activity (0.86 to 0.89 a_w). Histamine in the mango pickles was quantified using HPLC and noticed a lesser histamine content (3.60 to 7.46 mg kg^{-1}) which makes mango pickle safer for consumption. Nutritional property and histamine content concludes that the mango pickle does not lead to any histamine related toxication and are biologically safer fermented product.

Keywords: Mango pickle; Histamine; Histidine Decarboxylase gene (HDC); HPLC.

Histamine is one of the weak known biogenic amine found in several foods such as fish products, cheese, wine, and fermented products and it is a product of microbial decarboxylation of histidine present in the foods (Bakirci *et al*., 2000). Insufficiency in histamine regulatory mechanism or excess accumulation of histamine via foods or disordered function of histamine metabolism by diamine oxidase inhibitors leads to histamine toxication (Joosten *et al*., 1988; Fox *et al*., 1996). Biogenic amines are naturally found in vegetables and fruits in small quantities while it is formed as the result of fermentation in cheese, wine, and sauerkraut. Histamine at its elevated levels generates food poisoning or food allergic condition. Histamine intoxication is possibly the best known food-borne illness which gives a range of symptoms such as rash, nausea, urticaria, diarrhea, vomiting, tingling, flushing and itching of the skin. The severity of the symptoms varies with the amount ingested and the individual's sensitivity (Lehane *et al*., 2000). Salted products are known to contain histamine forming bacteria. These bacteria's on proliferation under favorable conditions may contribute to the increase of toxic amines, leading to histamine poisoning (Jeyasekaran *et al*., 2003). Numerous microorganisms were reported to produce histamine; the major group of histamine forming bacteria includes *Morgonella morganii*, *Proteus* spp., *Klebsiella sp*. and *Hafnia alvei* (Jay *et al*., 1992).

Processing of vegetables by pickling involves brine water soakage followed by sun-drying. Pickling concept was started many centuries ago and apparently, for preserving food

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to consume during off seasons the benefits of eating pickles, can increase your appetite; they are low in cholesterol and saturated fat, a good source of iron, vitamin A, and fibers. Pickle has both the spice and sour flavor which alone can enhance the taste of any food you eat. Pickles have a shelf life of several months to years under efficiently stored conditions.

Though the studies with relevance to histamine in foodstuffs are vast, there have been limited information on the presence of histamine producing bacteria and histamine content in many of traditional Indian foods. Thus the present study was executed to examine the histamine-forming bacteria and its role in the accumulation of histamine in fermented mango pickles from west coast of India which are brine based and are fermented and consumed over an year after fermentation.

MATERIALS AND METHODS

Sample collection

Total of 10 fermented mango pickle samples were collected from Mangalore, India. Samples were collected in sterile containers, transferred to the laboratory and subjected to microbiological, physicochemical and proximate analysis.

Isolation of histamine forming bacteria

Fermented mango pickle samples were homogenized and serially diluted using sterile phosphate buffer saline (pH 7.0) and vortexed for two minutes. Histamine-forming bacteria were isolated on histamine-forming bacterium isolation agar fortified with L-histidine using spread plate method (Niven's *et al*., 1981) at 37°c for 6 to 7 days incubation. The colonies formed were counted and the bacterial counts were expressed in colony forming units (CFU/g). Colonies with blue or purple color on the plates were picked and further inoculated in Niven's broth for the confirmation of histamine producing.

HDC gene identification

Isolated cultures from pickle samples were used for HDC gene identification. The isolated pure culture was then inoculated in LB broth for further studies and incubated at 37°c for 24 hours. Genomic DNA from selected bacterial isolates was extracted using HipurA™Bacterial Genomic DNA kit (HIMEDIA, Mumbai, India) as per the instructions of the manufacturer. Histamine forming bacteria was identified by HDC gene amplification. Gene encoding histidine decarboxylase (HDC) was amplified from the above said genomic DNA using HDC specific primers [JV16 HD 5'-AGATGGTATTGTTTCTTATG-3' and JV17 HD 5'-AGACCATACACCATAACCTT -3'], as described by (Jeune *et al*., 1995). PCR Amplification was performed in 50 µl reactions that included 25 µl Hi-Chrom PCR Master Mix (Himedia, Mumbai, India), 2 µl of each primer and 2 µl DNA templates. PCR reaction was performed using Eppendorf Gradient Thermocycler (Germany), with following program: Initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 48.1°C for 10 sec, extension at 72°C for 15 sec and final extension at 72°c for 5 min. The PCR products were separated on a 1% agarose gel and visualized by Ethidium bromide staining under UV transilluminator. Product size was confirmed using Gene Ruler 1kb DNA ladder (Thermo Scientific).

Molecular characterization of selected bacterial isolate

Total genomic DNA from isolates showed HDC gene was isolated by HipurA Bacterial Genomic DNA kit (HIMEDIA, Mumbai, India) as per the instructions of manufacture. PCR amplification with forward primer 27F (5´AGA GTT TGA TCM TGG CTC AG3´) and reverse primer 1492R (5´TACGGYTACCTTGTTACGACTT3´) was carried out with the optimized cycles conditions. The amplified product was confirmed by agarose gel electrophoresis, and further sequenced by gene sequencer.

pH, moisture, ash, and nitrogen content determination

Fermented mango pickle samples (10 g) were vortexed in sterile 50ml centrifuge tubes with 10 mL of distilled water to make thick slurry. The pH of this slurry was then measured using pH meter. Moisture and ash contents were determined by employing the standard methods of analysis (AOAC, 2000). The nitrogen content of the sample was determined by the standard Kjedhal procedure of the (AOAC, 2000). The water activity of the samples was determined by aqua lab dew point water activity meter (series 4TE, Decagon Devices, Inc., USA) as per manufacturer instructions.

Histamine quantification

Standard preparation

The Stock solution of Histamine dihydrochloride was prepared by dissolving 1mg/ 10ml of 0.1M HCl and used as the standard stock solution. A serial dilution of the stock solution was used to make the calibration curve.

Sample preparation

Five grams of fermented mango pickle samples were mixed with 20ml of 6% trichloroacetic acid (TCA) and then transferred to 50ml centrifuge tubes. The sample was homogenized for 3 min and centrifuged (10,000 rpm, 10 min, 4 ºC) and filtered through Whatman No.1 filter paper. TCA was added to the filtrates to bring a final volume of 20 ml.

Benzoylation

Standard histamine solutions and 2 ml aliquots of the food sample extracts were derivatized with benzoyl chloride according to (Hwang *et al*., 1997). In brief 2 ml of extract, 1 ml of 2M NaOH and 10µl of benzoyl chloride were mixed using vortex mixture and incubated for 60 min at 60°C. Then 2ml of saturated NaCl, 3ml of diethyl ether were added and centrifuged (10,000rpm, 10 min, 4°C). The organic phase was separated and dried by using Nitrogen (N2) gas. The benzoyl derivatives were dissolved in 1 ml of methanol, and 20µl aliquots were used for HPLC analysis.

HPLC Conditions

The histamine content in test samples was determined according to (Hwang *et al*., 1997) method with slight modification. The detection of histamine was performed using Prominence Ultra Fast Liquid Chromatography (UFLC) system (Model LC20AD, Shimadzu, Japan) with RP-C18 column (250×4.6mm) and PDA detector (set at 233 nm). The gradient elution program began with 50:50 (v/v) methanol: water at a flow rate of 1ml/min for 0.5 min, followed by a linear increase to 85:15 methanol: water during the next 6.5 min, methanol: water mix held constant at 85:15 for another 5 min, and then decreased to 50:50 (0.8ml/min) during the next two min. Histamine standards were analyzed with test samples to check the chromatographic consistency. The samples were injected twice. Histamine concentrations in test samples were calculated based on the comparison against peak area as noticed from the histamine standard.

Statistical analysis

All statistical analysis of the results was performed using Statistical Software IBM SPSS Statistics, version 20.0.

RESULTS AND DISCUSSION

Microbiological analysis of fermented mango pickle

Microbial analysis of fermented mango pickle revealed aerobic microflora, LAB (Lactic acid bacteria) and histamine forming bacteria on NA, MRS and Niven's agar respectively (Table 1). Bacterial counts on APC in fermented mango pickle samples ranged from 6.96 to 8.69 log CFU/g. The tested pickle samples had higher bacterial counts when compared to an earlier study reported by (Kung *et al*., 2006) where the count was <1.0–4.2 log CFU/g of APC in mustard pickle sample. The pickle associated bacterial count on MRS and Niven's agar media ranged from 4.40 to 4.73 log CFU/g and 2.4 to 3.7 log CFU/g. Among the bacterial colonies noticed on Niven's agar medium plates, 12 isolates were identified as histamine producers. The lower pH range (<4.2) of these pickle samples had some inhibitory effect on the growth of microbes. Based on the morphological and biochemical observation, they were characterized as *Enterobacter* sp. and *Bacillus* sp. It has been observed that the majorities of microflora in the fermented mango pickle belonged to *Enterobacteriaceae*, and *Bacillus* bacterial group and are potential histamine producers as reported

Table 1. Total culturable microbial population from mango pickle

Samples	Aerobic Plate Count on NA (log CFU/g)	LAB count on MRS agar (log CFU/g)	Histamine forming bacteria on Niven's agar (log CFU/g)
MMPO1	7.79 ±0.13^{b}	4.73±0.21^{a}	2.4±0.20^{b}
MMPO2	8.69±0.26^{a}	4.64±0.16^{a}	3.7±0.17^{a}
MMPO3	7.63±0.18^{b}	4.54±0.14^{a}	2.6±0.12^{b}
MMPO4	6.96 ±0.13^{c}	4.40±0.14^{a}	2.6±0.11^{b}

by (Sumitha *et al*., 2013).

PCR amplification of histidine decarboxylase (HDC) gene

Promising histamine producing isolates were further screened for histidine decarboxylase (HDC) gene. PCR-based screening of 12 bacterial isolates as noticed as histamine producer on Niven's agar media were tested and revealed amplification of HDC gene from two isolates Fig: 1 (PUFSTP86, PUFSTP92). Previous studies reported histamine producing *Staphylococcus capitis* and *Staph. pasteuri* from mustard pickle; *Raoultella ornithinolytica*, *Enterobacter aerogenes* from various marine source (Kung *et al*., 2012). Histamine-producing *Oenococcus oeni* IOEB 9204, *Lactobacillus hilgardii* IOEB 0006, *L. buchneri* DSM 5987, *L. sakei* LTH 2076 and *Tetragenococcus muriaticus* LMG 18498 were reported from fermented foods wine, cheese, sauerkraut, squid liver sauce and confirmed with amplification of HDC gene (Lucas *et al*., 2008).

Amplification of 16S rRNA gene

The isolate which showed amplification of HDC gene for which biochemical and molecular characterizations were done and identified as *Enterobacter cloacae*, *Enterobacter cloacae* (Showing 99% similarity in BLAST search to *Enterobacter cloacae*, *Enterobacter cloacae*). The sequence size was 958 bp, 953 bp. The phylogenetic tree of *Enterobacter cloacae* strains PUFSTP86, PUFSTP92 (GenBank Accession numbers KT921425, KT921426) is shown in Fig: 2. Early studies are reported *E. cloacae* from salted anchovies by (Jerez *et al*., 1994). *Pantoea* sp. and *E. cloacae*, from salted mackerel, *Bacillus* sp., from fermented fish (Tsai *et al*., 2005, 2006) and *S. epidermidis* and *S. capitis* (Hernandez-Herrero *et al*., 1999) were identified as histamine forming bacteria in salted products.

pH, moisture, ash, and nitrogen content of fermented mango pickle

The moisture, ash, nitrogen contents among the various mango pickle samples ranged from 67.17 to 74.76 %, 85.20 to 88.72 %, 3.60 to 7.46 % respectively (Table 2). Tested samples are showing the water activity fairly 0.86 to 0.89, and these ranges are not favorable for microbes. The previous report states that histamine production was increased as the pH rose from 5.3 to 6.4. Sodium chloride plays a major role in microbial growth and therefore influences the activity of their amino acids decarboxylase (Zaman *et al*., 2009). The present study revealed that the pH of mango pickle samples ranged from 3.00 to 4.29 (Table 2), which was unfavorable for decarboxylase involved in histamine synthesis. Survival of bacteria requires a relatively high water activity with the range of 0.9 or higher (Battcock and Ali., 1999). A few species which can tolerate water activities lower than this, but usually the yeasts and fungi will predominate on foods with a lower water activity. The percentage of protein and nitrogen content in mango pickle sample ranged 3.60 - 7.46 %. In general, a positive correlation existed among protein and nitrogen content and a negative correlation existed between pH and histamine content in the tested samples.

Histamine content of fermented mango pickle

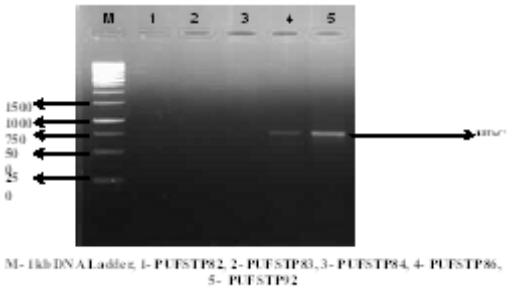
The mango pickles were analyzed for their histamine concentration using HPLC. The average histamine content in pickle samples were 3.0 – 3.2 mg kg^{-1} (Table 2). Earlier reports demonstrated that the average histamine content of 10mg 100 kg^{-1} as biologically safe (Kunsch *et al*., 1989). The present study revealed marginal histamine content over other fermented foodstuffs as reported in the earlier studies (Huang *et al*., 2010; Kuda *et al*., 2007). It has also been reported that histamine content of

Table 2. pH, moisture, ash, and nitrogen content of mango pickle

Source	pH	Moisture (%)	Ash (%)	Nitrogen content (%)	Water activity (a_w)	Histamine level (mg/kg)
MMPO1	3.49 ± 0.17^{b}	70.66 ± 0.04^{b}	86.20 ± 0.32^{b}	7.46 ± 0.41^{a}	0.87 ± 0.002^{b}	3.2 ± 0.03^{a}
MMPO2	4.29 ± 0.12^{a}	67.17 ± 1.41^{c}	85.20 ± 0.65^{b}	6.66 ± 0.41^{b}	0.86 ± 0.002^{c}	3.2 ± 0.01^{a}
MMPO3	$3.40\pm0.30^{b,c}$	71.28 ± 0.31^{b}	88.72 ± 0.73^{a}	4.84 ± 0.44^{c}	0.89 ± 0.004^{a}	3.3 ± 0.02^{a}
MMPO4	3.00 ± 0.29^{c}	74.76 ± 0.27^{a}	87.78 ± 0.24^{a}	3.60 ± 0.17^{d}	0.89 ± 0.001^{a}	3.0 ± 0.01^{a}

The presented values are the means of three determinations, with standard deviations indicated. The mean values (standard deviations) within the same column followed by different superscript letters differ significantly ($p < 0.05$).

200 mg kg^{-1} may be sufficient to cause the symptoms of scombroid poisoning (CDC, 2000). Histamine content in mixed, hot pepper and cucumber pickles were ranged from 16.54 to 57.89 mg kg^{-1}, 19.78 to 74.97 mg kg^{-1}, 26.66 to 44.72 mg kg^{-1} (Ekici *et al*., 2004). (Taylor *et al*., 1978) Analyzed histamine contents in 50 sauerkraut samples obtained from retail markets, and noticed that histamine contents ranging from 0.91 mg 100g^{-1} to 13.0 mg 100g^{-1}. Earlier study by Kunsch *et al*., 1989 reported a histamine level of 229mg kg^{-1}, whereas the latter study reported a histamine level of 10mg kg^{-1} in sauerkraut (Kalac *et al*., 1999) a safer limit as recommended by Kunsch *et al*., 1989. Histamine content of our test samples were less than 3.0mg kg^{-1}, which is the lower level for causing clinical symptoms. The present study report on histamine content in the histamine content in the mango pickle and the concentrations were found to be significantly lower than the (USFDA., 2001) guidelines.



M- 1kb DNA Ladder, 1- PUFSTP82, 2- PUFSTP83, 3- PUFSTP84, 4- PUFSTP86, 5- PUFSTP92

Fig. 1. PCR amplification of HDC gene

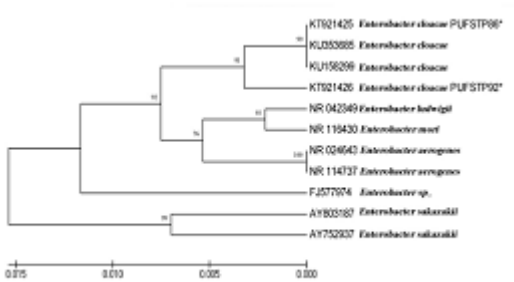


Fig. 2. Phylogenetic tree showing the relatedness of 16SrRNA of two bacterial isolates, with accession no KT921425 and KT921426 with already genes in the Genbank data base

CONCLUSION

Accumulation of elevated levels of histamine in foodstuffs leads food poisoning or food allergic conditions. Mango pickle is an important integral part of Indian meal, especially southern India. There are several varieties of pickles made out of different vegetables all across India. Thus it is of important to study the histamine level in mango pickle and its associated bacterial population. Results in this study showed a very limited amount of histamine and also marginal presence of histamine-producing bacteria in fermented mango pickles of India, indicating that the fermentation microflora have potential to produce histamine and cause a risk to health in case optimum conditions prevail.

REFERENCES

1. A.O.A.C. Official methods of analysis of the association of the analytical chemists, Maryland, USA. 2000.
2. Bakirci, I. Peynirlerde biyojen amin olusumu ve etkili faktörler. In Süt mikrobiyolojisi ve katki maddeleri sempozyumu kitabi. (Tekirdag), 2000; pp: 328-336.
3. Elici, K., and Coskun, H. Histamine content of some commercial vegetable pickles. *P. j. n.*, 2004; **3**(3): 197-198.
4. Fox, P. F., O'connar, T. P., Mcseeney, P. L. H., Guinne, T. P., & O'Brien, N. M. Cheese: Phisical, biochemical and nutritional aspects. *Adv. Food. Nutr. Res.*, 1996; **39**: 163-328.
5. Huang, Y. R., Liu, K. J, Hsieh, H. S., Hsieh, C. H., Hwang D. F., & Tsai, Y. H. Histamine level and histamine-forming bacteria in dried fish products soldin Penghu Island of Taiwan. *Food.Control.*, 2010; **21**(9): 1234-1239.
6. Hwang, D. F., Chang, S. H., Shiau, C. Y, & Chai, T. High-performance liquid chromatographic determination of biogenic amines in fish implicated in food poisoning. *J. Chromatogr. B.*, 1997; **693**: 23-30.
7. Jay, M. J. Modern food microbiology. Van Nostrand Reinhold, New York. 1992.
8. Jerez. J. J. R., Ventura. M. T. M., Sabater. E. I. L., Herrero. M. M. H. Histidine, Lysine and Ornithine Decarboxylase Bacteria in Spanish Salted Semi-preserved Anchovies. *J. Food. Protect.*, 1994; **57**(9): 784-787.
9. Jeune. C. L., Funel, A. L., Brink, B. T., Hofstra, H., Vossen, J. M. Development of a detection system for histidine decarboxylating lactic acid bacteria based on DNA probes, PCR and activity test. *J. Appl. Bacteriol.*, 1995*;* **78**: 316–326.
10. Jeyasekaran, G., & Jeyashakila, R., Occurrence of biogenic amine forming bacteria in cured

fishery products of Thoothukkudi region of Tamil Nadu, India. *Asian. Fish. Sci.,* 2003; **16**: 195-202.

11. Joosten, H. M. L. J. The biogenic amine contents of Dutch cheese and their toxicological significance. *Neth. Milk. Dairy. J.,*1988; **42**: 25-42.
12. Kalac, P., Špicka, J., Krizek, M., Steidlova, Š., & Pelikanova, T. Concentrations of seven biogenic amines formation in sauerkraut. *Food. Chem.,*1999: **67**: 275-280.
13. Kuda, T., Mihara, T., & Yano, T. Detection of histamine and histamine-related bacteria in fish-nukazuke, a salted and fermented fish with rice-bran, by simple colorimetric micro plate assay. *Food.Control.* 2007; **18**: 677-681.
14. Kung, H. F., Lee, T. M., Lin, C. S., Hong T. Y., & Tsai, Y. H. Polymerase Chain Reaction for the Detection of Histamine - Producing Bacteria Isolated from Taiwanese Foods. *J. Food. Drug. Anal.,* 2012; **20**(1): 74-82.
15. Kung, H. F., Lee., Y. H., Teng., D. F., Hsieh., P. C., Wei., C. I., Tsai., Y. H. Histamine formation by histamine-forming bacteria and yeast in mustard pickle products in Taiwan. *Food. Chem*., 2006; **99**: 579-585.
16. Kunsch, U., Scharer, H., Temperli, A. Biogene Amine als Qualitatsindikatoren von Sauerkraut. XXIV Vortragstagung der Deutschen Gesellschaft für Qualitätsforschung: Qualitätsaspekte von Obst und Gemüse, Ahrensburg, Germany, 1989.
17. Lehane, L., & Olley, J. Histamine fish poisoning revisited. *Int. J. Food. Microbiol.,* 2000; **58**: 1-37.
18. Lucas, P. M, Claisse, O., & Funel, A. L. High Frequency of Histamine-Producing Bacteria in the Enological Environment and Instability of the Histidine Decarboxylase Production Phenotype. *Appl. Enviro. Microb.,* 2008; **74**(3): 811–817.
19. Niven, C. F., Jeffreg, M. B., & Corlett, D. A. Differential plating medium for quantitative detection of histamine-producing bacteria. *Appl. Enviro. Microb.,* 1981; **41**: 321-322.
20. Sumitha, D., Sundar, K., Shetty., H. P. Occurrence of Histamine Forming Bacteria and Histamine in Dried Fish. *Adv. J. Food Sci. Technol.,* 2014; **6**(4): 428-432.
21. Taylor, S. L., Leatherwood, M., & Cieber, E. R. Histamine in sauerkraut. *J. Food. Sci.,* 1978; **43**: 1030-1032.
22. Tsai, Y. H., Kung, H. F., Lee, T. M., Chen, H. C., Chou, S. S., Wei, C. I., & Hwang, D. F. Determination of histamine in canned mackerel implicated in a food born poisoning. *Food Control.*, 2005; **16**(7): 579-585.
23. Tsai, Y. H., Kung, H. F., Lee, T. M., Chen, H. C., Chou, S. S., Wei, C. I., & Hwang, D. F. Histamine contents of fermented fish products in Taiwan and isolation of histamine-forming bacteria. *Food Chem.*, 2006; **98**: (1), 64-70.
24. USFDA (US Food and Drug Administration). Scombrotoxin (histamine) Formation. In: Fish and Fishery Products Hazards and Controls Guide. 3rd Edn., Department of Health and Human Services, Public Health Service, Food and Drug Administration, Center for Food Safety and Applied Nutrition, Office of Seafood, Washington, DC., 2001, pp: 73.
25. Zaman, M. Z., Abdulamir, A. S., Bakar, F., Selamat, A. J., & Bakar, J. A review: Microbiological, physicochemical and health impact of high level of biogenic amines in fish sauce. *Am. J. Appl. Sci.,* 2009; **6**: 1199-1211.