HISTAMINE PRODUCING *MICROCOCCUS* AND *FLAVOBACTERIUM* SPP. FROM FISH

by

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ABSTRACT

Histamine produced by bacteria on fish cause allergic reactions in some consumers. Histamine producting bacteria were isolated and identified in this study.

Of 103 bacterial cultures isolated from fish, 30 produced histamine. They consisted of *Micrococcus* sp. (3) of the family Micrococcaceae and *Flavobacterium* sp. (4), *Hafnia* sp. (5), *Enterobacter* sp. (1), *Klebsiella pneumoniae* (3), *Klebsiella* sp. (8), *Hafnia alvei* (1), *Morganella* sp. (4) and *Proteus* sp. (1) of family Enterobacteriaceae. They produced mean histamine concentrations of 234±88, 110±6, 950±50, 209±8, 354±50, 480±127, 382±10 and 700±100 mg/kg, respectively, in Niven's medium. *Micrococcus* and *Flavobacterium* spp have not previously been reported to produce histamine. *Micrococcus* and *Flavobacterium* spp carry intermediate and low potential to produce histamine, respectively. Low and intermediate potential histamine producers represent only 7%, while high potential histamine producers represent 22% of the total spoilage bacterial flora of the 103 isolates.

INTRODUCTION

Fish carry a variety of microorganisms responsible for spoilage of fish. They include *Morganella morganii*, *Hafnia alvei*, *Clostridium perfringens*, *Aeromonas aerogenes*, *Klebsiella pneumoniae* and *Vibrio alginolyticus*. A major food safety problem associated with spoilage of fish is the production of histamine. Histamine is produced more commonly by *Morganella morganii*, *Hafnia alvei* and *Klebsiella pneumoniae* (Taylor, *et al.*, 1984).

Histamine producing bacteria usually grow rapidly at temperatures of 20-45 °C with optimum production at 38 °C (Yoshinaga and Frank, 1982). Several growth media have been investigated to isolate and identify histamine producing bacteria. The formulation of Niven's medium is considered most suitable to identify these bacteria currently (Niven *et al.*, 1981). In the studies where Niven's medium was developed *Proteus morganii*, *Klebsiella pneumoniae*, *Escherichia coli*, *Enterobacter aerogenes*, *Enterobacter cloacae*, *Proteus* sp, *Edwardsiella* sp and *Vibrio* sp have been identified (Niven *et al.*, 1981).

Modifications of Niven's medium have been tested by others for the ability to isolate histamine producing bacteria. They found that Niven's medium was superior to three other modifications in achieving the isolation of bacteria (Chen *et al.*, 1989). Estimation of histamine concentrations in fish and isolation and identification of histamine producing bacteria continue to be an important approach in assessing safety of fish. Further studies reported several new histamine producing bacteria (Ababouch *et al.*, 1991; Lopez-Sabater *et al.*, 1994; Lopez-Sabater *et al.*, 1996). This study examines the histamine producing bacteria in fish.

MATERIALS AND METHODS

Isolation of bacteria

From fish available at the market, approximately 2 g of inside with the skin was aseptically transferred into 10 ml of sterile 1% peptone water and shaken for two min. to mix it well. A loopful of this solution was inoculated into violet-red-bile-glucose-agar (VRBGA) in petri dishes and incubated at 37°C for 5 days. Colonies showing a pink reaction were transferred into Niven's agar medium and incubated at and 37°C for 2 days (Niven *et al.*, 1981). Colonies showing a purple halo, indicating probable histamine production, were isolated and maintained on nutrient agar slants at 5°C for further studies.

Histamine production by bacteria

The bacteria isolated from Niven's agar were activated in sterile trypticase soy broth supplemented with histidine (TSBH) at ambient temperature of (25±2) °C for 24 h. A loopful of activated bacteria were inoculated into 25 ml of sterile Niven's broth in triplicate and incubated at 37 °C for 10 days (Chen *et al.*, 1989; Ababouch *et al.*, 1991). Histamine concentration in 5 g of the incubated broth was estimated by TLC (Lieber and Taylor, 1978) and fluorometry (AOAC, 1990).

Identification of bacteria

The cultures showing histamine production were maintained on nutrient agar at 37°C and identified using the following tests; Gram's stain, motility, oxidase, catalase, MR-VP, Koty, Hugh and Leifson test for cocci and bacillii, presumptive urease, citrate, indol ring, indol nitrate, nitrate reduction, utilization of sugars (for bacillii), gelatin liquefaction, growth at 10 °C and 44 °C (Cowan, 1974; Baird Parker, 1974; Baird Parker, 1979; Chinivasagam, 1988). The identified bacteria were subcultured and stored at -10 °C.

RESULTS AND DISCUSSION

Identification of histamine producing bacteria

One hundred and three (103) cultures showing positive reaction on the VRBGA medium were transferred to Niven's agar and 63 out of them showed a purple halo indicating probable production of histamine. These bacteria were tested for histamine production in Niven's broth. Thirty (30) histamine producing cultures were identified based on biochemical tests. Of the 30 cultures identified, three (3) were *Micrococcus* sp from the family Micrococcaceae while 27 belonged to the family Enterobacteriaceae. In the family Enterobacteriaceae, the bacteria identified were *Flavobacterium* sp (4), *Hafnia* sp (5), *Enterobacter* sp (1), *Klebsiella pneumoniae* (3), *Klebsiella* sp (8), *Hafnia alvei* (1), *Morganella* sp (4) and *Proteus* sp (1).

Histamine producing ability of identified bacteria

Micrococcus sp, *Flavobacterium* sp, *Hafnia* sp, *Enterobacter* sp, *Klebsiella pneumoniae*, *Klebsiella* sp, *Hafnia alvei*, *Morganella* sp and *Proteus* sp produced mean histamine concentrations of 234±88, 110±6, 950±50, 209±8, 354±50, 480±127, 382±10 and 700±100 mg/kg, respectively when incubated in Niven's broth at 37 °C for 10 days (Table 1). Histamine production has not been reported, to our knowledge by the species *Micrococcus* and *Flavobacterium* isolated from fish.

Of the species examined *Flavobacterium* sp produced low concentrations of 110±6 mg/kg of histamine. In contrast *Micrococcus* sp which produced 234±88 mg/kg of histamine which is comparable with the histamine concentration produced by species commonly accepted as histamine producers in fish. *Micrococcus* and *Flavobacterium* represent 7% while other bacteria represent 22% of the total spoilage

bacterial flora of 103 isolated from fish in this study. The histamine contribution by commonly known bacteria in fish is higher than the two new histamine producers identified in this study. In this study, histamine concentrations were estimated quantitatively by fluorometry in most instances while at a later stage of the research estimations were done by TLC.

Table 1. Histamine producing bacteria isolated and identified from fish, their sources and concentrations of histamine produced in Niven's broth at 37° C.

Type of bacteria	No. of cultures	Code No.	Sources of cultures	Histamine (µg/ ml) Mean ± SD
Klebsiella pneumoniae	3	A1	dried anchovies	325±05
		A2	fresh tuna	318±18
		A3	fresh tuna	420±07
Klebsiella sp	8	A4	fresh tuna	409±08
		A5	maldive fish	200±12
		A6	fresh skipjack	500-600
		A7	fresh tuna	500-600
		A8	fresh tuna	500-600
		A9	fresh skipjack	500-600
		A10	dried skipjack	500-600
		A11	fresh skipjack	500-600
Hafnia alvei	1	B1	fresh tuna	382±10
Hafnia sp	5	B2	fresh crabs	900-1000
		B3	fresh prawns	900-1000
		B4	fresh tuna	900-1000
		B5	fresh tuna	900-1000
		B6	fresh tuna	900-1000
Enterobacter sp	1	C1	fresh tuna	209±08
Flavobacterium sp	4	D1	fresh carp	110±02
		D2	fresh flying fish	112±02
		D3	fresh skipjack	102±05
		D4	fresh mahseer	115±04
Morganella sp	4	E1	fresh skipjack	600-800
		E2	fresh tuna	600-800
		E3	fresh tuna	600-800
		E4	fresh herring	600-800
Proteus sp	1	F1	fresh tuna	500-600
Micrococcus sp	3	G1	fresh trevally	269±08
		G2	dried anchovies	313±13
		G3	fresh skipjack	121±09

Sources of histamine producing bacteria

The sources of the identified bacteria were mainly fresh fish, while several dried-fish samples and a Maldive fish sample also contained histamine producing bacteria. Three cultures of *Micrococcus* sp were isolated from fresh trevally (*Caranx stellatus*) and skipjack (*Katsuwonus pelamis*) and dried anchovies (*Thrissocles* sp). Four cultures of *Flavobacterium* sp were isolated from fresh carp (*Cyprinus carpio*), flying fish (*Exocoetus volitans*), skipjack and masheer (*Tor khudree longispinis*). The available evidence does not show any preference by the two new histamine producing species for any type of fish or processing method for fish such as drying or smoking.

Effectiveness of Niven's medium

The capability of Niven's agar medium to isolate histamine producing bacteria needs further investigation. Out of 65 cultures of bacteria which produced purple coloured colonies with a purple halo on the yellow background of Niven's agar medium, only 30 were able to produce histamine in Niven's broth at detectable concentrations of above 5 mg/kg. It has been reported that Niven's agar medium has a tendency to show positive results even though the bacterial cultures do not produce histamine concentrations in Niven's broth detectable either by TLC or fluorometry. This disparity between the reactions in Niven's agar medium and Niven's broth has led to the assumption that Niven's agar medium tends to give false positive results (Chen *et al.*, 1989; Ababouch *et al.*, 1991).

The reason for the difference between the histamine observed in Niven's broth and agar medium may be attributed to the fact that not only histamine but other alkaline products such as tyramine, putrescine, cadaverine, spermine and spermidine can be produced in Niven's medium and cause the colour change from yellow to purple. In such situations, when the Niven's broth is analyzed for histamine it may not show histamine (Lopez-Sabater *et al.*, 1996). On the other hand there could be low potential histamine producing bacteria which may show positive results in Niven's agar medium due to formation of several alkaline amines along with negligible quantities of histamine. If inoculated into Niven's broth these bacteria may not show positive results for histamine due to formation of low quantities which cannot be detected by TLC or fluorometry. Thus it is important to combine both the microbiological method (Niven's medium) and the chemical assessment in studies on histamine production by bacteria.

It appears that a wider variety of bacterial species, from among the spoilage micro-organisms, commonly found in fish, are capable of histamine production. Therefore more efficient mechanisms are needed in minimizing bacterial activity to reduce formation of histamine in fish during handling and storage.

CONCLUSIONS

Flavobacterium sp and *Micrococcus* sp which have not previously been reported as histamine producing bacteria before, were isolated from fish. *Micrococcus* sp appears to produce comparable histamine concentrations as other histamine producing bacteria in fish. *Flavobacterium* sp appears to be a low histamine producer.

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