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Inhibition of Actinomycetes to Histamine Producing Bacteria Associated with Indian Mackerel Fish (*Rastrelliger kanagurta*, Cuvier, 1816)

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Abstract: Actinomycetes and Histamine Producing Bacteria (HPB) were isolated and identified from the Indian mackerel fish, *Rastrelliger kanagurta* from three different parts viz., skin with muscle, gills and gut. Among the three parts, gill harboured the highest number of actinomycetes (2.9×10⁵ cfu g⁻¹) and HPB (2.47×10⁵ cfu g⁻¹) than the other parts. At the same time, the fish harboured more number of actinomycetes than HPB. During the present investigation, a total of 29 HPB strains were isolated and identified as *Bacillus* sp., *Pseudomonas* sp., *Vibrio* sp. and *Aeromonas* sp. Among them, *Pseudomonas* sp. contributed more (45%) followed by *Vibrio* sp. (22%), *Bacillus* sp. (20%) and *Aeromonas* sp. (13%) in all the three parts of fish. In the present investigation, 41 strains of actinomycetes also isolated and all belonged to the genus, *Streptomyces*. Out of these 41 strains, 32 strains showed inhibitory activity to one or more HPB at varying levels. Among them, 3 strains viz., ASP-2, ASP-11 and ASP-15 showed good inhibitory activity against all 4 HPB and these 3 actinomycete strains (ASP-2, ASP-11 and ASP-15) were tentatively identified as *S. aureofasciculus* (ASP-2), *S. chattanoogenesis* (ASP-11) and *S. hawaiiensis* (ASP-15). The study indicates that the actinomycetes could be used to control HPB.

Key words: Indian mackerel fish, histamine producing bacteria, actinomycetes, antagonistic activity

INTRODUCTION

Fish is a highly perishable commodity, which starts to spoil soon after the death of the fish, if not properly preserved. Consumption of spoiled fish results in the outbreaks of food poisoning and histamine fish poisoning is one such food poisonings. Scombroid or histamine toxicity is nothing but food borne chemical intoxication caused by the consumption of spoiled or bacterially contaminated fishes. Scombroid poisoning is usually a mild illness with a variety of symptoms including rash, urticaria, nausea, vomiting, diarrhea, flushing and tingling and itching of the skin (Taylor, 1986). Scombroid fish poisoning results from eating the spoiled fishes of the family Scombroidae. These fish contain characteristically high level of free histidine in their muscle tissue, which will be converted to histamine under conditions conductive to bacterial growth and the synthesis of histidine decarboxylase (Eitenmiller *et al.*, 1982; Taylor *et al.*, 1989). These fishes include tuna, mackerel, skipjack and bonito. However, non-scombroid fishes such as mahi-mahi, blue fish, amberjack, herrings, sardines and anchovies have also been implicated in histamine fish poisoning.

India being one of the major exporters of fish from Indo-Pacific region, studies on histamine in the home country assumes greater dimension from public health point of view as well as export earning. This can be done by introducing stringent quality control measures with regard to incidence and concentration of histamine, which will be beneficial for both the food processing industry as well as the consumer. Though the total annual fish catch in India during 2006-2007 accounts to 253 million tonnes, but a major part of it gets spoiled due to lack of proper preservation technology. Hence, assessing the magnitude of fish spoilage through histamine formation aids in maintaining the quality and microbiological safety of fishery products as rejection of export oriented product causes considerable financial loss and poor reputation for fishery products thereby affecting the sales.

However, there are considerable options to prevent and reduce the histamine contamination through quality processing techniques including of ice storage, salt and other preservative techniques. But in the present study, an attempt has been made to identify some of the natural agents like actinomycetes which could control the growth of histamine producing bacteria.

MATERIALS AND METHODS

Collection of Fish Samples

To determine the occurrence of actinomycetes and histamine producing bacteria (HPB), a commercially important and most commonly eaten fish viz., Indian Mackerel (*Rastrelliger kanagurta*) was collected a live from the Parangipettai coastal area, southeast coast of India (Lat. 11° 29'N, Long. 79° 46'E). For enumeration of actinomycetes and histamine producing bacteria, the samples (gills, gut and skin with flesh) were prepared by the methods described by Paramasivam (2002) and Sivakumar *et al.* (2005).

Enumeration of Histamine Producing Bacteria (HPB)

For the enumeration of HPB, 1 mL of serially diluted homogenates was poured on to petri plates containing Niven's medium (Niven *et al.*, 1987) and Modified Niven's medium as described by Buck and Clevendon (1960). To this, 5-10 mL of molten agar was poured over to prevent the swarming of some spreading colonies. The colonies showing positive results (purple hollow around Niven's medium and pink hollow around in Modified Niven's medium) were enumerated after 24 h of incubation at 25±2°C. The positive colonies in Niven's medium were picked up and streaked on Trypticase soy agar plates supplemented with 0.1% histidine. The isolated colonies were maintained as pure cultures in duplicate slants of the same composition for further confirmation of their histidine decarboxylase activity. Bacteria isolated using Niven's medium and Modified Niven's medium were subjected to confirmation by the methods described by Yoshinaga and Frank (1982), Yoshinaga and Frank (1982) and Smith *et al.* (1982). The morphological and biochemical examinations of HPB strains were carried out as described by Okuzumi *et al.* (1982, 1994).

Enumeration of Actinomycetes

For the enumeration of actinomycetes, 1 mL of serially diluted homogenates was poured on to petri plates containing Actinomycetes Isolation Agar medium in duplicate petri plates after suitable dilution. To minimize bacterial and fungal contaminations, all agar plates were supplemented with 20 mg L⁻¹ of nystatin and cycloheximide (100 mg L⁻¹), respectively (Kathiresan *et al.*, 2005). The actinomycete colonies that appeared on the petri plates were counted from 5th day onwards, upto 28th day. All the colonies that were growing on the petri plates were separately streaked in petri plates, subcultured, ensured for their axenicity and maintained in slants.

Inhibitory Activity of Actinomycetes Against HPB

Inhibitory activity of actinomycetes strains isolated during the present study from the different parts of Indian mackerel fish was tested against HPB. The inhibitory activity was studied by using the cross streak method (Waksman and Lechevalier, 1962). Single streak of actinomycetes was made on

the surface of the modified nutrient agar (Sivakumar *et al.*, 2005) and incubated at room temperature (28±2°C). After observing a good ribbon-like growth of the actinomycetes on the petri plates, the histamine producing bacteria were streaked at right angles to the original streak of actinomycetes and incubated at 28±2°C. The inhibition zone was measured after 24 and 48 h. A control plate was also maintained without inoculating the actinomycete, to assess the normal growth of bacteria.

Taxonomic Investigation of Potential Actinomycete Strains

The genus level identification was made for the actinomycetes using cell wall composition analysis and micromorphological studies (Lechevalier and Lechevalier, 1970). Species level identification of the strains which showed inhibitory activity was made by following the methods described by Shirling and Gottlieb (1966), Key of Nonomura (1974) and Bergey's manual of determinative bacteriology (Buchanan and Gibbons, 1974).

RESULTS AND DISCUSSION

Generic Composition of HPB

During the present investigation, a total of 29 strains were isolated. All these strains were preliminarily investigated for their ability to produce histamine in Niven's medium and among them, 12 strains showed positive results in modified Niven's medium. The isolated strains showed positive reaction-presence of purple colonies surrounded by a purple halo on the Niven's medium after 24-48 h of incubation and 12 strains yielded a positive result in modified Niven's medium-this being detected by the presence of red colonies surrounded by red halos in agar surface-streak cultures-after 24, 48 and 72 h of incubation. These changes in colour were due to the increase in the pH of the media probably derived from the decarboxylation of L-histidine of other amino acids present in them as suggested by Ben-Gigirey *et al.* (2006).

The isolated histamine producing bacteria were identified upto the generic level. They belonged to four genera viz., *Bacillus*, *Pseudomonas*, *Vibrio* and *Aeromonas*. Among them, *Pseudomonas* contributed more (45%) followed by *Vibrio* (22%), *Bacillus* (20%) and *Aeromonas* (13%) in all the three parts of the fish. Yatsnami *et al.* (1992) recorded halotolerant histamine bacteria viz., *Staphylococcus* sp., *Vibrio* sp. and *Pseudomonas* sp. from the fermented and salted fish products. Though the genus *Staphylococcus* is frequently reported as histamine-former in fermented salted fish, accounting nearly 50% of histamine-forming microorganisms (Yatsnami *et al.*, 1992), the present study did not encounter any *Staphylococcus* whereas other genera, *Vibrio*, *Pseudomonas* and *Aeromonas* were isolated. However, Paramasivam (2002) isolated six different bacterial genera from different parts of fin and shell fishes and recorded *Vibrio* as the dominant histamine producer.

Many researchers have studied the presence of microbial forms in fishes and the diversity of microbes varied from species to species of fish. Fujii et al. (1997) isolated two species of gram-positive bacteria (*Photobacterium damsela* and *P. histaminum*) which are histamine-producers from the skin lesions of damsel fish. *Tetrogenococcus muriaticus* sp. nov., a halophilic lactic acid bacterium was isolated from fermented squid liver sauce (Satomi et al., 1994). Kim et al. (2001) have isolated *Mogonella moganii*, the most predominant and prolific histamine former from Pacific mackerel during storage. Such differences in the diversity of histamine producing bacteria could be attributed to differences in the species of fish, handling procedures, holding time and temperatures.

Population Density of HPB

Populations density of HPB in different parts viz., skin with muscle, gills and the gut of the Indian mackerel fish varied largely.

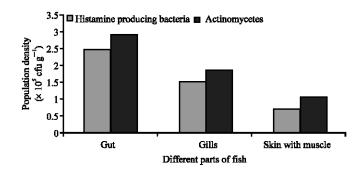


Fig. 1: Population density of histamine producing bacteria and actinomycetes in the different parts of Indian mackerel fish

Density of HPB in different parts of the fish varied from 0.79 to 2.47×10^5 cfu g⁻¹ (Fig. 1). The highest density was recorded in gut contents $(2.47 \times 10^5$ cfu g⁻¹) followed by gills $(1.5 \times 10^5$ cfu g⁻¹) and skin with muscle $(0.79 \times 10^5$ cfu g⁻¹). The apparent residence of maximum histamine formers in the fish intestine has also been reported by Lerke *et al.* (1978), Frank *et al.* (1981). The reason for this could be that the gills and the gut contents are exposed to the entry of variety of microbial populations including histamine producing bacteria along with diverse food particles and more numbers of microbes could have been retained in the guts than the gills as the latter is continuously washed off with large volumes of water as suggested by Sahu *et al.* (2006).

Population Density of Actinomycetes

Like the population density of HPB, the population density of actinomycetes in different parts viz., skin with muscle, gills and the gut of the Indian mackerel fish varied from 1.07 to 2.9×10^5 cfu g⁻¹ with the minimum $(1.07 \times 10^5$ cfu g⁻¹) in the skin with muscle sample and maximum $(2.9 \times 10^5$ cfu g⁻¹) in gut sample (Fig. 1). Kundu *et al.* (2006) and Sahu *et al.* (2007) observed maximum and minimum population densities in gut and skin respectively from different estuarine fishes viz., *Mugil cephalus, Etroplus suratensis* and *Chanos chanos*. Such occurrence of actinomycetes could be beneficial to the fishes either in the production of (microbial) enzymes useful for the digestion or in the secretion of growth factors and vitamins (by microbes) which are useful for fishes. Higher percentage of occurrence of actinomycetes in the guts could be also due to the production of mycoid slime in the guts which can act as a nutrient source for actinomycetes. This would help in symbiotic or commensal relationship between the host and actinomycetes. In a recent study from the Veli Lake of Kerala state, this has been well emphasized (Dhevendaran and Annie, 1999).

Comparison of Population Density of HPB and Actinomycetes

To compare the population density of histamine producing bacteria and actinomycetes, 10 different sizes of Indian mackerel fishes were analyzed and results are presented in Fig. 2-4.

Figure 2 present that among the 10 gut samples of mackerel fishes, in 7 guts of fishes viz., samples No. 1, 3, 4, 7, 8, 9 and 10, population density of actinomycetes was higher than the histamine producing bacteria. But, in the case of other 3 gut samples of fishes (samples No. 2, 5 and 6), density of actinomycetes and histamine producing bacteria was more or less same. Similar trend was noticed in the case of gills and skin with muscle samples (Fig. 3 and 4).

The above results show that actinomycetes population density was higher than the HPB in all the three parts viz., gut, gill and skin with muscle of almost all the fishes. This would indicate that actinomycetes for the dominant microflora in the body parts of the Indian mackerel fish. This suggests that actinomycetes could produce some metabolites which could suppress the growth of histamine

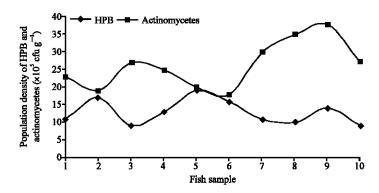


Fig. 2: Population density of histamine producing bacteria and actinomycetes in the gut of ten different mackerel fish samples

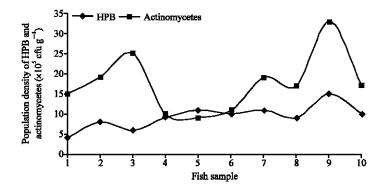


Fig. 3: Population density of histamine producing bacteria and actinomycetes in the gills of ten different mackerel samples

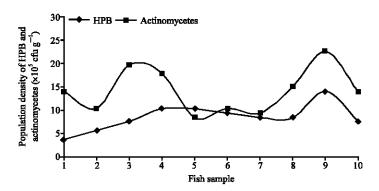


Fig. 4: Population density of histamine producing bacteria and actinomycetes in the skin with muscle of ten different mackerel samples

producing bacteria. For confirmation of the statement, the isolated 41 strains of actinomycetes were investigated for their inhibitory activity against HPB viz., *Bacillus* sp., *Pseudomonas* sp., *Vibrio* sp. and *Aeromonas* sp. which are found to be histamine producers in the Indian mackerel fish.

Table 1: Inhibitory activity of actinomycetes against histamine producing bacteria associated with the Indian mackerel fish

fish					
Strain No.	Histamine producing bacteria				
	Bacillus sp.	Pseudomonas sp.	<i>Vibrio</i> sp.	Aeromonas sp	
Inhibition zones (in cm)					
ASP-1	1.5	-	1	-	
ASP-2	1.6	1.7	1.2	1.4	
ASP-3	0.2	-	-	0.3	
ASP-4	1.6	1.6	-	_	
ASP-5	-	-	-	-	
ASP-6	1.6	-	1.5	1.4	
ASP-7	0.5	-	-	_	
ASP-8	-	-	-	_	
ASP-9	0.6	0.9	1	-	
ASP-10	1.6	-	1.5	_	
ASP-11	1.0	1.6	1.4	1.7	
ASP-12	-	1.6	1	-	
ASP-13	-	1	-	_	
ASP-14	-	- -	-	_	
ASP-15	0.9	1.5	0.7	1.1	
ASP-16	-	0.2	•	0.3	
ASP-17	-	-	_	-	
ASP-18	1.3	1.5	_	_	
ASP-19	-	-	_	_	
ASP-20	1	1.5	_	_	
ASP-21	0.5	-	0.8	_	
ASP-22	0.7	_	0.5	_	
ASP-23	0.1	_	-	0.7	
ASP-24	0.8	0.5	_	-	
ASP-25	-	-	-	_	
ASP-26	0.7	_	1.1	0.9	
ASP-27	0.4	_	-	-	
ASP-28	-	_	-	_	
ASP-29	0.4	0.9	0.1	_	
ASP-30	0.7	· · · · · · · · · · · · · · · · · · ·	0.5		
ASP-31	-	1.1	0.7	_	
ASP-32	_	0.6	0.2		
ASP-33	- -	0.9	-		
ASP-34	- -	· ·	<u>-</u>	_	
ASP-35	0.1	0.5	0.2	0.5	
ASP-36	0.1 -	0.3	0.2	0.3	
ASP-37	-	0.1	-	0.1	
	0.4	0.5	-	-	
ASP-38		0.3	-	-	
ASP-39	-	-	-	-	
ASP-40	0.6	0.9	-	-	
ASP-41	-	-	-	-	

Inhibitory Activity of Actinomycetes Against HPB

In the present study, 41 strains were isolated and among them, 32 strains (80%) were found to be inhibitory to one or more histamine producing bacterial strains at varying levels (Table 1). Out of the 41 strains, 55% were effective against *Bacillus* sp., 45% against *Pseudomonas* sp. and 40% against *Vibrio* sp. and 5% of the strains were effective against *Aeromonas* sp. (Fig. 5). Sivakumar *et al.* (2005) reported that 41.67% of actinomycetes strains isolated from mangrove sediments were effective against human pathogens, while Sahu *et al.* (2006) reported that 12.5% of actinomycetes strains isolated from the Vellar estuary showed antagonistic activity against human pathogens.

From the above, it is quite clear that the antagonistic activity of actinomycetes varies widely based on the sources from where they are isolated and the pathogens against which the antagonistic activity is tested. Under these circumstances, in the present study, it was noted that 55, 45, 40 and 5% strains were effective against HPB viz., *Bacillus* sp., *Pseudomonas* sp., *Vibrio* sp. and *Aeromonas* sp., respectively.

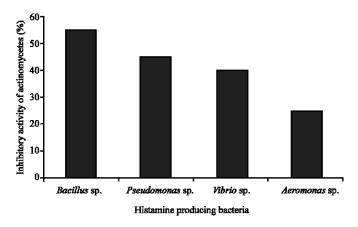


Fig. 5: Inhibitory activity of actinomycetes (%) against histamine producing bacteria

During the present investigation, out of the 32 strains, only 3 actinomycetes strains (ASP-2, ASP-11 and ASP-15) showed good activity against all the test HPB strains and hence, the strains ASP-2, ASP-11 and ASP-15 were considered as potential candidates and identified.

Taxonomic Investigation of Antagonistic Actinomycetes

All the 3 strains viz., ASP-2, ASP-11 and ASP-15 possessed LL-Diaminopimelic Acid along with glycine in their cell wall. Presence of LL-Diaminopimelic Acid along with glycine indicated the cell wall chemotype-1. The strains with chemotype -1 did not have any characteristic pattern of sugars (Lechevalier and Lechevalier, 1970).

The representatives belonging to the wall type 1 are *Streptomyces*, *Stretoverticillium*, *Chainia*, *Actinopycnidium*, *Actinosporangium*, *Elytrosporangium*, *Microellobosporia*, *Sporichthya* and *Intrasporangium*. The micromorphological observations of the strains ASP-2, ASP-11 and ASP-15 revealed that all these belong to the genus *Streptomyces*. Mathew *et al.* (1994), Dhevendaran and Annie (1999), Dhevendaran and Anithakumari (2002), Umamaheswary *et al.* (2005), Muthurayar *et al.* (2006), Sivakumar *et al.* (2006), Kundu *et al.* (2006), Sahu *et al.* (2007) and Murugan *et al.* (2007) screened the actinomycetes from the fin fishes and shell fishes and reported that all the isolated strains belong to the genus *Streptomyces*. The present study also leads support to this.

The morphological, micromorphological, physiological and biochemical characteristics of these strains (ASP-2, ASP-11 and ASP-15) are given in Table 2-4. These characteristics were compared with those of the *Streptomyces* species given in the key of Nonomura (1974) and those species described in the Bergey's Manual of Determinative Bacteriology (Buchanan and Gibbons, 1974).

Strain ASP-2 showed failure in the production of melanoid pigment and soluble pigment when compared to the reference strain, *S. aureofasciculus*. Except this, all the other characters are similar to those of *S. aureofasciculus*. Hence, the strain ASP-2 has been tentatively identified as *S. aureofasciculus* (Table 2). Strain ASP-11 differed from the reference strain, *S. chattanoogenesis* by utilizing the carbon compound viz., inositol and production of soluble pigment. However, the strain ASP-11 resembled the reference strain by showing close similarity in all the other characters and hence the strain ASP-11 has been tentatively identified as *S. chattanoogenesis* (Table 3). Strain ASP-15 differed from the reference strain, *S. hawaiiensis* by producing the reverse side pigment and also not utilizing the carbon source viz., mannitol. All the other characters were similar to those of *S. hawaiiensis*. Hence, the strain ASP-15 has been tentatively identified as *S. hawaiiensis* (Table 4).

Table 2: Comparison between the strain ASP-2 and Streptomyces aureofasciculus

Character studied (As per nonomura key)	Strain ASP-2	S. aureofasciculus
Colour of aerial mycelium	White	White
Melanoid pigment	-	+
Reverse side pigment	+	+
Soluble pigment	-	+
Spore chain	Rectiflexibiles	Rectiflexibiles
Cellulose degradation	-	-
Hydrogen sulphide production	+	+
Milk coagulation	-	-
Starch hydrolysis	-	-
Carbon source assimilation		
Arabinose	+	+
Xylose	+	+
Inositol	+	+
Mannitol	+	+
Fructose	+	+
Rhamnose	+	+
Sucrose	±	±
Raffinose	+	+

^{+:} denotes positive; -: denotes negative

Table 3: Comparison between the strain ASP-11 and Streptomyces chattanoogenesis

Character studied (As per Nonomura key)	Strain ASP-11	S. chattanoogenesis
Colour of aerial mycelium	White Yellow	White Yellow
Melanoid pigment	-	-
Reverse side pigment	-	-
Soluble pigment	-	+
Spore chain	Spiral	Spiral
Cellulose degradation	<u>-</u>	-
Hydrogen sulphide production	+	+
Starch hydrolysis	+	+
Carbon source assimilation		
Arabinose	-	-
Xylose	-	-
Inositol	-	+
Mannitol	+	+
Fructose	+	+
Rhamnose	-	-
Sucrose	+	+
Raffinose	+	+

^{+:} denotes positive; -: denotes negative

Table 4: Comparison between the strain ASP-15 and Streptomyces hawaiiensis

Character studied (As per Nonomura key)	Strain ASP-15	S. hawaiiensis
Colour of aerial mycelium	White Yellow	White Yellow
Melanoid pigment	+	+
Reverse side pigment	+	-
Soluble pigment	-	-
Spore chain	Spiral	Spiral
Cellulose degradation	-	-
Hydrogen sulphide production	+	+
Starch hydrolysis	-	-
Carbon source assimilation		
Arabinose	±	±
Xylose	±	±
Inositol	+	+
Mannitol	-	+
Fructose	+	+
Rhamnose	+	+
Sucrose	+	+
Raffinose	+	+

^{+:} denotes positive; -: denotes negative

The presently identified inhibitory actinomycetes viz., *Streptomyces aureofasciculus* (ASP-2), *S. chattanoogenesis* (ASP-11) and *S. hawaiiensis* (ASP-15) can be taken up for further detailed investigations to compare the anti-histamine producing activity and to isolate the compound responsible for the inhibitory activity.

CONCLUSION

The present study indicates that the Indian Mackerel fish harbour more actinomycetes population than histamine producing bacteria. This suggests that actinomycetes are the dominant microflora in the Indian Mackerel fish which could produce some metabolites and suppress the growth of histamine producing bacteria. These metabolites could be used as natural preservatives along with others to control the histamine producing bacterial growth after death of the fish. More studies are needed to extract the compounds from the actinomycetes and to use them as natural preservatives and also to determine the quality of the fish after the use of the compounds.

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