J. Bangladesh Agril. Univ. 9(2): 339–344, 2011 **ISSN 1810-3030**

Microbial quality of hilsa shad (*Tenualosa ilisha)* at different stages of processing

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Abstract

An attempt was made from October 2008 to March 2009 in a fish processing industry of Bangladesh (the Sea Resource Limited, Sadarghat, Chittagong) to determine microbial quality of Tenualosa ilisha at different stages of processing. During this investigation Escherichia coli, fecal coliform, vibrio cholerae, Salmonella and total load of bacteria were identified from 5 stages of processing. Those stages were receiving, primary washing stage with 5PPM chlorine water, final washing stage with 10 PPM chlorine water, after blast freezing at -40 ± 2^0C and finally after one month storage at -18 ± 2^0C. It was observed that 80.69 % of total bacterial load, 77.29% of total coliform and 58.33 % of fecal coliform were destroyed during different processing stages. There was no evidence of presence of Salmonella and Vibrio cholera at any stages of processing.

Keywords: *Tenualosa ilisha*, CFU, Chlorine water, Contamination, *Salmonella*

Introduction

Fishery plays a significant role in the field of nutrition, employment opportunity and foreign exchange earning for the people of Bangladesh. The role of fish and fishery products in supplying animal protein serve vital as evident from the past research. Basically fish take a prominent place in the mind of many people as a source of protein compared to other protein sources. Among the various types of fish river shad (*Tenualosa ilisha)* plays a very important role. It is one of the members of the genus *Tenualosa* of the family Clupeidae, order Clupeiformes. Locally known as Ilish, it has been designated as the national fish of Bangladesh. This fish is highly tasty and very much well known to the people of Bangladesh. It is rich in protein and poly unsaturated fatty acids. Its liver contains considerable amount of vitamin A, while its body oil contains vitamin C (Bhuiyan 1984). It also contains calcium, phosphorus and other mineral salts. It is estimated that one pound of *Tenualosa ilisha* fish has an average 300-1100 calories energy (Rahman 1976).

But fish is considered as one of the most perishable of all food stuffs. As soon as a captured fish dies, it begins to deteriorate. The deterioration of the flesh of the fish is caused by the action of enzymes, by micro-organism and by chemical action. Bacteria on the surface of fish skin, gills and in the guts are generally harmless in the living fish, but they start their destructive activities as soon as this fish dies. They grow and multiply rapidly at ordinary temperature, invade the flesh through the skin and breakdown the complex chemical construction of the flesh, producing the stale and later the putrid smells and tastes which are usually associated with spoilage of fish (Jadhev and Magar 1970). The activity of enzymes, bacteria and chemicals could be minimized by standards of cleanliness, careful handling technique, preservation, quality control and temperature reduction. The quality of frozen fish is determined mainly by the total number of bacteria present and by the individual count of bacteria of public health significance such as *Escherichia coli, Fecal coli*, coagulate positive staphylococcus, *Vibrio cholera*, *Salmonella etc.* The quality of fish and fishery products depend on various factors i.e. the freshness of the raw fish, method of handling and processing factories, pre-and post- process temperature etc. Strict control of every stage of processing is necessary to prevent bacterial multiplication and the various chemical changes. A regular assessment of the quality of raw material is essential especially in view of the variation in the freshness of raw materials like *T. ilisha*, where the rate of spoilage may be high and will depend on the size and species (Rahman 1976).

Although frozen fish is being exported in large scale there is lot of information available from Bangladesh export promotion bureau that, the exported fish and fishery products are sometimes rejected by the foreign countries due to high load of bacteria and presence of undesired pathogenic type of micro-organism. Further, several ingredients are now added to seafood as additives, antioxidants, preservatives, emulsifiers, cryoprotectants and coloring materials. There are also problems of pesticide residues, toxic metals, mycotoxins, biotoxins, antibiotic residues etc. Under these circumstances, the responsibility of the processor has become increasingly complex and hence, there is a global shift from food quality to food safety. In this context, the present study was undertaken to estimate total bacterial load and specific pathogen of *T. ilisha* and to compare microbiological load at different stages of their processing.

Material and Methods

Sampling stages and time

The research was conducted during the period of October 2008 to March 2009 at a processing industry named Sea Resource Limited, Sadarghat, Chittagong. Experimental samples were collected from 5 stages of processing viz., receiving, after primary washing with 5 ppm chlorine, after final washing with 10 ppm chlorine, after blast freezing and after 1 month of frozen storage. Nine fishes were taken from each stage which were divided into 3 groups. Approximately 150g sample was taken randomly from each fish and ground in a previously sterilized grinder. After grinding, 50g sample from each group were taken for determination of total bacterial load, 25 g for *Salmonella* and 25g for *Vibrio cholerae*, Fecal coliform and total coliform according to standard laboratory procedure (US food and drug administration bacteriological analytical manual January 2001).

Determination of total plate count for hilsa shad

Fifty grams of fish muscle was blended with 0.1% of 450ml peptone water in a sterile blender jar for 5 minutes. To make decimal dilution, 1ml mixture was transferred with the help of sterilized pipette to a test tube containing 9 ml of sterilized peptone water. In this way decimal dilutions of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} were prepared. One ml of sample from 10^{-3}, 10^{-4} and 10^{-5} dilution were pipetted into previously prepared agar plates. After mixing and solidification the media, the petri-dishes were incubated at inverted position for 72 hours at 30ºC**.** After 72 hour of incubation, forming colonies were counted which had a range between 30 and 300. As there is sufficient evidence that 10^{-1} and 10^{-2} dilution contain more than 300 colonies, these two dilutions were ignored for determining total bacterial load.

Determination of total coliform for hilsa shad

Nine test tubes of lauryl sulfate triptose broth (LSTB) with Durham's tube ware taken to determine total coliform which were grouped into 3 divisions. Tubes were taken for first 3 dilution i.e. dilution no 10^{-1}, 10^{-2}, 10^{-3}, but not for 10^{-4}, and 10^{-5} dilution. First 1 ml of sample was inoculated from 10^{-1} dilution by sterile pipette to first test-tube of first group. Again 1 ml of sample was inoculated from 10^{-1} dilution by sterile pipette to second test-tube, and lastly it was again repeated for 3 test-tubes of first group. By this way rest of the 6 test-tubes from 10^{-2} and dilution10^{-3} dilution were inoculated. All nine tubes were incubated at 37 ºC for 48 hours. After incubation, gas producing tubes were marked and recorded. After that, total coliforms were counted from the most probable number (MPN) chart from USFDA, Bacteriological analytical manual 6th edition 1984.

Determination of fecal coliform for hilsa shad

To determine fecal coliform, 2 test tubes for each gas forming LSTB were taken. Among those two test-tubes one contained Brilliant green lactose bile broth (BGLBB) and another test tube contained peptone water/tripton water. Now to identify fecal coliform 1 loop (minimum 3 mm diameter) of sample from every gas forming test-tube was transferred to the test tube of BGLBB and the test-tube containing tripton water. Both two test-tubes were incubated in circulating water bath at 44.5±2 ºC for 48 hours. After incubation period, the gas producing tubes were identified and marked. Then tubes which produced gas on both BGLBB and peptone/tripton water was counted and the fecal coliform from most probable number (MPN) chart was identified.

Determination of *Salmonella* for hilsa shad

At first 25g of raw sample was taken in a sterile bottle with 225ml of buffered peptone water and incubated at 37ºC for 48 hrs. After incubation 1ml of sample was transferred into 10ml of Salenite Cystene Broth (SCB) and 1 ml in Tetra Thionet broth (TTB). Both TTB and SCB bottle were incubated at 37 ºC for another 24 hrs. Then 3mm loopful incubated sample was streaked from SCB and TTB into the sterile petridish containing Xylen deoxycholate agar (XLDA) and Brilliant green agar (BGA). Both petridishes were incubated at 37ºC for another 48 hours. On XLDA petridish, *Salmonella* will appear pink or yellow colonies with or without black centers. On the other hand on BGA petridish typical *Salmonella* colonies appears as dark to deep black color. The colonies that were found positive by observing XLDA and BGA petridish were inoculated into Triple Sugar Iron (TSI) agar by streaking the slant and stabbing the butt without flaming the sterile loop. The colony was again touched by serial loop and inoculated into lysine Iron Agar (LIA) by stabbing the butt and streaking the slant. Then the TSI and LIA test-tubes were incubated at 37 ºC for 24 hrs. *Salmonella* cultures typically produce an alkaline (red) slant and acid (yellow) with or without production of hydrogen sulphides. In TSI and in LIA *Salmonella* culture typically produce an alkaline (purple) reaction in the butt tube. Only a distinct yellow coloration in the butt of the tube will be considered as an acidic (negative) reaction. All positive presumptive *Salmonella* culture of TSI and LIA were retained for biochemical characterization. For positive TSI culture, they were streaked on to Macconkey agar and nutrient agar incubated at 37ºC for 24 hrs.

Determination of *Vibrio cholerae* for hilsa shad

At first 25g of sample were taken in a sterile bottle with 225ml of peptone water and incubated at 37ºC for 48 hours. After incubation they were streaked on thiosulphate citrate bile salt sucrose agar (TCBS), and incubated at 37ºc for 24 hrs. If suspected colony was found they were marked. Lightly touched to the center and picked with a sterile needle, and inoculated into TSI test tube and Kiglar iron Agar (KIA) test tube. After inoculating sample into TSI test tube they were incubated overnight at 37ºC. *Vibrio cholerae* will manifest in TSI as acid slant and acid butt (yellow) and will produce neither gas nor blacking in the butt i.e. hydrogen sulphid negative. In KIA as alkaline slant (red) and acid butt (yellow).

To ensure the biochemical identity of *Vibrio cholerae* the following tests were performed according to standard laboratory procedures as described by US food and drug administration bacteriological analytical manual, January 2001.

Test	Reaction	Result
Oxidize test	Purple	Positive
Hugh-leifson test using dextrose	Fermentation (acid gas aerobically and anaerobically)	Positive
Fermentation Reaction		
a. Glucose	Acid , no gas	Positive
b. Sucrose	Acid , no gas	Positive
c. Manitol	Acid , no gas	Positive
d. Inositol	No Acid , no gas	Negative
Lysine decarboxylase	Purple	Positive
Growth in 1% tryptophane	Turbid	Positive

Results and Discussion

From this investigation it was identified that in the receiving area total bacterial load in *T. ilisha* was 1.827×10^6 CFU/g and before shipment it was reduced to 2.94×10^5 CFU/g. In the receiving area the load of total coliform was 28 /g and before shipment it was 6.13/g which was 4.67 times lower than the receiving area. In case of fecal coliform, it was 7.33/g during receiving stage and before shipment it was 3/g which was 2.44 times lower than the fishes for receiving area. There was no evidence of *Vibrio cholerae* and *Salmonella* in selected *T. ilisha* samples.

During the study the total bacterial load of unwashed freshly caught *T. ilisha* at receiving center was 17.5 $\times 10^5$ CFU/g to 18.65 $\times 10^5$ CFU/g (Table 3) and the average was 18.27 $\times$ 10^4 CFU/g (Table 3), which was almost 8 times higher than Alam (1980) who found bacterial load to be 2.8$\times$ 10^5 CFU/g and recorded bacterial load ranging from 1$\times$ 10^3 CFU/g to 1$\times$ 10^5 CFU/g , 1.7$\times$ 10^5 CFU/g to 2.5$\times$ 10^5 CFU/g and 1.5 $\times$ 10^5 CFU/g to 2.9 $\times$ 10^5 CFU/g at freshly caught flat fishes, fresh sardines and pomfrets muscle, respectively. Total bacterial load of *T. ilisha* at different processing steps were presented in Table 3. It was found that total bacterial load at receiving area was higher than the previous investigation, which might be due to seasonal variation, degrading water quality, untreated industrial wastage discharge into fish habitat and human recreation. After washing with 5 ppm chlorine water bacterial load reduced to 11.38 $\times$ 10^5 CFU/g from 18.27$\times 10^5$ CFU/g (Table 3), ie.37.71% lower and it was 1.61 times lower from receiving area. Alam (1980) reported after washing bacterial load reached 6.2 $\times 10^4$ CFU/g from 8.8 $\times 10^4$ CFU/g which was 29.54 % lower. That was also supported by the present research. The rate of bacterial reduction in present study was a bit higher; this may be due to careful and standard washing technique. In a further washing by 10 ppm chlorine after grading, the bacterial load reached 7.55$\times 10^5$ CFU/g (Table 3) which was 1.51 time lower than previous step and 2.42 time lower then receiving step. This result is supported by Alam (1980). Furthermore Green (1949) reported that washing the whole fish reduced the bacterial load per gram by 29% and 18.19 %, respectively and the present study found total bacterial load to be reduced to 33.66% when fish were washed with 10 ppm chlorine. Alam (1980) also reported that during deep freezing (-40$\pm 2^0$C) there was a reduction of 92 % of total bacterial load in *T. ilisha* **,** which was in agreement with results reported by Shewan and Ehrenberg (1977) and Iyer and Chaudury (1969), who found about 90% reduction in the bacterial load during deep freezing. It has been stated by Shewan and Ehrenberg (1977) that as soon as freezing occurs the bacterial growth is arrested due to restricted moisture condition. The present research found that total bacterial load dropped to 3.57 $\times 10^5$ CFU/g from 7.55 $\times$ 10^5 CFU/g (Table 3) that was 2.11 fold or 52.72 % lower than washing with 10 ppm chlorine. According to this research, total bacterial load fall 3.57 $\times 10^5$ CFU/g to 2.98 $\times 10^5$ CFU/g (Table 3) after one month storage where temperature was -18$\pm 2^0$C. This 1 month storage reduced total bacterial load to 0.59 $\times$ 10^5 CFU/g. So there was an expectation to reduce bacterial load by preserving fish in cold temperature for a longer period of time. This hypothesis was agreed by Alam (1980) and Bhuiyan (1984). Alam (1980) found initial bacterial load of *T. ilisha* (after blast freezing) 2.52 $\times 10^4$ CFU/g which come 1.52$\times 10^4$ CFU/g after 6 month storage at -18 ^{0}, as well as Bhuiyan (1984), observed that total bacterial load of shrimp was 3.22$\times$ 10^5 CFU/g just after freezing and after 6 month stock at -18 ^{0}C it was reduced to 0.62 $\times 10^5$ CFU/g. Although the present study found that bacterial load in the first 3 stage of processing (receiving, primary washing with 5 ppm chlorine and final washing with 10 ppm chlorine,) exceeded the acceptable limit, after blast freezing and subsequent storage, the load was reduced to the acceptable limit.

In case of total coliform, the load was 28 /g at receiving stage , 20.33 /g at primary washing by 5 ppm chlorine , 12 /g at final washing by 10 ppm chlorine ,7.33 /g after blast freezing and 6.13/g after one month storage at -18 $\pm 2^o$C (Table 2). From this result it was observed that by different processing steps, total coliform load at frozen storage stage was reduced 4.57 fold then receiving stage. It was almost 17 % lower than primary condition. Mukherjee *et. al.,* (1991) recorded 3.1$\times 10^2$ *E. coli.* He also recorded that washing reduced 35.5 % *E. coli* which is in agreement with the present study where washing with 10 ppm chlorine water and blast freezing at -40 $\pm$ 2^0C was very effective in reducing *E. coli* load. The natural habitat for *E. coli* is the intestines of human and vertebrate animals. In temperate waters this organism is absent from fish and crustaceans at the time of capture (except in grossly polluted waters). Moreover, fish and shellfish should always be held at temperatures below those which support growth. This organism is, therefore, particularly useful as indicator of contamination (small numbers) or mishandling such as temperature abuse in product handling (large numbers). Contamination of food with *E. coli* implies a risk that one or more of enteric pathogens may have gained access to the food. However, failure to detect *E. coli* does not assure the absence of enteric pathogens. Bhuiyan, 1984) reported that total coliform load was 109.3/g after taking this fish in market which exceeds the accepted limit (accepted limit is < 100 coliform/g) (Table 1).

Table 1. Acceptable limit of different bacteria in Bangladesh for frozen fish

Systematic name	CFU/g	Total coliform/g	Fecal coliform/g	*Salmonella* spc./25g	*Vibrio cholerae* spc./25g
Acceptable limit	5×10^{-5}	< 100	< 10	Absent	Absent

Table 2. Microbiological analyses *of T. ilisha* samples

Stages	Total bacterial load $\times10^5$ CFU/g	Total coliform/g	Fecal coliform/g	*Vibrio cholerae* (25 g sample)	*Salmonella* (25 g sample)
Receiving	18.27	28	7.33	Absent	Absent
Primary washing (with 5 PPM chlorine water)	11.38	20.33	6.13	Absent	Absent
Final washing (with 10 PPM chlorine water)	7.55	12	3.4	Absent	Absent
Blast freezing (-40 ± 2 ^{0}C)	4.30	7.33	3.4	Absent	Absent
Stocking (Before shipment after 1 month stock) (-18 ± 2 ^{0}C)	2.94	6.13	3.0	Absent	Absent

Table 3. Total bacterial load of *T. ilisha* at different stages of processing

Sampling area	Sample no.	Result for 10^{-3} CFU/g	Result for 10^{-4} CFU/g	Result for 10^{-5} CFU/g	Result $\times10^5$ CFU/g	Average $\times10^5$ FU/g
Receiving	1	>300 >300	180 170	19 18	17.50	18.27
	2	>300 >300	190 183	18 19	18.65	
	3	>300 >300	187 186	19 19	18.65	
Primary washing (Before Grading with 5 PPM chlorine water)	1	>300 >300	110 115	12 12	11.25	11.38
	2	>300 >300	117 111	13 11	11.40	
	3	>300 >300	117 113	12 21	11.50	
Final washing (After Grading with 10PPM chlorine water)	1	>300 >300	78 72	7 7	7.50	7.55
	2	>300 >300	73 70	7 7	7.15	
	3	>300 >300	80 80	8 8	8.00	
Blast Freezing (-40 ± 2 ^{0}C)	1	>300 >300	38 36	4 3	3.70	3.57
	2	>300 >300	36 35	3 3	3.55	
	3	>300 >300	37 32	3 3	3.45	
Stocking at -18 ± 2 ^{0}C for shipment (after 1 month stock)	1	298 297	28 27	3 3	2.98	2.98
	2	297 297	26 26	2 2	2.97	
	3	299 297	29 28	3 3	2.98	

Source: Microbiological analysis of food with good lab practices by Elias S. U. 2005

Fecal coliform is a hazardous pathogen for human. For Bangladeshi fishery product its acceptable limit is <10/g. If this limit exits then the foreign buyer rejects, the shipment. In the present study the load of fecal coliform was 7.33/g at receiving stage, 6.13 /g after washing with 5 ppm chlorine, 3.4/g when samples were washed with 10 ppm chlorine, 3.4/g after sample freezing at -40±2^{o}C and 3/g after one month storage at -18±2^{o}C (Table 2). From this result it was identified that by different processing technique fecal coliform load at stocking stage was reduced 2.44 fold then receiving sage. It was almost 59% lower than primary condition. Mukherjee *et. al.,* (1991) recorded that after icing fecal coliform load was only 0.8×10^{2} which was 37.5 % lower than washing stage. Iyer and Chaudury (1967) observed that during frozen storage (-20^{0}C) *E. coli* was fully destroyed within 5 to 6 months. Coagulase positive Staphylococcus was destroyed after 6 month storage but 10–15% of the residual fecal coliform was destroyed in a period of 6 month storage. The higher resistance of fecal coliform to cold storage temperature indicates its superiority as an index of fecal coliform in frozen fish.

Table 4. Result of total coliform and fecal coliform of *Hilsa ilisha* at after one month storage stage

Sampling area	Sampling no.	Broth base	Concentration		
			10^{-1}	10^{-2}	10^{-3}
Stocking at -18 ± 2 ^{0} C for shipment (after 1 month stock)	1	LSTB	0	1	1
		BGLBB	1	0	0
		TW	0	1	0
		TC	6.1 /g		
		FC	3 /g		
	2	LSTB	0	2	0
		BGLBB	0	2	0
		TW	0	1	0
		TC	6.2 /g		
		FC	3 /g		
	3	LSTB	0	1	1
		BGLBB	1	0	0
		TW	0	0	0
		TC	6.1 /g		
		FC	<3.0 /g		
Average load of T.C. /g	6.13 /g				
Average load of F.C. /g	3.0 /g				

It is worth mentioning that although the quality of water and surrounding environment is degrading day by day, no *Vibrio cholerae* and *Salmonella* was identified from selected samples during this investigation.

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