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Changes in the Nitrogenous Compounds during Fermentation of Hilsa Steaks

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Lona ilish, a traditional salt fermented fish product, made exclusively from hilsa (*Tenualosa ilisha*), is widely consumed in Bangladesh and adjoining Northeastern parts of India. No detailed scientific studies have been made on *lona ilish*. To understand the science behind this traditional preservation process, the product was prepared in the laboratory following traditional method, and biochemical changes during fermentation period of 150 days, were studied. At fortnightly intervals, important parameters such as the protein and protein degradation as well as lipid and its degradation in the product were studied. Increase in non-protein nitrogen (NPN), free alpha amino nitrogen (FAN) and total volatile basic nitrogen (TVBN) contents during fermentation indicated hydrolysis of protein. However, the decrease in protein nitrogen content was not significant. A value of NPN/TN of 18% in the fish muscle was found indicative of the fermentation point. High salt content (15.48%), intermediate moisture content (49.89%) and low pH (5.28) of the final product were found to be satisfactory for stability of the *lona ilish* at ambient temperature.

Key words: Hilsa, traditional technology, salting, fermentation, biochemical composition

Hilsa (*Tenualosa ilisha*) has a universal appeal to the consumers of Eastern India and Bangladesh. *Lona ilish*, a salt fermented product is prepared exclusively from this fish. The product very popular due to its distinctive flavour and texture, is used as both condiment as well as main dish. The fermented fish products find an important place in the dietary lists of Southeast Asian countries (Amano, 1962; Mackie *et al*., 1971). These products are commonly used as a food condiment which are strictly traditional and limited to local consumption (Saisithi *et al*, 1966). This method of preservation still enjoys popularity in many developing and underdeveloped countries owing to its simplicity and low cost of processing (Zenitani, 1955).

The name *lona ilish* means salted fish ('*lona*' means salted in Bengali). The product is sliced hilsa (hilsa steaks) about 1.00 to 1.50 cm in thickness, distinguishing itself from other fermented fish products by having a soft but firm texture, very pleasant flavour and aroma and attractive fresh fish colour even after maturation. It is kept immersed in saturated brine till consumption. A typical *lona ilish* has a uniform pink colour with glossy appearance immediately after taking out from the brine. The pink colour and glossiness of properly matured *lona ilish* vanish after a few minutes of taking out of the brine and colour gradually becomes light greyish to deep greyish. The flesh does not easily separate from bone and it has a characteristic strong aroma constituted chiefly by the typical aroma of hilsa fish mixed with some sweet, fruity and acidic note and saltiness.

The physical and chemical changes that occur during fermentation determine the overall sensory qualities of salted products (Voskresensky, 1965). These changes are induced by enzymes which break down both

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proteins and fats. The hydrolysis of proteic substances is brought about by the action of peptide hydrolases of cathepsin A, B and C types as there exists a close agreement between their optimum pH values and those in the fish muscle tissues (Shenderyuk & Bykowski, 1990). Van Klaveren & Legendre (1965) reported that endo- and exo- peptidases from fish were affected differently by gradually increasing salt concentrations. They showed that when salt concentrations exceed 15%, the catheptic activity slows but continues at a reduced rate even in saturated brines. The visceral enzymes of herring were also affected by high salt concentrations, but a lesser extent than cathepsins (Voskresenski, 1965). Uyenco *et al.* (1953) studied the influence of different salt concentrations on the hydrolysis of fish protein and pH of the fish sauce and found that the amount of amino nitrogen formed varied inversely with the salt concentrations below 6 pH. The aim of the presnt study was to observe the changes of nitrogenous compounds in hilsa steaks during the fermentation period of 150 days.

Materials and Methods

Freshly landed fish, hilsa (*Tenualosa ilisha*) was purchased from Chandpur market of Bangladesh and transported in ice packs within 20 hours. The weight of the fishes ranged from 600 to 800 g. *Lona ilish* was prepared following the traditional method. The fishes were washed thoroughly with potable water, descaled, beheaded without removing gut and then cut diagonally into steaks of 1.25 to 2.0 cm thickness. The fish steaks were immediately dry salted (fish to salt ratio was 4:1 or 5:1) by thorough rubbing of salt and kept in a bamboo basket layer after layer with sprinkling of salt between each layer and covering the top layer with salt. The bamboo basket was well covered with black polythene sheet to prevent entry of light and allowed to stand. During this period the self-brine from the fish was allowed to drain. After 48 hours, the salted fish steaks were made free from adhering salt and packed tightly in tin-coated steel container of 5 l capacity. When the containers were almost filled, previously boiled and cooled saturated brine prepared from good quality salt was poured slowly to fill the voids between the steaks to a level about 2 cm above the fish. The containers were then closed with lid and stored in a room for maturation.

Five steaks from different depths of each container were taken and allowed to drain the brine for one minute giving little pressure by hand and macerated in a blender for about 10 sec. The mixtures were used as representative sample of each container. Moisture, pH, ash, and salt contents were measured following standard methods (AOAC, 1995). Total nitrogen was measured by using the micro-Kjeldahl method of AOAC (1995). Ten percent trichloroacetic acid (TCA) extract was used to estimate non-protein nitrogen (NPN), total volatile basic nitrogen (TVBN) and free alpha amino nitrogen (FAN) by using micro-Kjeldahl method (AOAC, 1995), Conway's micro-diffusion method (Conway, 1947) and by copper method (Pope & Stevens, 1939) respectively.

Analysis of variance (one way - ANOVA) was performed to determine the differences between experimental periods of maturation. The tests for differences were done by using Tukey's Multiple Comparison Test. All statistical analyses were performed using Statistical Package for Social Sciences (SPSS, version 11.0 for windows). The level of significance for the experiments was set at 5%.

Results and Discussion

The changes in moisture, pH, ash and salt are presented in Table 1. After dry salting the moisture content decreased significantly ($p< 0.05$) from 55% of fresh fish to 46% and further decreased to 42% after first 15 days in saturated brine. However, after 150 days of fermentation, the moisture increased slightly and reached to about 50%. In this study, during fermentation of hilsa a slight increase in the moisture content was

Table 1. Change in moisture, pH, ash and salt in muscle during fermentation of hilsa

Period	Fresh	D-0	D-15	D-30	D-45	D-60	D-75	D-90	D-105	D-120	D-135	D-150
Moisture (%)	55.55^{c} (2.02)	46.46^{ab} (0.25)	42.46^{a} (0.25)	44.86^{ab} (2.16)	45.83^{ab} (5.64)	47.72 ^{ab} (2.68)	45.83 ^{ab} (0.84)	46.57 ^{ab} (1.73)	45.14 ^{ab} (1.33.)	47.32 ^{ab} (1.86)	48.57^{abc} (1.92)	49.89^{bc} (2.19)
pH	6.24^{a} (0.06)	5.88^{b} (0.05)	5.63^{c} (0.04)	5.60^{c} (0.01)	5.48^{d} (0.01)	5.45^{de} (0.01)	5.43^{de} (0.01)	5.35^{eg} (0.01)	5.35^{eg} (0.03)	5.17^{ih} (0.05)	5.22^{hf} (0.02)	5.28^{fg} (0.05)
Ash (%)	1.74^{a} (0.12)	14.98^{b} (0.39)	17.06^{c} (0.64)	16.84^{c} (0.65)	16.41^{bc} (0.46)	16.01 ^{bc} (0.35)	16.57 ^{bc} (0.33)	16.44 ^{bc} (0.69)	16.93^{c} (0.66)	16.58 ^{bc} (0.40)	16.78^{c} (0.17)	16.37 ^{bc} (0.17)
Salt (%)	0.18^{a} (0.03)	13.65^{b} (0.33)	15.78 ^{b} (0.44)	15.59 ^{b} (0.70)	15.20 ^{b} (0.42)	14.89 ^{b} (0.28)	15.40 ^{b} (0.13)	15.31 ^{b} (0.55)	15.86 ^{b} (2.18)	15.59 ^{b} (1.48)	15.85 ^{b} (1.73)	15.48 ^{b} (1.21

Fresh : Raw, dressed fish steaks, D-0 : Samples after dry salting and before packing and brining.
D-15….D-150 : Samples at 15 days interval.
Figures in brackets indicate SD, n = 3.
Means with same superscript in the same row do not differ significantly ($p < 0.05$).

noticed during the later part of fermentation which can be attributed to the absorption of water by fish tissues as they swell when immersed in salt solution during a prolonged storage (Wheaton & Lawson 1985, Hernandaz-Herrero *et al.,* 1999). During salting, moisture is rapidly removed from the fish and on the other hand salt penetrates in to the flesh (Clucas, 1982). Hernandaz-Herrero *et al.* (2002) also observed similar trend during fermentation of salted anchovy. The average composition of moisture, protein, lipid and ash content of hilsa (from Chandpur area of Bangladesh) was reported by Rahman *et al.* (1999) in the order of 52.98%, 17.37%, 26.93% and 1.68% respectively. The loss in moisture was accompanied by increase in salt and ash. Salt and ash content, from the initial value of 0.18% and 1.74 % came down to 15.48 % and 16.37 % respectively at the end of 150 days. A similar relation has been reported by Srikar *et al.* (1993) during fermentation of salted mackerel and pink perch (*Nemipterus japonicus*). No significant difference was observed in the salt and ash content after dry salting upto the end of fermentation.

The pH of fresh fish decreased significantly ($p<0.05$) from 6.24 to 5.88 after dry salting and thereafter slowly declined to 5.17 after 120 days and then slightly increased and reached to 5.28 at the end of 150 days. Further decrease in pH observed may infer that samples were undergoing fermentation and not spoilage. Increase in pH during the later part of fermentation has been attributed to the formation of volatile basic compounds (Yatsunami & Takenaka, 1996). The gradual decrease in pH was accompanied by the gradual increase in TTA. The TTA reached to 0.98% after 105 days and then slowly decreased and attained 0.88% after 150 days of fermentation. The decrease in TTA started after 105 days and the increase in pH was observed after 120 days. As expected, there was a negative correlation between the pH and TTA

($r = - 0.41$)

The changes in the nitrogenous compounds are presented in Table 2. The total nitrogen (TN) increased significantly ($p < 0.05$) from 3.12% to 3.58% after dry salting and reduced significantly ($p<0.05$) to 2.90% at the end of fermentation. The changes in nitrogenous compounds of the fish during fermentation are associated with denaturation of proteins, increase of free amino acids and other forms of non-protein nitrogen in the muscle tissue of the fish. Increase of protein nitrogen (PN) after dry salting may be due to the fact that the loss of water has been more than the loss of PN during this time. On the other hand, after addition of brine, greater contact of brine and tissue facilitated a high rate of loss of

Table 2. Change in nitrogenous compounds during fermentation of hilsa

Period	Fresh	D-0	D-15	D-30	D-45	D-60	D-75	D-90	D-105	D-120	D-135	D-150
TN (%,muscle)	3.12^{abc} (0.05)	3.58^{b} (0.03)	3.02^{c} (0.03)	3.18^{abc} (0.06)	3.25^{abc} (0.05)	3.20^{abc} (0.25)	3.16^{abc} (0.05)	3.03^{c} (0.07)	2.95^{c} (0.27)	3.06^{abc} (0.26)	3.04^{abc} (0.27)	2.90^{c} (0.17)
PN (%,muscle)	2.78^{abc} (0.03)	3.15^{c} (0.01)	2.79^{abc} (0.03)	2.88^{bc} (0.09)	2.95^{bc} (0.08)	2.86^{bc} (0.17)	2.82^{abc} (0.03)	2.72^{abc} (0.06)	2.66^{abc} (0.18)	2.69^{abc} (0.20)	2.60^{ab} (0.22)	2.37^{a} (0.15)
NPN (%,muscle)	0.34^{abc} (0.01)	0.43^{cd} (0.04)	0.23^{a} (0.01)	0.29^{ab} (0.05)	0.30^{ab} (0.09)	0.34^{abc} (0.06)	0.34^{abc} (0.03)	0.31^{ab} (0.01)	0.29^{ab} (0.03)	0.37^{bc} (0.04)	0.44^{cd} (0.02)	0.53^{d} (0.02)
FAN (mg %)	44.46^{a} (3.5)	59.59^{abc} (0.58)	69.20^{ab} (0.7)	90.18^{bcd} (8.03)	99.0^{cdef} (25.7)	99.86^{cdef} (12.7)	100.3^{cdef} (11.3)	101.2^{cdef} (6.27)	109.6^{cd} (9.5)	117.6^{cdef} (13.6)	122.4^{cdef} (5.37)	140.0^{f} (7.0)
TVBN (mg %)	16.77^{a} (1.65)	23.07^{abc} (2.26)	19.80^{ab} (2.10)	22.90^{abc} (1.48)	26.43^{cd} (3.71)	40.80^{g} (2.65)	36.10^{fg} (2.26)	33.95^{efg} (2.16)	30.33^{def} (2.70)	29.26^{cde} (2.7)	25.8^{bcd} (3.36)	23.38^{bc} (2.24)
NPN/TN	0.11^{ab} (0.01)	0.12^{ab} (0.01)	0.09^{a} (0.02)	0.09^{a} (0.01)	0.09^{a} (0.02)	0.11^{ab} (0.01)	0.11^{ab} (0.01)	0.10^{a} (0.01)	0.10^{a} (0.01)	0.12^{ab} (0.01)	0.15^{bc} (0.01)	0.18^{c} (0.02)

Fresh : Raw, dressed fish steaks, D-0 : Samples after dry salting and before packing and brining.
D-15…….D-150 : Samples at 15 days interval. Values are mean (SD), n=3.
Means in same row superscript by common letters were not significantly different ($P < 0.05$).
TN- Total nitrogen, PN- Protein nitrogen, NPN- Non-protein nitrogen, FAN- Free alpha-amino nitrogen, TVBN-Total volatile basic nitrogen.

salt-soluble protein in the initial 15 days. Following day 15 there was a stabilization of the loss of PN before a steady decrease on the PN in the later period. Though we have not come across any literature explaining why the decrease of PN in the final stage takes place, similar observations have been reported by other workers (Uyenco *et al.*, 1953; Mathew & Raghunath, 1996; Hernandaz-Herrero *et al.*, 2002). TN also followed the same pattern as the PN. This may be due to the effect of PN. However, it is generally known that salt soluble protein is leached out from fish body due to osmosis. A higher percentage of PN was lost during fermentation relative to dry salting. As it was observed, the variation of TN on each sampling day of fermentation may be due to continuous exchange of salt and moisture, hydrolysis of protein by intrinsic and microbial enzymes, and also due to leaching of low molecular weight nitrogen compounds in to the brine. The microbial enzymes have a role in flavour and aroma development of fermented fish products (Beddows *et al.*, 1979; Thongthai & Siriwongpairat, 1990). After dry salting, the PN increased from 2.78% to 3.15% in fresh fish and then slowly decreased and finally reached 2.37% after 150 days.

In the present study, the initial NPN value of 0.43 after dry salting increased to 0.53 after 150 days ($p < 0.05$). There was marked variation in the NPN content throughout the maturation period and also between the samples, which may be attributed to the fact that a fraction of NPN was continuously diffusing into the surrounding salt solution at different intensities. Mathew & Raghunath (1996) also reported similar findings. During salting, the exchange of matter in the system is accomplished mainly by the movement of salt molecules; but during fermentation period, nitrogenous substances, mainly of low molecular weight (NPN compounds) diffuse from the fish into the brine (Voskresensky, 1965). While studying fermentation of salted anchovy, Hernandaz-Herrero *et al.* (2002) reported an initial decrease and then gradual increase of NPN during the period of fermentation.

The NPN/TN in the fish muscle showed a slight decrease initially and then increased slowly and later on markedly to 0.18 after 150 days. The NPN/TN in the fish muscle has already been proposed as a method to assess the fermentation process of anchovy (Perez-Villarreal & Pozo, 1992) and proposed a value of NPN/TN between 33% and 40%

as indicative of the fermentation point. However, in the present study with hilsa, a value of 18% (NPN/TN) in the fish muscle would indicate the fermentation point.

The TVBN increased steadily ($p < 0.05$) to 40.8 mg % till 60 days and thereafter slowly decreased ($p < 0.05$) to 23.3 mg % at the end of 150 days. The observed pattern of change in the TVBN content in the tissue might have been influenced by the factors like rate of enzymatic activity, bacterial action as well as simultaneous diffusion into the surrounding liquid. The intial steady increase of TVBN is probably due to the enzymatic and bacterial action that superceded the diffusion of TVBN into surrounding solution. During the later part, the diffusion of TVBN could have been more than its generation. A similar trend of TVBN was observed by Hernandaz-Herrero *et al.* (2002) during fermentation of salted anchovy. Connell (1978) suggested the limit of acceptability of TVBN in salt fermented fish to be below 100 to 200 mg/100g.

The free alpha amino nitrogen (FAN) increased markedly ($p < 0.05$) throughout the fermentation period and reached 140 mg % from the initial value of 59.59 mg % after dry salting (Table 2). The increase in FAN throughout the period of maturation could be attributed to the combined effects of autolysis and microbial degradation of the fish muscle (Ijong & Ohta, 1996). According to Voskresensky (1965) the increase in alpha-amino nitrogen after curing indicates degradation of protein. In the present study, the steady increase of FAN may be attributed to the action of enzymes from all the three sources, i.e. muscle, gut and bacteria. Since, the muscle enzymes of fish play a less active role during fermentation than that of gut enzyme (Voskresensky, 1965); it is probable that the retention of gut is important in providing the proteolytic enzymes during fermentation of hilsa. In addition, unlike muscle cathepsins, the endopeptidases of the alimentary tract have not been shown to be severely affected by sodium chloride at rather high concentrations (Reddi *et al.*, 1972). The other factor that determine the FAN content of the muscle may be the diffusion of a fraction of FAN to the surrounding brine.

From the study, it appears that fermentation of high fat fish hilsa is mostly accompanied by protein degradation. The increase in NPN and decrease in PN indicated the hydrolysis of proteins presumably by the gut enzymes. The role of gut enzymes in this process must be very important.

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