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Biochemical and Microbial Changes Associated with Fermentation of *Setipinna phasa*

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Abstract

Salt-free fermented *Setipinna phasa*, locally known as '*phasa shidal*' is a traditional fish product of north-east India. This study was conducted to assess the biochemical and microbial changes during fermentation of *S. Phasa.* Moisture content, pH and crude protein of the fish product decreased, whereas, lipid and total titratable acidity increased during fermentation. There was no significant change in the total nitrogen content, however, protein nitrogen decreased significantly with the increase of non-protein nitrogen and alpha-amino nitrogen. The non-protein nitrogen to total nitrogen ratio also increased gradually from 16.62 to 37.96%. The lipid degradation products showed considerable increase during the fermentation process. The volatile nitrogenous bases showed a rapid increase in the beginning followed by a slow but gradual increase throughout the fermentation period and the value reached 231.94 mg of nitrogen 100 g^{-1} on 90th day. During the same period the microbial count increased rapidly in the beginning and reached 6.79 log cfu g^{-1} on 90th day. *Staphylococcus* spp. and *Bacillus* spp. were found to be the predominant groups of bacteria during fermentation. On 90th day, the product was moist with very soft surface and texture, moderate stickiness with strong characteristic smell of *shidal*.

Keywords: *Setipinna phasa*, traditional fish product, north-east India, *Bacillus*, *Staphylococcus*

Introduction

Fermentation is an age-old food preservation method which was evolved to develop desirable physicochemical and sensory characteristics in food. Micro-organisms decompose organic constituents in order to secure energy for their own growth (Majumdar, 2005). The fermented fish products perform a beneficial role in human nutrition (Muller-Paludan et al., 2002) and are rich in amino acids, nitrogen and various trace-elements, including sodium chloride, phosphorus, calcium and fluoride (Huss, 1988). The hydrolytic products of fish protein and partially hydrolyzed substrate are readily assimilated than the native protein (Ninawe & Rathnakumar, 2008). In addition to preservation, fermented foods can also have an added benefit of enhanced flavour, increased digestibility, improved nutritional value and pharmacological values (Jeyaram et al., 2009).

North-east region of India is bestowed with many fermented fish products such as *shidal, ngari, hentak, lona ilish, tungtap* etc. *Shidal* is very popular due to its strong flavour and made exclusively either from *Puntius* spp. or *Setipinna phasa*. The typical strong flavour is due to break down of fish protein and lipid, which produce some peptides, amino acids, fatty acids, indole, skatole etc. producing a strong characteristic odour of *shidal*.

Literature regarding chemical and microbiological changes during fermentation of *S. phasa* is very limited. The objective of the present study was to understand the biochemical and microbiological changes during traditional fermentation process of *S. phasa*.

Materials and methods

Dried *S. phasa* was procured from Agartala, Tripura dry fish market. Other materials like earthen *matka* (fermenting container), mustard oil, cover paste, bamboo baskets etc. were procurred from local market. Fermented *S. phasa* was prepared in the laboratory following traditional practices (Fig. 1). Sampling was done fortnightly for biochemical and microbial analysis. Fermentation was set in three

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containers which could hold approx. 5 kg fish. The fermenting container was cleaned to remove adherent dirt. The mud layer and cover paste were first removed very carefully in the laboratory. Three to five fish from the centre of each container were taken and transferred to a polythene pouch. Ten gram of flesh from the sampled fish from each container were asceptically collected in a sterile petridish for microbiological analysis. For biochemical analysis, the fishes from each container were mixed together and crushed in a pestle mortar to make a representative sample. All the biochemical parameters were analysed in triplicate for each container.

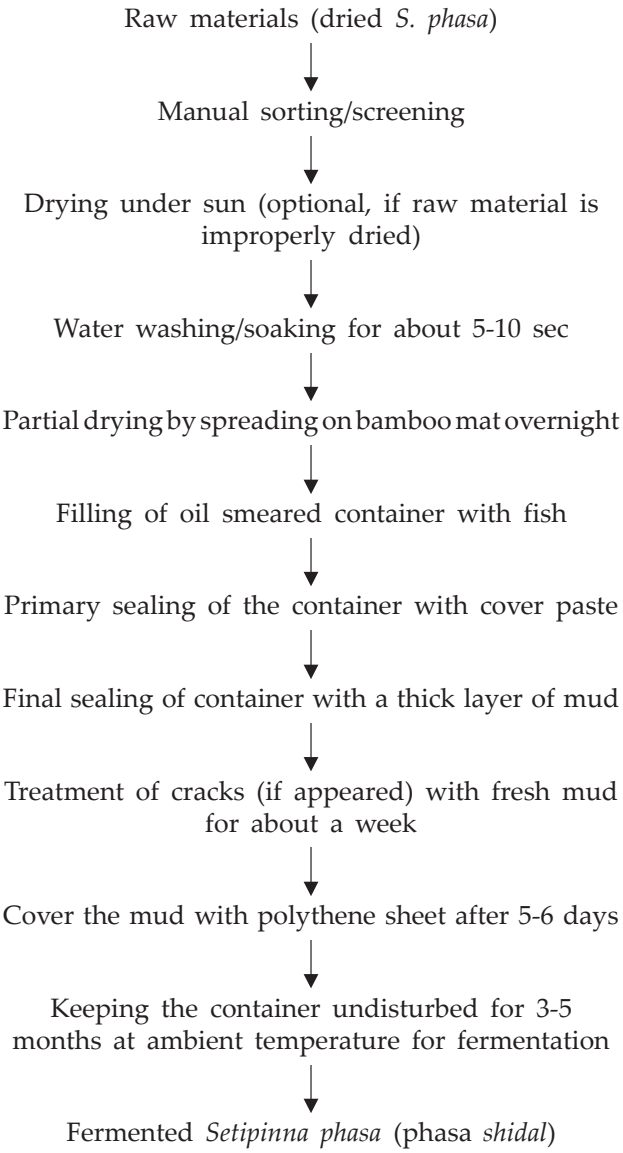

Fig. 1. Production of fermented *Setipinna phasa* following traditional method

The proximate composition of dried *S. phasa* before and during fermentation were analysed according to the method of AOAC (1995; 2000). The total tritratable acidity (TTA) and pH were determined according to AOAC (1995). Total nitrogen and non-protein nitrogen (NPN); total volatile base nitrogen (TVBN) and alpha amino nitrogen (AAN) was determined by using the micro-kjeldahl method (AOAC, 1995), Conway's micro-diffusion method (Conway, 1974) and copper method (Pope & Steven, 1939) respectively. Protein nitrogen was estimated by subtracting non-protein nitrogen from the total nitrogen. The peroxide value (PV) and free fatty acid (FFA) were determined on the chloroform extracts of tissues as per the method suggested by Jacob (1958) and Takagi et al. (1984) respectively. Thiobarbituric acid reacting substance (TBARS) was determined by the method given by Tarladgis et al. (1960). Minerals were analysed from the acid soluble ash using Atomic Absorption Spectrophotometer (AOAC, 1995). Total plate count (TPC) and total fungal count (TFC) were done using nutrient agar (Khuntia, 2011) and potato dextrose agar (APHA, 1976) respectively by spread plating method. Identification of predominant groups of bacteria during fermentation was made up to the generic level following the identification scheme given for Gram-positive bacteria by Surendran et al. (1981). Bacteria were identified based on biochemical tests and standards given by Austin & Austin (2007). The changes of sensory characteristics of *S. phasa* during fermentation was evaluated.

All statistical analyses were performed using SPSS (version 16.0 for windows). Analysis of variance (one way - ANOVA) was performed to determine the differences between experimental periods of fermentation. The tests for differences were done by using Duncan's Multiple Comparison Test. Significance was tested as ($p<0.05$).

Results and Discussion

Proximate composition, biochemical and microbiological quality parameters of dried *S. phasa* are given in Table 1. Mean value of moisture, ash, crude protein and total lipid were found to be 21.24, 7.03, 50.12 and 21.04% respectively. The pH of dried *S. phasa* was 6.43. Kakati & Goswami (2013) reported almost similar values for dried *S. phasa* collected from Assam. The *S. phasa* collected during winter (October-December) was considered to be the best raw material to obtain quality fermented product.

This may be attributed to the fact that this period coincides with the time of the maximum fat content of the fish (Kakati & Goswami, 2013). Azam et al. (2003) reported higher values of fat in dried *S. phasa* in winter (4.5%) compared to summer (3.67%). Total ash content was found to be 7.03%. Minerals like calcium, magnesium, iron and copper were estimated and found as 131.12, 9.85, 9.06 and 0.13 (mg 100 g^{-1}) respectively. Higher calcium content estimated in dried *S. phasa* could be due to the presence of the pin bones which could not be separated during collection of flesh for analysis.

Biochemical quality parameters of dried *S. phasa* were estimated as NPN (1.24%), PV (26.52 mmoles O_2 kg^{-1} fat), TBARS (0.12 mg malonaldehyde kg^{-1} fish), FFA (53.10 as % oleic acid) and TVBN (113.19 mg 100 g^{-1}). Sarojnalini & Suchitra (2009) reported 1.59 % NPN, 66% FFA, 117.30 mg% TVBN and TBA value of 0.45 mg malonaldehyde kg^{-1} fish for sundried *Setipinna* spp. collected from Imphal, Manipur. TBA number less than 3.0 mg malonaldehyde kg^{-1} of cured fish is considered to be in good condition (Sinhuber & Yu, 1958). The mean values of TPC (log cfu g^{-1}) and TFC (log cfu g^{-1}) were found to be 5.87 and 3.90 respectively. For sundried *Setipinna* spp. TPC and TFC values were reported as 4.77 and 3.25 log cfu g^{-1} respectively (Sarojnalini & Suchitra, 2009). Higher values of TPC and TFC in dried *S. phasa* may be due to traditional method of packing in gunny bags while storing and unhygienic handling.

Table 1. Biochemical and microbial quality of raw material (dried *Setipinna phasa*)

	Mean ± SD (n=3)
pH	6.43 ± 0.02
Moisture (%)	21.24 ± 2.07
Ash (%)	7.03 ± 0.13
Crude Protein (%)	50.12 ± 1.48
Total nitrogen (%)	8.02 ± 0.24
Protein nitrogen (%)	6.78 ± 0.21
Non-protein nitrogen (%)	1.24 ± 0.06
Crude Lipid (%)	21.04 ± 0.67
Peroxide Value, PV (mmoles O_2 kg^{-1} fat)	26.52 ± 1.07
Thiobarbituric acid value, TBA (mg malonaldehyde kg^{-1} meat)	0.12 ± 0.00
Free fatty acid, FFA (as % oleic acid)	53.10 ± 2.60
Free alpha amino nitrogen, AAN (mg 100 g^{-1})	79.69 ± 2.89
Total volatile basic nitrogen, TVBN (mg 100 g^{-1})	113.19 ± 3.86
Calcium (mg 100 g^{-1})	131.12
Magnesium (mg 100 g^{-1})	9.85
Iron (mg 100 g^{-1})	9.06
Copper (mg 100 g^{-1})	0.13
Total Plate Count, TPC (log cfu 100 g^{-1})	5.87 ± 0.03
Total Fungal Count, TFC (log cfu 100 g^{-1})	3.90 ± 0.02

The changes in biochemical characteristics during fermentation are given in Table 2. The pH of fish meat decreased gradually during fermentation from 6.48 to 6.14; on the other hand the TTA level was increased from 0.12 to 0.38. The results corroborate the observations of Muzaddadi (2002). The gradual decrease of pH and increase of TTA could be due to formation of lactic acid during anaerobic break-down of stored carbohydrate in the fish muscle.

The moisture content of dried *S. phasa* (21.24 ± 2.07%) increased to 28.82 ± 0.37% due to washing and water soaking. During fermentation, the moisture content increased ($p<0.05$) from 28.82 to 30.52% after 30th days of sampling and then decreased thereafter. Similar trends of moisture content changes during fermentation of fish were also reported by Majumdar et al. (2006); Hernandaz-Herrero et al. (2002) and Wheaton & Lawson (1985). The increase in moisture content at initial period may be attributed to the fact that dried *S. phasa* absorbed water during the process of water soaking and may also be due to uptake of moisture from the cover paste during initial days of fermentation. The ash content (%) increased ($p>0.05$) slowly throughout the fermentation period from 6.55 and finally reached to 7.20 on 90th day with slight fluctuation in between. The loss in moisture content was accompanied by increase in ash content. Similar trend was also reported by Suchitra & Sarojnalini (2012) during fermentation of '*Ngari*'. The protein content decreased slowly from 45.5 to 44.12% after 90 days of fermentation. The decrease in protein content could be due to hydrolysis of protein by intrinsic and microbial enzymatic action (Majumdar et al., 2006). Total nitrogen (TN) content decreased from 7.28 to 7.06% after 90 days of fermentation,

which was similar to that reported by Majumdar et al. (2006) during fermentation of *Hilsa*.

Protein nitrogen (PN) content decreased ($p<0.05$) gradually from 6.07 to 4.38% after 90 days of fermentation period. This could be due to the breakdown of protein to low molecular weight compounds by enzymatic and microbial activity with the progress of fermentation. Similar trends have been reported by Majumdar et al. (2006); Hernandaz-Herrero et al. (2002); Uyenco et al. (1953) and Mathew & Raghunath (1996) during fermentation of fish. The NPN content increased gradually throughout the fermentation period from 1.21 to 2.68%. The NPN/TN ratio also increased gradually from 16.62 to 37.96%. The increasing trends of NPN during fermentation was also reported by Yatsunami & Takenaka (1996); Ahmed & Mahendrakar (1996) and Majumdar et al. (2006). The gradual and significant ($p<0.05$) increase in NPN content could be due to the enzymatic breakdown of protein to peptides and amino acids resulting in high NPN (Mahanta & Muzaddadi, 2012). The lipid content increased ($p>0.05$) gradually but slowly from 18.58 to 22.24 after 90 days of fermentation period. Kakati & Goswami (2013) reported an increased and high value of lipid in fermented *S. phasa* (24.1%) and fermented *puntius* (20.31%). The increasing trend of lipid content in the present study corresponds to the decrease in moisture content and decrease in crude protein content throughout the fermentation period in addition to leaching of mustard oil used for lining the containers.

The TVBN content experienced a very rapid increase ($p<0.05$) in the beginning and the initial value reached 170.89 mg nitrogen 100 g^{-1} after 15 days. This was followed by a slow but gradual increase throughout the fermentation period and the value reached 231.94 mg of nitrogen 100 g^{-1} after 90 days. Similar trends of TVBN changes were also reported by Hernandaz-Herrero et al. (2002) and Saisithi et al. (1986) during the fermentation of fish. The increase of TVBN values at initial as well as in later stages coincides with the rate of growth of bacteria. The AAN content increased gradually ($p<0.05$) from

Table 2. Biochemical and microbiological changes during fermentation of *Setipinna phasa* (mean ± SD, n=3)*

Parameters	Day 1	Day 15	Day 30	Day 45	Day 60	Day 75	Day 90
Moisture (%)	$28.82^{abc} \pm 0.37$	$29.05^{bc} \pm 1.91$	$30.52^{c} \pm 1.87$	$28.57^{abc} \pm 1.18$	$26.68^{ab} \pm 2.29$	$26.81^{ab} \pm 2.68$	$25.49^{a} \pm 0.71$
pH	6.48	6.41	6.37	6.32	6.26	6.19	6.14
TTA (g lactic acid)	ND	$0.12^{b} \pm 0.0$	$0.20^{c} \pm 0.01$	$0.26^{d} \pm 0.0$	$0.29^{e} \pm 0.01$	$0.34^{f} \pm 0.01$	$0.38^{g} \pm 0.01$
Ash (%)	$6.55^{a} \pm 0.11$	$6.77^{a} \pm 0.19$	$6.90^{a} \pm 0.11$	$7.37^{a} \pm 0.10$	$7.52^{a} \pm 0.13$	$7.34^{a} \pm 0.25$	$7.20^{a} \pm 0.18$
Crude protein (%)	$45.5^{bc} \pm 0.32$	$45.06^{abc} \pm 0.64$	$44.6^{ab} \pm 0.40$	$45.5^{bc} \pm 0.45$	$45.87^{c} \pm 0.29$	$44.56^{ab} \pm 0.69$	$44.12^{a} \pm 0.57$
Total lipid (%)	$18.58^{a} \pm 0.17$	$18.32^{a} \pm 0.19$	$17.84^{a} \pm 0.15$	$18.20^{a} \pm 0.36$	$19.81^{ab} \pm 0.18$	$20.43^{ab} \pm 0.33$	$22.24^{b} \pm 0.57$
Protein nitrogen, PN (%)	$6.07^{e} \pm 0.08$	$5.77^{d} \pm 0.10$	$5.49^{c} \pm 0.14$	$5.34^{bc} \pm 0.10$	$5.10^{b} \pm 0.08$	$4.57^{a} \pm 0.06$	$4.38^{a} \pm 0.10$
Total nitrogen, TN (%)	$7.28^{bc} \pm 0.05$	$7.21^{bc} \pm 0.07$	$7.14^{ab} \pm 0.06$	$7.28^{bc} \pm 0.07$	$7.34^{c} \pm 0.05$	$7.13^{ab} \pm 0.02$	$7.06^{a} \pm 0.05$
PN/TN	83.38	80.03	76.89	73.35	69.48	64.09	62.04
Non-protein nitrogen (%)	$1.21^{a} \pm 0.04$	$1.44^{b} \pm 0.00$	$1.65^{c} \pm 0.07$	$1.94^{d} \pm 0.04$	$2.24^{e} \pm 0.11$	$2.56^{f} \pm 0.07$	$2.68^{g} \pm 0.03$
NPN/TN	16.62	19.97	23.11	26.65	30.52	35.90	37.96
AAN (mg 100 g^{-1})	$73.03^{a} \pm 1.38$	$82.94^{b} \pm 0.79$	$91.50^{c} \pm 2.10$	$100.00^{d} \pm 1.37$	$109.74^{e} \pm 0.80$	$133.99^{f} \pm 2.12$	$153.08^{g} \pm 0.00$
TVBN (mg 100 g^{-1})	$109.02^{a} \pm 0.33$	$170.89^{b} \pm 6.84$	$185.29^{c} \pm 0.00$	$196.35^{d} \pm 3.95$	$209.81^{e} \pm 3.99$	$218.45^{f} \pm 4.02$	$231.94^{g} \pm 4.02$
PV (mmoles O_2 kg^{-1} fat)	$21.53^{a} \pm 1.21$	$33.00^{b} \pm 1.00$	$36.67^{c} \pm 0.36$	$41.76^{d} \pm 0.59$	$44.67^{e} \pm 0.58$	$51.02^{f} \pm 0.83$	$62.96^{g} \pm 3.21$
FFA (as % oleic acid)	$57.80^{a} \pm 2.94$	$65.65^{b} \pm 2.16$	$71.41^{c} \pm 0.61$	$85.32^{d} \pm 2.02$	$95.70^{e} \pm 3.30$	$114.40^{f} \pm 1.91$	$123.63^{g} \pm 3.60$
TBA (mg malonaldehyde kg^{-1} meat)	$0.09^{a} \pm 0.00$	$0.13^{b} \pm 0.00$	$0.58^{c} \pm 0.01$	$0.63^{d} \pm 0.00$	$0.67^{e} \pm 0.01$	$0.71^{f} \pm 0.00$	$0.79^{g} \pm 0.01$
TPC (log cfu 100 g^{-1})	$4.13^{a} \pm 0.05$	$4.23^{a} \pm 0.03$	$5.21^{b} \pm 0.06$	$7.24^{e} \pm 0.17$	$6.36^{b} \pm 0.31$	$6.72^{d} \pm 0.08$	$6.79^{d} \pm 0.03$
TFC (log cfu 100 g^{-1})	$3.96^{a} \pm 0.02$	$4.56^{e} \pm 0.04$	$4.52^{e} \pm 0.02$	$4.33^{d} \pm 0.05$	$4.15^{c} \pm 0.03$	$4.05^{b} \pm 0.02$	$4.04^{b} \pm 0.04$
Bacillus spp.	NE#	3.71 (0.05)	3.90 (0.04)	-	-	-	-
Staphylococcus spp.	NE	Very less	Very less	5.93 (0.04)	5.15 (0.03)	5.68 (0.06)	5.67 (0.06)

*Mean values with different superscripts (a, b, c) in a row are significantly different ($p<0.05$) # NE= Not enumerated genus wise

73.03 to 153.08 mg of nitrogen 100 g^{-1} after 90 days of fermentation period. Significant increase of AAN content with the progress of fermentation period indicated the degradation and breakdown of protein by microbes. Similar trends of changes in AAN contents were also reported by Majumdar et al. (2006); Ahmed & Mahendrakar (1996).

The FFA (as % oleic acid), the lipid hydrolysed product of the present study increased gradually ($p<0.05$) from 57.80 to 123.63 after the fermentation process which could be due to enzymatic activity like lipase, lipoxidase. Similar trends of changes in FFA contents were also reported during fermentation of fish (Srikar et al., 1993; Roldan et al., 1985). Peroxide value (PV, mmoles O_2 kg^{-1} fat), the primary lipid oxidised product increased gradually ($p<0.05$) from 21.53 to 62.96 during the fermentation process. Similar trends of changes in PV were also reported by Hernandaz-Herrero et al. (2002); Srikar et al. (1993) and Ahmed & Mahendrakar (1996) during fermentation of fish. Increased PV in the present study could be due to lipid oxidation in microaerophilic condition. Secondary lipid oxidative products TBARS (mg malonaldehyde kg^{-1} meat) increased significantly ($p<0.05$) from 0.09 to 0.79 after 90 days of fermentation period. Very low TBARS in the present study could be due to microaerophilic condition as well as absence of metallic container, salt etc.. Increase of TBARS value during fermentation of fish was also reported by Hernandaz-Herrero et al. (2002); Karacam & Boran (1996) and Hernandaz-Herrero et al. (1999).

Changes in microbial counts and predominant group of bacteria during fermentation are presented in Table 2. TPC was found to increase rapidly in the beginning and reached 6.79 log cfu g^{-1} after 90 days with slight fluctuation in between ($p<0.05$). Reason for such fluctuation in total bacterial count during fermentation of *phasa* may be attributed to different growth phases of bacteria. Washing of dried *S. phasa* reduced TPC on day-1 fish and thereafter rapid growth of bacteria could be attributed to increase of moisture content and contamination from the cover paste. This shows that bacteria was possibly involved and played a significant role in fermentation. Increase of bacterial count during fermentation of fish was also reported by Thapa et al. (2004); Jiang et al. (2007); Suchitra & Sarojnalini (2012) and Sarojnalini & Suchitra (2009). The TFC value increased slightly on day-15 and thereafter decreased ($p<0.05$) gradually to 4.04 log cfu g^{-1} on day-90. Anaerobic condition caused the decline of mould during the aging process of fermented fish (Chou et al., 1988). Suchitra & Sarojnalini (2012) reported declining trend of TFC count at all temperature level during fermentation of *'Ngari'*.

During fermentation it was observed that *Bacillus* spp. was the dominant bacterial group up to 30 days followed by *Staphylococcus* spp. TPC counts along with the number of dominant group of bacteria are

Table 3. Changes in sensory characteristics during fermentation period

Fermentation Period (days)	Characteristics observed
D-1	Dry with hard surface, hard texture, pale white to yellowish in colour, characteristic smell of dry fish
D-15	Semi-moist with moderately soft surface, moderately soft texture, almost no changes in colour and smell
D-30	Semi-moist with moderately soft surface, texture becomes more soft, colour changing starts towards dull yellowish with mild smell
D-45	Little bit moist with moderately soft surface, moderately soft texture, colour becomes yellowish to light yellow, mild smell
D-60	Moderately moist and soft surface with soft texture, colour becomes slightly dark yellow, moderately strong smell
D-75	Moist and soft surface, texture becomes softer and slight sticky, colour becomes little bit reddish brown, smell stronger
D-90	Moist with very soft surface, soft texture with moderate stickiness, reddish brown in colour, characteristic strong smell

given in Table 2. During the initial period of fermentation, the count of *Staphylococcus* spp. was very less. After 45 days onwards, *Staphylococcus* spp. was found to be the dominant bacterial group and comprised of 81.9%, and it continued as dominant group up to 90th days of fermentation. On 90th day, both TPC as well as Staphylococcal count reduced and *Staphylococcus* spp. contributed 83.5% of TPC. Such reduction of microbial count with the progress of fermentation may be explained as the declining phase of microbes due to accumulation of metabolites. During this period the count of *Bacillus* spp. was found negligible. For further confirmation the suspected *Staphylococcus* colonies were streaked into Baird Parker Agar (BPA) medium. The colonies showed growth on BPA medium along with coagulase test. Staphylococcal count exceeding 6 log cfu g^{-1} is considered to be hazardous (Bergdoll, 1979). However, in the present study the Staphylococcal count was below the hazardous level. Thapa et al. (2004); Achinewhu et al. (2004) and Anihouvi et al. (2007) also reported *Staphylococcus* and *Bacillus* species as the predominant group of bacteria in different fermented fish products.

The changes in sensory characteristics during fermentation period are given in Table 3. At the beginning of fermentation, the product retained hard surface and texture, pale white to yellowish in colour and smell characteristics to dry fish. Thereafter, there were changes in all quality attributes with the progress of the fermentation period. After 90 days the product was moist with very soft surface, soft texture with moderate stickiness, reddish brown in colour along with strong smell characteristic of the product.

This study would provide a simple and standard method of production of fermented *S. phasa* (phasa *shidal*) and can easily be adopted by dry fish traders. The traditional process yields variable quality end product due to inferior raw material and inadequate fermentation for early returns. However, a complete study on this traditional fermented fish product and the data would provide base line information for shortening of period of fermentation by using starter culture or improvement of post fermentation shelf life through packaging. Though *Bacillus* and *Staphylococcus* were identified as dominant bacterial groups, their numbers were below the hazardous level. In future studies, these bacteria may be identified up to species level so that these strains can be used as starter culture during fermentation of *Setipinna phasa*.

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