



Enzymatic and Fermentatively Produced Rohu (*Labeo rohita*) Sauce and its Biochemical and Microbiological Quality

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Abstract

Rohu (*Labeo rohita*) sauce was produced by enzymatic and fermentative method using optimized conditions carried out through response surface method. Solar salt (20%, w/w) and commercial papain (3%, w/w) were used in the sauce produced by enzymatic method. The optimized conditions for fermentative production of sauce were 25% salt (w/w), 7.5% sugar (w/w) and 10% (w/v) inoculum (*Pediococcus pentosaceus* FSBP4-40) with the cell concentration of approximately $8 \log \text{ cfu ml}^{-1}$. The sauces produced by enzymatic and fermentative method were stored at room temperature and 37°C , respectively for 180 days. Changes in yield, water activity (a_w), total volatile base nitrogen (TVB-N), total soluble nitrogen (TSN), non protein nitrogen (NPN), titratable acidity (TA), degree of hydrolysis (DH) and fatty acid composition of both the sauce samples were observed. The result suggested that TSN, NPN, TA and DH significantly increased ($p < 0.05$) in treated samples compared to control (sample without added papain and *P. pentosaceus* FSBP4-40). However, a_w , pH, moisture, fat, TVB-N, fatty acid concentration and L^* , a^* , b^* values showed different changes. Sensory properties of enzyme treated sauce had slightly higher score than the lactic acid bacteria fermented sauce. Bacterial counts after 180 d were significantly lower ($p < 0.05$) in the fermentatively produced rohu sauce than enzymatically prepared sauce. The study emphasizes the importance of optimized use of enzyme and native

lactic acid bacteria for the acceleration of fermentation process to produce biofunctionally and bacteriologically superior rohu sauce.

Keywords: Rohu sauce, papain, *Pediococcus pentosaceus*, fermentation, bio-functional

Introduction

Fish sauce is an amber-colored salty liquid in Southeast Asian cuisine used as an important condiment for improving the taste of foods. Researchers have also focused on effective inhibition of biogenic amines in fish sauces using novel bacterial starter cultures, apart from effectively deciphering the formation of different biogenic amines and their toxicity in fish sauces (Siddegowda et al., 2016). The annual production of fish sauce is estimated to be >400 million l with 20 out of 100 fish sauce producers contributing more than 80% of the global production (Vidanarachchi et al., 2014). The leading fish sauce producer in the world is Thailand. Nampla, a traditional Thai fish sauce has become popular among Western consumers, especially in the United States (Lopetcharat et al., 2001).

Papain is the most common commercial protease from plant sources used for the hydrolysis of fish protein (Hoyle & Merritt, 1994). Every country in Southeast Asia has their own formulation for the production of fish sauce and the physico-chemical characteristics vary from one formulation to other. Along with this variation the biochemical characteristics of fish sauce largely depend on the type and quality of raw materials, salt to fish ratio, method of processing and time of fermentation. Mueda (2015) conducted a study on physiochemical properties of salt fermented fish sauce from anchovy

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Stolephorus commersonii. Yashikawa et al. (2010) prepared the fermented chum salmon (*Oncorhynchus keta*) sauce using halotolerant microorganisms and its chemical properties were analyzed. The changes in chemical components of fish sauce obtained by a bioprocess of aerobic fermentation with *Aspergillus oryzae* OAY1 was conducted by Sun et al. (2016). Yu et al. (2014) investigated the biochemical properties of fish sauce prepared with low salt by solid state fermentation using anchovy by-products.

The halophilic bacteria not only increase the rate of protein solubilisation, but also contribute to flavor development. The lactic acid bacterial cultures *Pediococcus pentosaceus* FSBP4-40 and *Pediococcus acidilactici* FSBP28-50, isolated from salt fermented fish hydrolysates exhibited excellent proteolytic activity and acidifying ability. The fermentative properties of these halotolerant isolates have the potential for the application in acceleration of fermentation as starters to produce salted fish products (Siddegowda et al., 2017). Two strains of lactic acid bacteria isolated from natural plaa-som fermentation were used as starter culture: *Lactobacillus plantarum* IFRPD P15 and *Lactobacillus reuteri* IFRPD P17. These strains have great potential for use as a mixed starter culture in plaa-som fermentation and may possibly help to reduce fermentation time (Saithong et al., 2010). Use of native proteolytic lactic acid bacteria from salted and fermented fish and fish products effectively hydrolyse fish proteins during fermentation of fish sauce (Siddegowda et al., 2016). Against this background, the objective of the present work to compare the enzymatically and fermentatively produced rohu sauce with reference to the biochemical and microbiological properties.

Materials and Methods

The eviscerated freshwater fish Rohu (*Labeo rohita*) collected from local fish market (Mysuru, India) was brought to the laboratory in iced condition. *Pediococcus pentosaceus* FSBP4-40, a native proteolytic lactic acid bacteria (LAB) starter isolated from salt fermented fish hydrolysate. Papain (Loba Chemie Pvt. Ltd.), the protease used for the enzymatic hydrolysis. Plate count agar (PCA), eosin methylene blue (EMB) agar, de-Man Rogosa and Sharpe (MRS) agar, Baird Parker agar (BPA), Salmonella-Shigella agar (SSA) and potato dextrose agar (PDA) were purchased from M/s Hi-media Laboratories (Mumbai, India). All the other chemicals used in different analysis were of analytical grade unless otherwise mentioned.

The preparation of rohu sauce is schematically represented in Fig. 1. Briefly, eviscerated rohu sliced into small pieces and washed in potable water was weighed and bottled into sterile food grade plastic containers. The sample was mixed with papain (3%, w/w), and was kept at room temperature for 4 h before adding 20% (w/w) salt. A control for the enzymatic production of sauce was maintained by adding only salt without papain. *P. pentosaceus* FSBP 4-40, a proteolytic halotolerant native LAB which was previously isolated from salt fermented fish hydrolysates by our group (GenBank accession no: KU933533) was added (10%, v/w) along with 7.5%, w/w sugar (dextrose), 2%, w/w solar salt for fermentative production of rohu sauce. This mixture was incubated for 24 h at 37°C and remaining salt of 23%, w/w was added to make up the total salt concentration of 25%, w/w. Fish with salt without added LAB was the control for the fermentation method. The liquid filtered through cheese-cloth every 15 d till 6 months and further filtered using Whatman no. 1 filter paper was considered as fish sauce. Fish sauce was used for chemical, biochemical, microbiological and sensory studies.

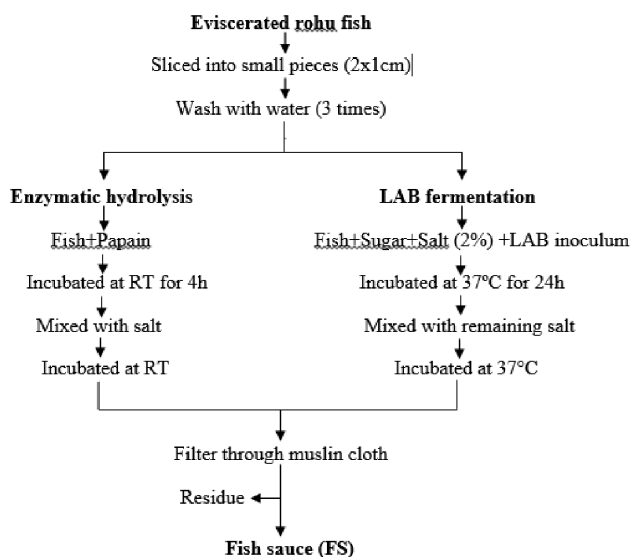


Fig. 1. Schematic flow of sauce production from rohu (*Labeo rohita*) using papain and *P. pentosaceus* FSBP4-40

Proximate composition (protein, fat and moisture) of the fermented fish sauce samples were determined as per AOAC method (AOAC, 2002). pH measurements using pH meter (Cyberscan 1000,

Eutech, Singapore). Titratable acidity (TA) was determined as per the method described in Sachindra et al. (2007). Total soluble nitrogen (TSN) content of samples was measured using Kjeldahl method (AOAC, 1999) and non-protein nitrogen (NPN) by precipitation of the proteins with trichloroacetic acid (TCA), followed by analysis by the Kjeldahl method. Salt content was determined according to the Mohr method (AOAC, 2000) and water activity (a_w) was measured using water activity meter (Aqua Lab Model CX-3T, Decagon Devices Inc., Pullman Washington, USA). TVB-N content of the fermented rohu sauce samples was measured using the method of Conway & Byrne (1936). The degree of hydrolysis of the fermented fish sauce was Hoyle & Merritt (1994). Fermented rohu sauce samples were analysed for non-enzymatic browning by measuring melanoidin pigment formation using the method of Hendel et al. (1950).

The colour of the rohu sauce samples was measured in the L^* , a^* and b^* mode using a Hunter Lab instrument (Minotia CM-5, Konica Minolta Optics Inc., Japan) according to CIE Lab scale. The instrument provides the values for three components: lightness (L^*), redness/greenness (a^*) and yellowness/blueness (b^*). The 10-15 ml of samples in quartz cuvettes were illuminated with D65-artificial daylight (10° standard angle) according to the manufacturer's protocol.

Total lipids of the rohu sauce were extracted by Folch method and the fatty acid composition of the

extracted lipids was determined by preparing the fatty acid methyl ester (FAME) as outlined in Majumdar et al. (2015). The values of fatty acids are presented in area percentage of total identified fatty acids. The microbial load of rohu sauce was determined using standard methods (APHA, 2001).

The fish sauce obtained after 180 d of fermentation as well as commercial Thai fish sauce were evaluated for acceptance by a panel of nine members. The panelists were research scholars in meat and marine sciences department, CSIR-CFTRI, Mysuru, of age group ranging from 25-30 years. Panelists were asked to give acceptance scores for five attributes: colour, flavour, taste, appearance and overall quality using the nine-point hedonic scale.

Results and Discussion

Changes in yield, a_w and pH of the rohu sauce produced by enzymatic and fermentative methods from day-1 to day-180 with a time interval of 15 to 30 d is given in the Table 1. There was a significant change in the yield, a_w and pH of papain treated and LAB treated sauce samples. The treated samples gave higher sauce yield during processing than the control after 180 d of fermentation. There was almost 10-12% difference in the yield of treated samples than the untreated one (Table 1). This might be due to the processing method followed, fermentation condition such as temperature employed, hydrolysing property of papain and acidifying ability of the LAB culture used for the production.

Table 1. Changes in yield, a_w and pH of papain and LAB treated rohu sauce during fermentation

Day	Yield (%)			a_w			pH		
	C	PT	LT	C	PT	LT	C	PT	LT
0	35.86±0.3 ^a	40.72±1.8 ^a	50.89±0.5 ^a	0.801±0.0 ^{b,c,d}	0.792±0.0 ^f	0.824±0.0 ^g	5.50±0.0 ^c	5.30±0.0 ^{a,b,c}	5.85±0.1 ^e
15	39.94±1.4 ^b	48.08±0.1 ^b	52.01±0.2 ^b	0.803±0.0 ^{c,d}	0.784±0.0 ^c	0.819±0.0 ^f	5.45±0.1 ^c	5.25±0.1 ^{a,b}	4.40±0.0 ^{a,b,c}
30	42.49±0.5 ^c	50.47±0.7 ^c	54.81±0.3 ^c	0.801±0.0 ^{b,c,d}	0.788±0.0 ^e	0.819±0.0 ^f	5.40±0.0 ^{b,c}	5.25±0.1 ^{a,b}	4.65±0.0 ^d
45	42.24±0.6 ^c	52.77±0.1 ^d	55.24±0.0 ^c	0.806±0.0 ^d	0.788±0.0 ^{d,e}	0.812±0.0 ^{c,d}	5.30±0.0 ^b	5.30±0.0 ^{a,b,c}	4.45±0.1 ^{b,c}
60	42.11±0.7 ^d	55.87±0.9 ^e	56.88±0.5 ^d	0.800±0.0 ^{b,c}	0.787±0.0 ^{d,e}	0.816±0.0 ^e	5.15±0.1 ^a	5.25±0.1 ^{a,b}	4.50±0.0 ^c
90	44.85±0.1 ^d	56.98±0.7 ^e	58.25±0.0 ^e	0.792±0.0 ^a	0.782±0.0 ^b	0.813±0.0 ^d	5.40±0.0 ^{b,c}	5.20±0.0 ^a	4.40±0.0 ^{a,b,c}
120	46.38±0.3 ^e	60.19±0.6 ^f	59.71±0.2 ^f	0.802±0.0 ^{b,c,d}	0.786±0.0 ^d	0.811±0.0 ^{b,c}	5.75±0.1 ^d	5.40±0.0 ^c	4.30±0.0 ^a
150	48.08±0.3 ^f	61.91±0.3 ^f	60.24±0.0 ^f	0.802±0.0 ^d	0.778±0.0 ^a	0.810±0.0 ^{a,b}	6.25±0.1 ^f	5.35±0.1 ^{b,c}	4.35±0.1 ^{a,b}
180	51.06±0.8 ^g	64.18±0.6 ^g	63.38±0.7 ^g	0.798±0.0 ^b	0.778±0.0 ^a	0.808±0.0 ^a	5.90±0.0 ^e	5.65±0.1 ^d	4.50±0.0 ^c

Values in column are mean±SD, C-control, PT-papain treated and LT-lab treated.

Different superscripts, column-wise, indicate statistically significant differences ($p<0.05$).

Hjalmarsson et al. (2007) reported $42\pm1\%$ and $59\pm5\%$ liquid yield in ground winter capelin and summer capelin, respectively and filter through cheese cloth with approximately 850 psi after 270 d. There was slight decrease in the water activity (a_w) of all the fish sauce samples treated with and without papain and LAB after 180 d of fermentation (Table 1). Water activity values of fermented fish sauce samples made from sardine with different ingredients were in the range of 0.93 to 0.84 (Kilinc et al., 2006). The pH value of the sauce treated with LAB decreased during fermentation, from the original 5.8 to 4.5 whereas, in the papain treated sauce and control, the pH values were increased to 5.7 and 5.9 from the initial 5.3 and 5.5, respectively (Table 1). The decrease in pH may be due to the production of acids by LAB and other autochthonous organisms. The pH values were well suitable for the sourness of the fish sauce. The fish sauce made of squid processing by-products using low salt also showed the pH range 5.26 ± 0.14 to 5.48 ± 0.12 (Xu et al., 2008). Udomsil et al. (2015) reported that the pH values for the fish sauce prepared by added *Staphylococcus* sp. CMC5-3-1 and CMS5-7-5 were 5.38 and 5.36, respectively. The pH of mahyaveh, a traditional Iranian fish sauce samples from different locations was in the range of 4.89-7.55 (Zarei et al., 2012).

Proximate composition of the rohu sauce is specified in the Table 2. Statistically significant differences were observed in the moisture, protein and fat content of treated and control (without added papain and *P. pentosaceus* FSBP 4-40) samples. The

moisture content was decreased after 180 d of storage in both treated and control. Our findings were in agreement with the results of Hjalmarsson et al. (2007) who found 72-75% and 81-83% moisture content in fish sauce produced from summer capelin and winter capelin respectively at different storage period between 5 to 270 d. The papain treated rohu sauce samples showed 3 fold increased protein content than control, whereas the LAB treated samples comparatively showed lower protein content (Table 2). This difference was due to high degree of hydrolysis of fish protein by papain and insufficient production of proteolytic enzymes produced by the LAB. The protein content ranged from 12.5 ± 1.3 - $22.3\pm0.0\%$, which is high when compared to Kilinc et al. (2006) who documented crude protein content of the fish sauce made from whole *Gambusia* was 12.37%. This difference could be attributed to differences in the protein content of the raw material and condition of fermentation such as pH, temperature and salt concentration. Fat content of the papain treated sauce samples was almost unchanged throughout the storage period but, decreased content of fat was found in the LAB treated sample after 180 d of fermentation (Table 2). The decrease in total lipid content in all the groups of sauce samples produced from sardine (*Sardina pilchardus*) using different ingredients was documented in Kilinc et al. (2006).

The salt content of the papain treated sauce samples showed 2% increase towards the end of 180 d of storage but, in the LAB treated samples showed

Table 2. Changes in proximate composition of papain and LAB treated rohu sauce during fermentation

Day	Moisture (%)			Protein (%)			Fat (%)		
	C	PT	LT	C	PT	LT	C	PT	LT
0	74.03 $\pm$ 0.0 ^c	68.95 $\pm$ 0.5 ^c	68.45 $\pm$ 0.1 ^a	5.86 $\pm$ 0.1 ^a	12.54 $\pm$ 1.3 ^a	5.04 $\pm$ 0.0 ^a	0.04 $\pm$ 0.0 ^{a,b}	0.10 $\pm$ 0.0 ^{b,c}	0.18 $\pm$ 0.0 ^d
15	73.39 $\pm$ 0.3 ^{b,c}	66.25 $\pm$ 0.2 ^{c,b}	71.83 $\pm$ 0.3 ^{b,c}	7.14 $\pm$ 0.3 ^b	17.01 $\pm$ 0.3 ^b	6.91 $\pm$ 0.0 ^b	0.05 $\pm$ 0.0 ^{b,c}	0.10 $\pm$ 0.0 ^{a,b}	0.10 $\pm$ 0.0 ^b
30	71.42 $\pm$ 2.6 ^{b,c}	63.51 $\pm$ 1.6 ^{b,c}	72.68 $\pm$ 0.4 ^c	7.00 $\pm$ 0.0 ^b	18.78 $\pm$ 0.0 ^{c,d}	8.03 $\pm$ 0.1 ^c	0.09 $\pm$ 0.0 ^e	0.11 $\pm$ 0.0 ^{b,c}	0.08 $\pm$ 0.0 ^a
45	72.55 $\pm$ 1.0 ^{b,c}	62.25 $\pm$ 3.9 ^{b,c}	71.00 $\pm$ 0.3 ^b	9.31 $\pm$ 0.1 ^{d,e}	18.64 $\pm$ 0.0 ^{c,d}	7.96 $\pm$ 0.2 ^c	0.07 $\pm$ 0.0 ^{c,d}	0.12 $\pm$ 0.0 ^d	0.10 $\pm$ 0.0 ^{b,c}
60	72.82 $\pm$ 0.1 ^{b,c}	60.19 $\pm$ 3.9 ^a	71.09 $\pm$ 0.5 ^b	9.24 $\pm$ 0.2 ^d	18.55 $\pm$ 0.2 ^c	7.80 $\pm$ 0.0 ^c	0.05 $\pm$ 0.0 ^b	0.13 $\pm$ 0.0 ^e	0.10 $\pm$ 0.0 ^{b,c}
90	64.76 $\pm$ 3.7 ^a	60.56 $\pm$ 3.0 ^a	71.63 $\pm$ 0.3 ^{b,c}	9.43 $\pm$ 0.2 ^{d,e}	18.93 $\pm$ 0.3 ^{c,d,e}	9.47 $\pm$ 0.0 ^d	0.06 $\pm$ 0.0 ^{c,d}	0.11 $\pm$ 0.0 ^c	0.11 $\pm$ 0.0 ^b
120	69.84 $\pm$ 1.3 ^b	63.23 $\pm$ 2.1 ^{b,c}	71.33 $\pm$ 0.1 ^b	9.56 $\pm$ 0.0 ^e	19.81 $\pm$ 0.0 ^{d,e}	12.39 $\pm$ 0.0 ^f	0.03 $\pm$ 0.0 ^a	0.09 $\pm$ 0.0 ^a	0.12 $\pm$ 0.0 ^c
150	72.02 $\pm$ 0.1 ^{b,c}	63.51 $\pm$ 1.4 ^{b,c}	72.68 $\pm$ 0.0 ^c	8.73 $\pm$ 0.0 ^c	20.11 $\pm$ 0.8 ^e	10.34 $\pm$ 0.2 ^e	0.05 $\pm$ 0.0 ^{b,c}	0.15 $\pm$ 0.0 ^f	0.10 $\pm$ 0.0 ^{b,c}
180	73.11 $\pm$ 0.1 ^{b,c}	65.28 $\pm$ 0.0 ^{a,b,c}	71.24 $\pm$ 1.4 ^b	10.72 $\pm$ 0.0 ^f	22.34 $\pm$ 0.0 ^f	12.31 $\pm$ 0.2 ^f	0.07 $\pm$ 0.0 ^d	0.17 $\pm$ 0.0 ^g	0.10 $\pm$ 0.0 ^b

Values in column are mean $\pm$ SD, C-control, PT-papain treated and LT-lab treated

Different superscripts, column-wise, indicate statistically significant differences ($p<0.05$).

2.5% reduction whereas, the salt content was unchanged in control (Table 3). An increase in non-enzymatic browning was observed in the samples obtained after 180 d of fermentation. Greater browning was found in the LAB treated samples compared to enzyme treated and control. Increase in browning was observed in the papain treated and control samples till 150 d and 120 d of storage, respectively. Later, the browning intensity was decreased towards the end of storage period of 180 d (Table 3). According to Klomklao et al. (2006), the increase in browning depends on the concentration of salt, highest browning was observed in fish sauce produced with a low salt concentration. Peptides and amino acid release during proteolysis served as substrates for Maillard browning reaction (Yongsawatdigul et al., 2007). The Maillard browning reaction contributes brown colour in fish sauce yu-lu (Lopetcharat et al., 2001). The result of the present study was in correlation with the findings of Klomklao et al. (2006) and Yongsawatdigul et al. (2007). As shown in Table 3, the NPN levels of papain and LAB treated samples increased significantly from the original $1.31 \pm 0.01 \text{ g } 100 \text{ g}^{-1}$ and $0.92 \pm 0.01 \text{ g } 100 \text{ g}^{-1}$ to $5.14 \pm 0.01 \text{ g } 100 \text{ g}^{-1}$ and $1.97 \pm 0.06 \text{ g } 100 \text{ g}^{-1}$, respectively after 180 d of fermentation. NPN level increased after 12 h of fermentation from the original $0.06 \text{ g } 100 \text{ g}^{-1}$ to $0.69 \text{ g } 100 \text{ g}^{-1}$ after 48 h of fermentation in fermented surimi with *Actinomucor elegans* XH-22 as starter (Zhou et al., 2014).

Degree of hydrolysis (DH) analysed during the fermentation is shown in Fig. 2. DH was higher in case of papain treated sauce samples compared to LAB treated samples. DH of papain treated and untreated samples were found to 24.01 and 4.87% after 180 d of storage from the original 4.04 and 1.16%, respectively. Whereas, the LAB inoculated sample showed 8.47% of DH from the original 3.17%. The increase in DH of LAB fermented sample compared to the control may be due to the acid producing ability and proteolytic activity of the LAB employed in the fermentation. The changes in the total acid content of fermented fish sauce are presented in the Fig. 2. The total acid content showed almost 4 fold increase in papain treated sample ($2.3 \text{ g } 100 \text{ ml}^{-1}$) after 180 d of storage compared to the control ($0.45 \text{ g } 100 \text{ ml}^{-1}$). This was probably due to the hydrolysed proteins in the papain treated samples serve as the ingredients for the fermenting indigenous microbes. They in turn produce organic acids as secondary metabolite during storage. The acid content in the LAB treated sample was $1.2 \text{ g } 100 \text{ ml}^{-1}$ after 90 d but, it decreased to $1.0 \text{ g } 100 \text{ ml}^{-1}$ at the end of 180 d of fermentation. These values were in agreement with the findings of Xu et al. (2008). The measurement of TVB-N indicates the degree of protein degradation in samples by spoilage bacteria, autolytic enzymes, deamination and nucleotide catabolites (FDA, 2004). The increased content of TVB-N was observed in untreated control than in papain and LAB treated

Table 3. Changes in salt content, non-enzymatic browning and NPN of papain and LAB treated rohu sauce during fermentation

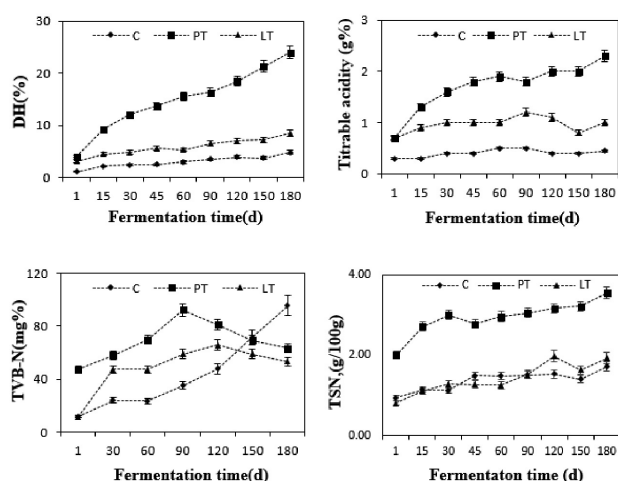
Day	Salt (%)			Non-enzymatic browning			NPN (g 100 g ⁻¹)		
	C	PT	LT	C	PT	LT	C	PT	LT
0	28.53±0.5 ^b	28.30±0.3 ^d	23.66±0.3 ^{a,b}	0.02±0.0 ^a	0.01±0.0 ^a	0.03±0.0 ^a	0.45±0.0 ^a	1.32±0.0 ^a	0.92±0.0 ^a
15	28.62±0.2 ^b	28.28±0.1 ^d	24.01±0.2 ^{b,c}	0.03±0.0 ^{b,c}	0.01±0.0 ^a	0.05±0.0 ^b	0.72±0.0 ^b	2.64±0.1 ^b	1.28±0.0 ^b
30	28.25±0.2 ^{a,b}	27.62±0.2 ^c	23.43±0.3 ^a	0.03±0.0 ^c	0.03±0.0 ^b	0.04±0.0 ^a	0.76±0.0 ^c	3.23±0.0 ^c	1.30±0.0 ^b
45	28.77±0.2 ^b	26.91±0.2 ^b	24.01±0.5 ^a	0.05±0.0 ^d	0.05±0.0 ^c	0.07±0.0 ^b	0.83±0.0 ^d	3.54±0.0 ^d	1.51±0.1 ^d
60	27.94±0.3 ^a	25.94±0.3 ^a	24.48±0.2 ^d	0.06±0.0 ^e	0.07±0.0 ^d	0.10±0.0 ^c	0.92±0.0 ^e	3.79±0.0 ^e	1.39±0.0 ^c
90	27.83±0.2 ^a	26.05±0.2 ^a	24.13±0.0 ^{c,d}	0.14±0.0 ^f	0.07±0.0 ^d	0.15±0.0 ^d	1.06±0.0 ^f	3.94±0.0 ^f	1.66±0.0 ^e
120	28.27±0.1 ^{a,b}	26.24±0.2 ^a	25.69±0.1 ^{c,d}	0.15±0.0 ^g	0.08±0.0 ^e	0.19±0.0 ^e	1.16±0.0 ^g	4.16±0.0 ^g	1.73±0.0 ^e
150	28.59±0.2 ^b	26.45±0.2 ^{a,b}	25.69±0.1 ^e	0.06±0.0 ^e	0.11±0.0 ^g	0.21±0.0 ^f	1.06±0.0 ^f	4.72±0.0 ^h	1.81±0.0 ^f
180	28.31±0.2 ^{a,b}	26.25±0.2 ^a	26.22±0.2 ^f	0.02±0.0 ^b	0.09±0.0 ^f	0.26±0.0 ^g	1.34±0.0 ^h	5.16±0.0 ⁱ	2.02±0.1 ^g

Values in column are mean±SD, C-control, PT-papain treated and LT-lab treated

Different superscripts, column-wise, indicate statistically significant differences ($p < 0.05$).

samples (Fig. 2). Comparatively lower content of TVB-N was found in the LAB treated sauce ($53.46 \text{ mg } 100 \text{ g}^{-1}$) than papain treated sauce ($64.64 \text{ mg } 100 \text{ g}^{-1}$). The TVB-N values were within the acceptable range ($14.1\text{--}338.6 \text{ mg } 100 \text{ ml}^{-1}$) which were found in most Southeast Asian fish sauces (Cho et al., 2000).

The total soluble nitrogen (TSN) content of the sauce samples increased after 180 d of fermentation (Fig. 2). Higher content of TSN was observed in papain treated sample ($3.56 \text{ g } 100 \text{ g}^{-1}$) than the LAB treated sample ($1.93 \text{ g } 100 \text{ g}^{-1}$). The changes of NPN were found to be similar to the changes in TSN. The increases of TSN and NPN content during processing of fish sauce could be attributed to the combined effect of autolysis, enzyme activity and microbial degradation of the rohu muscle. Udomsil et al. (2015) reported that the total nitrogen content ($1.89 \text{ g } 100 \text{ g}^{-1}$) in the fish sauce inoculated with *Staphylococcus* sp. CMS5-7-5 and incubated at 35°C for 180 d. The total nitrogen content of enzyme treated fish sauce exceeded the minimum value for second grade fish sauce (1.5–2.0%) set by the Thai Industrial Standards Institute after 120 d. The total nitrogen content reported for different types of fish sauce has been variable and may be based on the raw material or the processing condition. Total nitrogen content was in the range of 1.176 to 1.316 $\text{g } 100 \text{ g}^{-1}$ in the fish sauce prepared using low salt, solid state fermentation with anchovy by-products



(Yu et al., 2014).

Fig. 2. Degree of hydrolysis (DH), titrable acidity, TVB-N and total nitrogen content of papain treated and LAB treated rohu sauce during fermentation

Lipolysis played a major role in flavour formation during ripening of fermented rohu sauce. Fatty acid profile of papain treated and LAB fermented fish sauces is presented in Table 4. Overall composition of the fatty acids after 180 d of storage was reduced in LAB treated samples but, the composition was almost unchanged in papain treated samples. Amongst saturated fatty acids, palmitic acid (C16:0) was found to be dominant in LAB treated samples than papain treated samples. But, the vice versa was observed in case of palmitoleic acid (C16:1) concentration. Among the monoenoic fatty acid, oleic acid (C18:1n-9c) was increased in treated samples towards the end of storage. The saturated fatty acid (SFA) content of the enzymatically produced sauce was decreased to 36.51% after 180 d of storage from the original concentration (41.12%) on day-1 but, the unsaturated fatty acid (USFA) content was increased from the original concentration of 36.14% to 40.40% at the end of storage. Whereas in LAB treated sample the unsaturated fatty acid (USFA) concentration was decreased to 37.72% from 43.12% after 180 d of fermentation. EPA in papain and LAB treated samples were 0.95 and 0.43% respectively. The fatty acid profile of the rohu sauce samples are in correlation with results of Siddegowda et al. (2016). They observed similar trend in the rohu head sauce produced by enzymatic and fermentation method.

Rohu sauce obtained from different treatment had different colour characteristics (Table 5). The colour of the sauce samples intense gradually as the fermentation time increased. Generally, all the samples had decreased L^* values and increased a^* and b^* values during fermentation. From the result, the sauce samples treated with papain and LAB were showed decreased L^* values than untreated control. Papain treated sample had slightly greater a^* and b^* values as compared to LAB treated sample after 180 d of storage. This could be probably due to the decrease in the added sugar content in the fermented media. Usually, most of the small peptides and amino acids in the fish sauce contribute to the brown colour development (Lopetcharat et al., 2001). The colour intensity of salt fermented fish sauce changes according to fermentation period and also affected by the maturity of the product (Mueda, 2015). The study revealed that the papain treated and LAB fermented fish sauce samples after 180 d were ripened. It is based on the description that fish sauce is a clear brown liquid sauce, with straw yellow to amber in colour (PNS 413:1993).

Table 4. Changes in fatty acid profile of papain treated and LAB treated rohu sauce during fermentation

Fermentation time	DAY-1				DAY-90			DAY-180		
Name of Fatty acid methyl ester	RAW	C	PT	LT	C	PT	LT	C	PT	LT
Lauric acid (C12:0)	ta	0.748	0.262	ta	1.693	0.711	3.300	1.095	0.740	1.257
Tridecanoic acid (C13:0)	ta	0.845	ta	ta	1.175	0.991	1.311	0.647	0.869	0.948
Myristic acid (C14:0)	2.376	4.714	11.510	2.937	4.755	4.778	2.823	3.995	4.784	1.683
Myristoleic acid (C14:1)	1.187	1.892	0.873	ta	1.292	1.095	3.275	3.752	1.549	2.031
Pentadecanoic acid (C15:0)	1.228	1.494	0.682	1.369	1.856	1.848	0.737	1.368	1.689	0.676
Cis-10 Pentadecanoic acid (C15:1)	0.460	ta	ta	ta	0.578	0.472	ta	ta	ta	ta
Palmitic acid (C16:0)	24.610	21.152	25.319	22.172	24.265	22.024	16.950	18.070	21.493	24.480
Palmitoleic acid (C16:1)	1.715	9.288	12.466	4.545	10.249	11.404	3.087	8.151	11.009	3.900
Hepta decanoic acid (C17:0)	3.066	1.553	1.658	1.904	1.527	1.404	1.504	0.946	1.509	0.506
Cis-10 Hepta decanoic acid (C17:1)	0.508	0.618	ta	0.879	0.575	0.419	ta	0.974	1.299	ta
Stearic acid (C18:0)	5.850	3.445	ta	8.627	2.755	2.608	3.757	2.841	2.775	5.933
Oleic acid (C18:1n-9c)	10.132	11.845	8.730	14.479	12.196	14.129	13.738	10.955	12.934	19.530
Elaidic acid (C18:1n9t)	3.148	5.540	4.732	6.517	5.641	6.296	1.701	5.916	6.354	2.682
Linoleic acid (C18:2n-6c)	6.921	1.758	0.840	1.576	1.757	2.491	3.220	1.570	2.423	5.810
γ - Linolenic acid (C18:3n6)	7.587	ta	ta	0.360	1.994	3.051	1.201	1.357	2.868	2.062
α - Linolenic acid (C18:3n3)	0.529	2.155	0.790	ta	ta	ta	0.583	ta	ta	ta
Arachidic acid (C20:0)	0.975	ta	0.756	0.735	1.342	1.534	1.110	1.061	1.378	1.899
Eicosadienoic acid (C20:2)	1.407	ta	ta	0.676	ta	ta	ta	ta	ta	0.495
Eicosatrienoic acid (C20:3n6)	2.551	1.346	ta	1.098	1.150	1.142	0.521	0.632	1.012	0.784
Arachidonic acid (C20:4n6)	0.674	ta	ta	ta	ta	ta	ta	0.737	ta	ta
Eicosapentanoic acid (C20:5n3)	ta	1.129	6.652	ta	0.834	1.076	ta	0.496	0.949	0.427
Behenic acid (C22:0)	ta	ta	ta	ta	ta	ta	0.707	0.716	ta	ta
Docosahexanoic acid (C22:6n3)	0.680	ta	ta	1.901	ta	ta	ta	ta	ta	ta
Trichosonic acid (C23:0)	3.056	ta	0.935	ta	0.736	1.808	0.745	0.913	1.272	1.142
Nervonic acid (C24:1n9)	ta	ta	1.056	11.072	ta	ta	ta	ta	ta	ta
SFA	41.161	33.951	41.122	37.744	40.104	37.706	32.944	31.652	36.509	38.524
USFA	37.499	35.571	36.139	43.121	36.266	38.524	27.326	34.540	40.397	37.721

C-control, PT-papain treated and LT-lab treated, ta-trace amount

Table 5. Changes in L^* , a^* , b^* values of papain and LAB treated rohu sauce during fermentation

Day	L^*			a^*			b^*		
	C	PT	LT	C	PT	LT	C	PT	LT
1	99.24	98.39	99.37	-0.72	-1.49	-0.66	5.39	8.13	4.68
30	97.22	97.45	98.86	-2.54	-2.45	-1.77	15.88	15.59	8.87
60	94.44	94.92	96.88	-2.16	-2.71	-2.93	27.36	28.41	19.48
90	91.36	88.5	92.47	-1.14	1.17	-2.14	43.96	55.38	41.66
120	89.31	84.53	87.19	-0.19	5.37	2.32	57.69	68.59	59.07
150	87.26	80.41	85.82	1.15	10.32	3.97	58.61	78.10	63.84
180	83.87	78.89	80.39	5.97	12.20	10.38	73.40	82.09	78.18

C-control, PT-papain treated and LT-lab treated

The fermented fish sauce samples produced with different treatment were scored for color, flavor, taste, appearance and overall quality in comparison with the commercial fish sauce are shown in Table 6. The papain treated and LAB treated sauce samples prepared from rohu were in the acceptable range. LAB fermented rohu sauce had higher taste and color score when compared to enzyme treated sauce. This was probably due to the taste enhancing peptides and fatty acids produced during fermentation with *P. pentosaceus* FSBP4-40. The brown colored products of Maillard reactions have been considered as compounds contributing to the antioxidant activity (Moon et al., 2002). Appearance and overall quality scores of papain treated rohu sauce was superior than the LAB treated sauce. The overall quality of enzyme treated sauces had slightly higher than the LAB fermented sauces, probably due to the greater degree of hydrolysis which was associated with the formation of tasty products,

such as free amino acids. The slightly stronger flavor, taste and darker color of the commercial sauce could be due to longer aging. Additionally it may also results from the added ingredients before bottling as mentioned in Klomklao et al., (2006).

Changes in microbiological count ($\log \text{cfu g}^{-1}$) of all the sauce samples are presented in Figure 3A-D and Table 7. Initial counts of aerobic bacteria in papain and LAB treated samples were 7.53 ± 0.1 and 7.48 ± 0.5 and it has been decreased to 4.89 ± 0.4 and 2.14 ± 0.3 , respectively after 120 d of storage. The counts were found not detected towards the end of 180 d of storage in both the samples (Fig. 3A). *Escherichia coli* was not found within 15 d of fermentation from the initial count of 3.56 ± 0.2 in rohu sauce produced by fermentation method (Fig. 3B). Lactic acid bacteria count of papain treated sample was declined to 1.53 ± 0.2 from the initial count of 6.56 ± 0.2 after 180 d but, the count found not detected within 60 d of

Table 6. Sensory properties of papain and LAB treated rohu sauce after 180 d of fermentation

Parameter	Commercial sauce	C	Rohu sauce PT	LT
Color	7.5 $\pm$ 1.4	6.0 $\pm$ 1.7	6.6 $\pm$ 1.5	6.3 $\pm$ 1.2
Flavor	6.8 $\pm$ 0.8	4.5 $\pm$ 1.9	5.9 $\pm$ 2.1	6.5 $\pm$ 2.1
Taste	6.9 $\pm$ 1.0	5.3 $\pm$ 1.7	6.0 $\pm$ 1.7	6.5 $\pm$ 1.8
Appearance	7.0 $\pm$ 0.9	6.6 $\pm$ 0.7	7.3 $\pm$ 0.7	6.8 $\pm$ 1.0
Overall quality	7.2 $\pm$ 0.6	5.9 $\pm$ 0.8	7.4 $\pm$ 0.7	6.9 $\pm$ 0.6

Values in column are mean $\pm$ SD, C-control, PT-papain treated and LT-lab treated

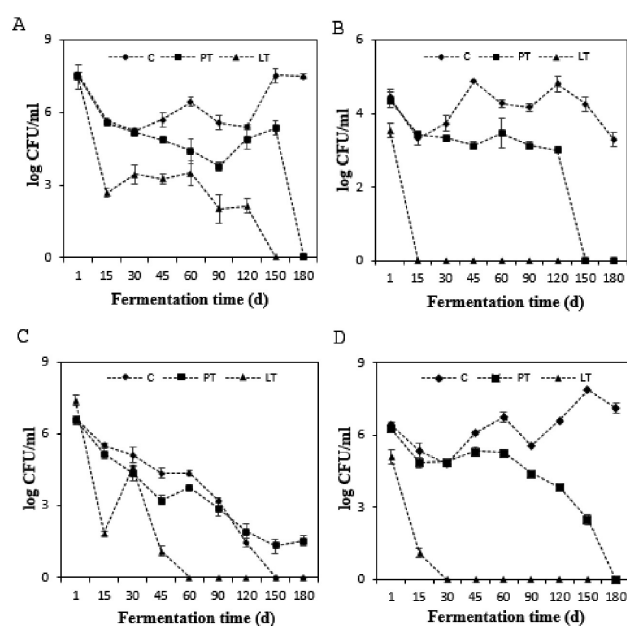
Table 7. Microbiological characteristics of papain and LAB treated Rohu sauce during fermentation

Parameter	DAY	1	15	30	45	60	90	120	150	180
<i>Salmonella-Shigella</i> count	C	2.36 $\pm$ 0.1	ND	ND	ND	ND	ND	ND	ND	ND
	PT	1.63 $\pm$ 0.3	ND	ND	ND	ND	ND	ND	ND	ND
	LT	2.76 $\pm$ 0.2	ND	ND	ND	ND	ND	ND	ND	ND
Halophile count	C	2.06 $\pm$ 0.1	4.74 $\pm$ 0.1	4.63 $\pm$ 0.1	5.71 $\pm$ 0.1	6.59 $\pm$ 0.1	5.64 $\pm$ 0.1	7.56 $\pm$ 0.1	7.57 $\pm$ 0.0	7.11 $\pm$ 0.2
	PT	2.06 $\pm$ 0.0	4.55 $\pm$ 0.2	4.73 $\pm$ 0.0	5.26 $\pm$ 0.0	4.74 $\pm$ 0.2	4.61 $\pm$ 0.0	5.40 $\pm$ 0.2	5.2 $\pm$ 0.1	4.59 $\pm$ 0.2
	LT	2.26 $\pm$ 0.2	1.46 $\pm$ 0.2	ND	ND	ND	ND	ND	ND	ND
Yeast and mold count	C	ND	ND	ND	ND	ND	ND	ND	ND	ND
	PT	1.40 $\pm$ 0.2	ND	ND	ND	ND	ND	ND	ND	ND
	LT	4.16 $\pm$ 0.1	ND	ND	ND	ND	ND	ND	ND	ND

C-control, PT-papain treated and LT-LAB treated, ND-not detected

Values in column are mean $\pm$ SD for triplicates and expressed in $\log \text{cfu/ml}$ of sample

fermentation in LAB treated sauce (Fig. 3C). The probable reason could be the rapid decrease in the pH of the fermentation medium with the production of more amount of acid leads to autolysis of the LAB. Bacterial colonies exhibited clear zone on MRS agar containing 0.3% (w/v) CaCO_3 were considered for total LAB count. CaCO_3 was used as an indicator for acid-producing LAB strains since it dissolved when interact with acid then a clear zone is observed around the colony (Onda et al., 2002). The staphylococci count was reduced to 1.10 ± 0.2 from the original count of 5.08 ± 0.3 within 15 d of fermentation (Fig. 3D). *Salmonella-shigella* and yeast and mould counts in all the samples were not detected within 15 d of storage (Table 7). The depletion in the nutrition ingredients and accumulation of metabolites with the progress of fermentation would be the reason for decrease in microbiological counts. There was gradual increase in the count of halophilic bacteria from 2.06 ± 0.0 and 2.26 ± 0.2 to 7.11 ± 0.2 and 4.59 ± 0.2 in papain and LAB treated samples, respectively towards the end of fermentation (Table 7). Paludan-Muller et al. (2002) have reported that the halotolerant bacteria will grow and



propagate after 5 days of fermentation and these halophiles mostly were LAB and yeasts.

Fig. 3A-D. Total plate count (A), *Escherichia coli* count (B), Lactic acid bacteria count (C), Staphylococci count (D) of papain and LAB treated rohu sauce during fermentation

The study suggested that rohu, a freshwater fish could be fermented in 180 days into fish sauce with acceptable quality in terms of nutrition. In addition, the commercially available papain and native halotolerant, proteolytic lactic acid bacteria were suitable agents for the acceleration of fermentation. Although the papain treated fish sauce samples appeared to have slightly better biochemical quality than the sauce prepared from LAB inoculated one, these fish sauces showed better microbiologically quality. Results of physicochemical properties such as, yield, pH and non-enzymatic browning in the sauce inoculated with *P. pentosaceus* FSBP4-40 were superior than the papain treated sauce. In summary, the addition of papain and LAB reduces the fermentation time in the effective utilization and preservation of over produced and harvested rohu fish. The developed product should also apply as flavouring condiment in wide variety of sea-foods.

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References

- AOAC (1999) Official method of analysis (Helrich, K., Ed), 15th edn., Washington, DC: Association of Official Analytical Chemists
- AOAC (2000) Official Methods of Analysis. 17th edn., Association of Official Analytical Chemists, Washington DC, USA
- AOAC (2002) Official Methods of Analysis, 16th edn., Association of Official Analytical Chemists, Washington, DC
- APHA (2001) American Public Health Association. Compendium of Methods for Microbiological Examination of Foods (Speak, M.L. Ed). Washington, USA
- Cho, Y.J., Im, Y.S., Park, H.Y. and Choi, Y.J. (2000) Quality characteristics of Southeast Asian salt fermented fish sauces. J. Korean Fish. Soc. 33(2): 98-102
- Conway, E.J. and Byrne, A. (1936) An absorption apparatus for the micro-determination of certain volatile substances I, the micro-determination of ammonia. J. Biochem. 27: 419-429
- FDA (2004) Code of federal regulations 21 CFR 161 Subpart B Section 161. 190. United State Food and Drug Administration, USA

- Hendel, C.E., Bailey, G.F. and Taylor, D.H. (1950) Measurement of non-enzymatic browning of dehydrated vegetables during storing storage. *Food Technol.* 3: 44-48
- Hjalmarsson, G.H., Park, J.W. and Kristbergsson, K. (2007) Seasonal effects on the physicochemical characteristics of fish sauce made from capelin (*Mallotus villosus*). *Food Chem.* 103: 495-504
- Hoyle, N.T. and Merritt, J.H. (1994) Quality of fish protein hydrolysate from Herring (*Clupea harengus*). *J. Food Sci.* 59: 76-79 & 129
- Kilinc, B., Cakli, S., Tolasa, S. and Dincer, T. (2006) Chemical microbiological and sensory changes associated with fish sauce processing. *Eur. Food Resour. Technol.* 222: 604-613
- Klomklao, S., Benjakul, S., Visessanguan, W., Kishmura, H. and Simpson, B.K. (2006) Effect of skipjack tuna spleen on the liquefaction and characteristics of sardine fish sauce. *Food Chem.* 98: 440-452
- Lopetcharat, K., Yeung, J., Dr. Park, J.W. and Daeschel, M.A. (2001) Fish Sauce Products and manufacturing. A review. *Food Rev. Int.* 17(1): 65-88
- Majumdar, R.K., Roy, D., Bejjanki, S. and Bhaskar, N. (2015) Chemical and microbial properties of shidal, a traditional fermented fish of Northeast India. *J. Food Sci. Technol.* 53(1): 401-410
- Moon, G., Leem M., Lee, Y. and Trakoontivakorn, G. (2002). Main components of soy sauce representing antioxidative activity. *Int. Congress Series.* 1245: 509-510
- Mueda, R.T. (2015) Physic-chemical and color characteristics of salt-fermented fish sauce from anchovy *Stolephorus commersonil*. *AACL Bioflux.* 8(4): 565-572
- Onda, T., Yanagida, F., Uchimura, T., Tsuji, M., Ogino, S., Shinohara, T. and Yokotsuka, K. (2002) Widespread distribution of the bacteriocin-producing lactic acid cocci in Miso-paste products. *J. Appl. Microbiol.* 92(4): 695-705
- Paludan-Muller, C., Madsen, M. and Sophanodora, P. (2002) Fermentation and micro flora Plaa-som, a Thai fermented fish product prepared with different salt concentrations. *Int. J. Food Microbiol.* 73: 61-67
- Sachindra, N.M., Bhaskar, N., Siddegowda, G.S., Sathisha, A.D. and Suresh, P.V. (2007) Recovery of carotenoids from ensilaged shrimp waste. *Bioresour. Technol.* 98: 1642-1646
- Saithong, P., Panthavee, W., Boonyaratanakornkit, M. and Sikkhamondhol, C. (2010) Use of a starter culture of lactic acid bacteria in plaa-som, a Thai fermented fish. *J. Biosci. Bioeng.* 110: 553-557
- Siddegowda, G.S., Bhaskar, N. and Shubha Gopal. (2016) Bacteriological properties and health related biochemical components of fermented fish sauce: An overview. *Food Rev. Int.* 32(2): 203-229
- Siddegowda, G.S., Bhaskar, N. and Shubha Gopal. (2016) Biochemical and bacteriological quality of rohu (*Labeo rohita*) head sauce produced by enzymatic and fermentation method. *Fish. Technol.* 53: 220 – 231
- Siddegowda, G.S., Bhaskar, N. and Shubha Gopal. (2017) Fermentative properties of proteolytic pediococcus strains isolated from salt fermented fish hydrolysate prepared using freshwater fish rohu (*Labeo rohita*). *J. Aquat. Food Prod. Technol.* 26(3): 341-355
- Sun, J., Yu, X., Fang, B., Ma, L., Xue, C., Zhang, Z. and Mao, X. (2016) Effect of fermentation by *Aspergillus oryzae* on the biochemical and sensory properties of anchovy (*Engraulis japonicus*) fish sauce. *Int. J. Food Sci. Tech.* 51: 133-141
- Udomsil, N., Rodtong, S., Tanasupawat, S. and Yongsawatdigul, J. (2015) Improvement of fish sauce quality by strain CMS5-3-1: a novel species of *Staphylococcus* sp. *J. Food Sci.* 80(9): M2015-M2022
- Vidanarachchi, J.K., Ranadheera, C.S., Wijarathen, T.D., Udayangani, R.M.C., Himali, S.M.C. and Pickova, J. (2014) Application of seafoods byproducts in the food industry and human nutrition. In: *Seafood processing by products: Trends and Applications* (Kim, S. K. Ed.). Springer, USA. pp 463-528
- Xu, W., Yu, G., Xue, C., Xue, Y. and Ren, Y. (2008) Biochemical changes associated with fast fermentation of squid processing by-products for low salt fish sauce. *Food Chem.* 107: 1597-1604
- Yongsawatdigul, J., Rodtong, S. and Raksakulthai, N. (2007) Acceleration of Thai fish sauce fermentation using proteinases and bacterial starter culture. *J. Food Sci.* 72: M382-M390
- Yoshikawa, S., Kurihara, H., Kawai, Y., Yamazaki, K., Tanaka, A., Nishikiori, T. and Ohta, T. (2010) Effect of halotolerant starter microorganisms on chemical characteristics of fermented chum salmon (*Oncorhynchus keta*) sauce. *J. Agric. Food Chem.* 58: 6410-6417
- Yu, X., Mao, X., He, S., Liu, P., Wang, Y. and Xue, C. (2014) Biochemical properties of fish sauce prepared using low salt, solid state fermentation with anchovy by-products. *Food Sci. Technol.* 23(5): 1497-1506
- Zarei, M., Najafzadeh, H., Eskandari, M.H., Pashmforoush, M., Enayati, A., Gharibi, D. and Fazlari, A. (2012) Chemical and microbial properties of mahyaveh, a traditional Iranian fish sauce. *Food Control.* 23: 511-514
- Zhou, X.X., Zhao, D.D., Liu, J.H., Lu, F. and Ding, Y.T. (2014) Physical, chemical and microbiological characteristics of fermented surimi with *Actinomucor elegans*. *Food Sci. Technol.* 59: 335-341