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Antioxidant Properties and Dominant Bacterial Community of Fermented Rohu (*Labeo rohita*) Sauce Produced by Enzymatic and Fermentation Method

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Abstract

Sauce was produced from Rohu (*Labeo rohita*) by enzymatic and fermentatively under conditions optimized by response surface method. Salt (20% w/w) and commercial papain (3% w/w) were employed for enzymatic production; while, salt (25%, w/w), sugar (7.5%, w/w) and lactic culture (10%, w/v; *Pediococcus pentosaceus* FSBP4-40) were used in case of fermentative production. Total antioxidant activity (as ascorbic acid, µg/ml), 2,2`-diphenyl-1-picryl-hydrazyl (DPPH, %) and 2,2`- azinobis-3-ethyl-benzothizoline-6 sulphonate (ABTS; %) scavenging activities of fermented sauce samples were higher at 3441±60, 49.57±1.7 and 99.0±0.3, respectively; as compared to enzyme treated samples which showed 803±13, 28.60±1.1 and 78.4±1.0, for the respective parameters, after 180 days storage at 37°C. Superoxide anion scavenging activity and reducing potential were found significantly higher ($P<0.05$) in both fermented as well as enzymatically prepared sauces in comparison to control. Fermented sauce exhibited excellent antibacterial property against *Listeria monocytogenes* ScottA. Bacterial counts such as total plate count and halophile count, after 180 days, were significantly lower ($P<0.05$) in the fermentatively produced sauce than enzymatically prepared sauce. *Pediococcus, Micrococcus, Enterococcus* and *Staphylococcus* were the dominant bacterial genera in fermented sauce. The study emphasizes the role of accelerating fermentation process, either enzymatically or fermentatively, to produce biofunctionally and bacteriologically superior rohu sauce.

Introduction

With the global fish production reaching 171 million tons in 2016 (FAO, 2018) and fish being considered as one of the important sources of protein, it has become important to look at processes that maintain nutritional value of fish. Fermentation of fish is a safe, environment-friendly and less energy consuming process which is traditionally used to increase shelf-life of fish and to develop fermented fish products (Marti-Quijal *et al*., 2020). Fermented fish products, apart from being an integral part of many food cultures, are also a source of interesting microbes and are an important industry in many countries (Zang *et al*., 2019). Fish sauce - one of the important fermented fish product - is consumed by over 80 –90% people in Southeast Asia (Longfils *et al*., 2008). Fish sauce is an amber-colored salty liquid in Southeast Asian cuisine used as an important condiment for improving the taste of foods. There is high divergence in manufacturing among fish sauce-producing countries throughout the world, although the fish and salt are the major raw materials in general production. The leading fish sauce producer in the world is Thailand, with the annual production

estimated to be >400 million litres with 20 out of 100 fish sauce producers contributing more than 80% of the global production (Vidanarachchi *et al.*, 2014).

The bioactive peptides from fermented fish products have been reported to act as antioxidants (Majumdar *et al*., 2015). Siddegowda *et al*., (2016) reviewed that the bioactive peptides may be involved in various biological functions, such as antihypertension, antagonist, immunomodulatory, antithrombotic, antioxidant, anticancer, and antimicrobial activities depending on the amino acid sequences. Aoshima and Ooshima, (2009) studied the antioxidant activity of Japanese liquid condiments, Shoyu (soy sauce) and Gyoshoyu (fish sauce). The *in vitro* antioxidant properties of the rohu head sauce produced by enzymatic and fermentation method were stated in Siddegowda *et al*., (2016). Antioxidants are very important for human health, since the production of reactive oxygen species is thought to be a significant cause of aging and carcinogenesis (Lambert and Yang, 2003).

Fish sauces without added starter cultures leads to the growth of halotolerant microorganisms due to the high salt conditions during the natural process of fermentation (Fukui *et al*., 2012). Various novel halotolerant bacterial cultures have been used to accelerate fermentation process, increase the α-amino content, enhance the sensory characteristics, and improve the microbiological quality. The proteolytic enzymes from halotolerant bacteria not only shorten the fermentation period in fish sauce production, but also in turn will help reducing the formation of biogenic amines (Siddegowda *et al*., 2016). These halotolerant organisms are the source of exogenous proteolytic enzymes, which hydrolyse the fish tissue in the fish sauce fermentation (Jung *et al*., 2013). Therefore, the study of bacteriological properties to understand the processes involved in fish sauce production has got some significance because the microbial communities reflect the overall quality of the sauce. Biochemical and microbial characteristics of both Ngari and Hentaak - traditional fermented fish products of India were analysed by Majumdar *et al*., (2015). Against this background, the objective of the present study was to compare *in vitro* antioxidant activities, antibacterial properties and dominant bacterial community in the enzymatically and fermentatively produced fish sauce from rohu.

Materials and Methods

Materials

Freshwater fish Rohu (*Labeo rohita*) was collected from local fish market (Mysore, India) form the material of the study. The material 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. The protease used for the enzymatic hydrolysis is papain (Loba chemie), 2,2`-diphenyl-1-picrylhydrazyl (DPPH), 2,2`- azinobis-3-ethyl-benzothizoline-6sulphonate (ABTS), peroxidase was purchased from sigma-Aldrich Chemie (Steinheim, Germany). The pathogenic bacterial strains (viz., *Micrococcus luteus, Staphylococcus aureus* FR1722*, Escherichia coli* MTCC118*, and Listeria monocytogenes* Scott A) used for the study of antibacterial activity were from the institute culture collection (CFTRI, Mysuru). Plate count agar (PCA) and brain heart infusion (BHI) 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.

Methods

Preparation of Rohu Sauce

The preparation of rohu sauce is schematically represented in Figure 1. Briefly, eviscerated rohu was sliced into small pieces of 2×1cm size and washed in potable water 3 times. The sliced rohu was weighed and bottled into clean sterile food grade plastic containers and mixed with commercial enzyme 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. The containers were closed with plastic lids and stored at room temperature. *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 24h 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 was filtered through cheese-cloth at every 15 d till 6 months and further filtered using Whatman no. 1 filter paper. The resulted liquid was considered as fish sauce and the yield of the same was measured as the ratio of original fish-salt and papain mass in the container to the weight of liquid after filtration. The fish sauce was lyophilized and used for *in vitro* antioxidant activity and antibacterial property.

Antioxidant Properties of Fermented Rohu Sauce

Preparation of Sample

Fish sauce samples were dissolved (50mg/ml) in double distilled water and homogenized at 10000 rpm for 2 min using homogenizer (Polytron PT 3100) followed by centrifugation at 7000 ×g for 15min. The supernatant was collected and filter through Whatman No. 1 filter paper and protein content in the filtrate was

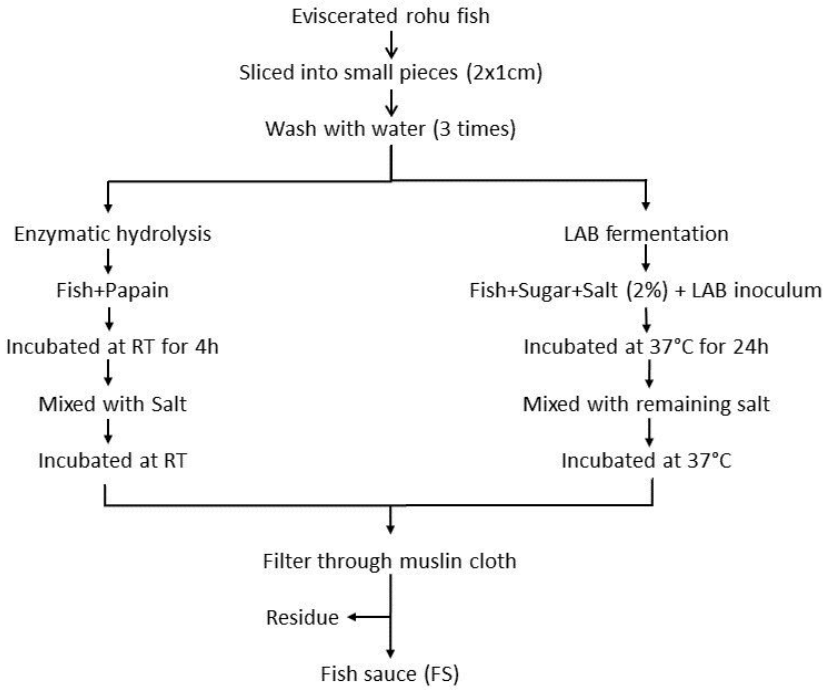

Figure 1. Schematic flow of sauce production from Rohu (*Labeo rohita*) using papain and *P. pentosaceus* FSBP4-40.

estimated by the method of Lowry *et al*., (1951). This filtrate was used for assaying various antioxidant activities.

Antioxidant Properties

Total Antioxidant Activity

The Total antioxidant activity (TAO) of fermented fish sauce sample was determined according to the method of Prieto *et al*., (1999). Briefly, 0.3 mL of sample was mixed with 3.0 mL reagent solution (1:1:1, v/v of 0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). Reaction mixture was heated at 95°C for 90 min in a water bath, cooled to room temperature. Absorbance of all the sample mixtures was measured at 695 nm and TAO was expressed as of ascorbic acid equivalents in micrograms per gram of sample.

DPPH Radical Scavenging Activity

The DPPH radical scavenging capacity of fish sauce samples was determined by the method described in Bijinu *et al*., (2011). Briefly, 2.0 mL of 0.16 mM DPPH solution (in methanol) was added to the test tube containing 100 µL of sample and made up to 2 mL with distilled water. The mixture was vortexed for 1 min and kept at room temperature for 30 min in dark. Sample blank was prepared by replacing DPPH with methanol and methanol along with DPPH served as positive control. The scavenging activity (%) was determined by measuring the absorbance of samples at 517 nm and calculated using the formula:

$$\text{Scavenging activity (\%)} = [1- \{(A_{sample} - A_{sample\ balnk})/A_{control}\}] \times 100$$

Super Oxide Scavenging Activity

Super oxide anion scavenging activity of the samples was determined by the method as described in Heo *et al*., (2005). An aliquot of 100 µL was made up to 0.3 mL and added with 2.6 mL of 50 mM phosphate buffer (pH 8.2), to this 90 µL of freshly prepared 3 mM pyrogallol dissolved in 10 mM HCl was added. Sample blank was prepared by mixing 0.3 mL of distilled water in 2.6 mL of phosphate buffer. The absorbance was measured at 325 nm from 0 min and 10 min.

ABTS Radical Scavenging Activity

ABTS radical scavenging activity of the samples was carried out as explained in Sachindra and Bhaskar (2008). ABTS radical solution was prepared by mixing ready to use ABTS solution with 100 mL of 0.05 M acetate buffer (pH 4.5) and 5 units of peroxidise and incubated at 37°C for 15h. ABTS (1.9 mL) was mixed with 0.1 mL sample and incubated at 37°C for 1 h. Buffer instead of ABTS served as sample blank and distilled water (0.1 mL) instead of sample was used as control. Scavenging activity was calculated as follows:

$$\text{Scavenging activity (\%)} = [1- \{(A_{sample} - A_{sample\ balnk})/A_{control}\}] \times 100$$

Reducing Power Assay

Reducing potential of the fermented fish sauce samples was assayed by the method as followed in Bijinu *et al*., (2011) and absorbance was measured at different concentration (50, 100, 150 and 200 µL). Briefly, samples at different concentrations were mixed with 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide. Reaction mixture was incubated at 50°C for 20 min and 2.5 mL of 10% trichloroacetic acid was added, centrifuged at 8000 rpm for 10 min. From the upper layer of the solution, 2.5 mL was taken for further reaction and mixed with 2.5 mL of distilled water and 0.5 mL of 0.1% ferric chloride. Absorbance of all the samples was measured at 700 nm by using distilled water as blank.

Antibacterial Properties

The antibacterial activity of fermented fish sauce samples was assayed by the agar well diffusion method (Geis *et al*., 1983). Selected human bacterial pathogens were overlaid on nutrient agar with brain heart infusion (BHI) soft agar (0.8%) and allowed to grow at 37°C for 4-6 h. Fish sauce samples for antibacterial activity were prepared by dissolving 50mg/ml sample in sterilized double distilled water and homogenized at 10000 rpm for 2 min using homogenizer (Polytron PT 3100) followed by centrifugation at 7000×g for 15 min. The resultant supernatant was added in a concentration of 50 µl into wells made on the plates containing pathogenic strains. The plates were then pre-incubated for 2-3 h at 4°C to allow the test material to diffuse into the agar and later they were incubated at 37°C a further for 18 h. The antibacterial spectrum of the samples was determined by measuring the diameter of the inhibition zones in mm.

Microbiological Analysis

In order to identify dominant microflora associated with fermentation, microbial load of fermented rohu sauce samples was determined monthly from day-1 to 180 d using standard methods (APHA 2001). Ten grams of sample was diluted in 90 ml of sterile physiological saline and then mixed by stomacher blender (Stomacher 400) for 2 min, the stock was then 10-fold serially diluted in sterile saline and 0.1ml of each proper diluted sample was spread with glass spreader on media plates for obtaining total plate count. The inoculated plates were incubated at 37ºC for 24-48 hours. Plate count agar with 10% (w/w) NaCl was used for determining total halophiles count and the plates were incubated at 37ºC for 10 days. Microbial count was expressed in log CFU/g. The dominant bacterial colonies were randomly selected by their similarity in morphological properties from the total plate count agar plates and biochemically characterized as per the protocol outlined in Bergey`s manual of determinative bacteriology (Bergey *et al*., 2002).

Results

Antioxidant Activity of Fermented Rohu Sauce

In vitro antioxidant activities such as TAO activity and ABTS radical scavenging activity of lyophilized powder are presented in the Table 1. Sauce samples inoculated with LAB (*P. pentosaceus* FSBP4-40) had better TAO activity and ABTS radical scavenging activity compared to the papain treated samples. There was 10-fold and 3-fold increase in TAO activity of LAB fermented and enzyme treated sauce samples, respectively than control at the end of 180 d. Initially, the TAO activity (Eq to ascorbic acid, µg/ml) of LAB fermented sauce sample was 1374±43 and reached 3441±60 towards the end of the fermentation. Almost maximum ABTS radical scavenging activity was exhibited by LAB treated sample at the 180 d. The ferric chloride reducing power of both treated and untreated samples are given in the Table 2. Higher ferric chloride reducing power was noticed in *P. pentosaceus* FSBP4-40 treated sauce sample than the control and enzyme treated samples. Over 3-fold increase in ferric chloride reducing power was observed in LAB fermented sauce than the enzyme treated sauce at all different concentrations of samples towards the end of 180 d. The sauce produced by fermentative method exhibited higher DPPH radical scavenging activity as compared to the enzyme treated and control after 180 d of storage (Figure 2A). The DPPH radicals

Table 1. Total antioxidant activity and ABTS scavenging activity of papain treated and LAB treated rohu sauce during fermentation

	TAO (Eq to ascorbic acid, µg/ml)			ABTS Scavenging (%)		
Day	C	PT	LT	C	PT	LT
1	208±15^{b}	604±30^{a}	1374±43^{a}	58.7±1.4^{a}	65.5±1.4^{a}	78.7±0.6^{a}
30	382±60^{d}	674±80^{b}	2271±52^{b}	65.0±1.4^{b}	70.9±0.7^{b}	87.4±0.6^{b}
60	374±16^{d}	722±30^{c}	2200±57^{b}	60.7±2.1^{a}	74.8±0.7^{c}	89.3±0.3^{c}
90	317±30^{c}	776±11^{d}	2423±90^{c}	58.7±2.1^{a}	72.6±1.0^{b,c}	93.3±0.6^{d}
120	275±30^{b}	739±24^{c}	2908±12^{d}	59.0±1.0^{a}	73.3±1.4^{b,c}	92.5±0.6^{d}
150	229±10^{a}	734±20^{c}	3223±18^{e}	57.0±1.7^{a}	72.3±2.1^{b,c}	97.7±0.3^{e}
180	314±12^{c}	803±13^{d}	3441±60^{f}	65.5±2.7^{b}	78.4±1.0^{d}	99.0±0.3^{f}

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).

Table 2. Reducing potential of papain treated and LAB treated rohu sauce during fermentation

	C				PT				LT			
DAY	25µl	50µl	75µl	100µl	25µl	50µl	75µl	100µl	25µl	50µl	75µl	100µl
1	0.05±0^{a}	0.10±0^{a}	0.15±0^{a}	0.20±0^{a,b}	0.06±0^{a}	0.15±0^{a}	0.19±0^{a}	0.26±0^{a}	0.24±0^{a}	0.43±0^{a}	0.62±0^{a}	0.83±0^{a}
30	0.07±0^{b}	0.15±0^{b,c}	0.23±0^{c}	0.28±0^{c}	0.11±0^{b,c}	0.18±0^{a,b}	0.27±0^{b}	0.34±0^{b}	0.34±0^{b}	0.63±0^{b}	0.88±0^{b}	1.27±0^{b}
60	0.08±0^{b}	0.15±0^{c}	0.22±0^{c}	0.28±0^{c}	0.10±0^{b}	0.18±0^{b}	0.28±0^{b}	0.38±0^{c}	0.41±0^{c}	0.69±0^{c}	0.99±0^{c}	1.37±0c
90	0.07±0^{a,b}	0.12±0^{a}	0.19±0^{bc}	0.29±0^{c}	0.12±0^{b,c}	0.22±0^{c}	0.33±0^{c,d}	0.43±0^{d}	0.56±0^{d}	0.89±0^{d}	1.20±0^{d}	1.54±0^{d}
120	0.05±0^{a}	0.12±0^{a}	0.17±0^{ab}	0.22±0^{b}	0.11±0^{b,c}	0.23±0^{c,d}	0.32±0^{c}	0.40±0^{c}	0.66±0^{e}	0.91±0^{e}	1.30±0^{e}	1.61±0^{e}
150	0.06±0^{a,b}	0.13±0^{a,b}	0.15±0^{a}	0.18±0^{a}	0.12±0^{b,c}	0.23±0^{c,d}	0.34±0^{c,d}	0.43±0^{d}	0.80±0^{f}	1.19±0^{f}	1.43±0^{f}	1.77±0^{f}
180	0.08±0^{b}	0.16±0^{c}	0.22±0^{c}	0.31±0^{c}	0.13±0^{c}	0.25±0^{d}	0.35±0^{d}	0.47±0^{e}	0.89±0^{g}	1.22±0^{g}	1.56±0^{g}	1.97±0^{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).

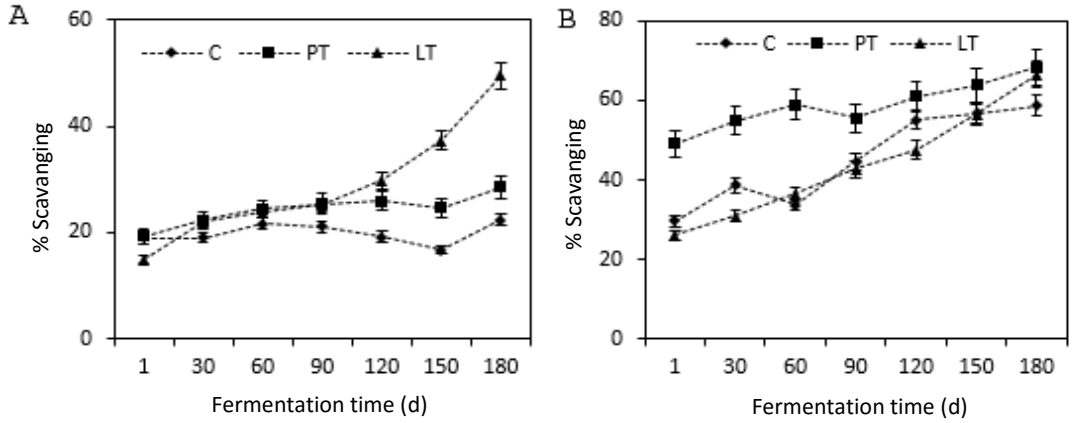

Figure 2. DPPH (A) and Superoxide (B) scavenging activity of papain treated and LAB treated rohu sauce during fermentation.

scavenging activity of LAB and papain treated sauce samples were 49.57±1.7% and 28.60±1.1%, respectively. The superoxide radicals scavenging activity of papain treated sample was slightly higher (68.40±1.0%) than LAB treated (66.50±0.8%) sample towards the end of fermentation period (Figure 2B). Overall, the antioxidant activities of LAB (*P. pentosaceus* FSBP4-40) treated samples were higher compared to papain treated samples except superoxide scavenging activity.

Antibacterial Properties of Fermented Rohu Sauce

Fermented rohu sauce produced using *P. pentosaceus* FSBP4-40 showed higher antibacterial activity against pathogen *L. monocytogenes* Scott A (Table 3). The enzyme treated and LAB fermented sauce samples shown the inhibition zone diameter of 16.0±0.0 mm and 26.5±2.1 mm, respectively. There was a marginal difference in the antibacterial activity of enzyme treated sample with that of untreated sauce samples. The antibacterial activity of papain treated and LAB fermented sauce samples exhibited slightly higher than the untreated sauce sample against *Micrococcus luteus, Staphylococcus aureus* FR1722 and *Escherichia coli* MTCC118.

Bacteriological Properties of Fermented Rohu Sauce

In the initial stages of the fermentation, total plate count of the treated and untreated samples of rohu sauce were in the range of 7.48±0.5 to 7.58±0.1 log cfu/ml (Figure 3A). There was gradual decrease in the count of treated samples and no bacterial load was found at the end of fermentation. The untreated rohu sauce showed 7.48±0.1 log cfu/ml after 180 d and the count was not detected after 150 d in LAB fermented rohu sauce. Almost continuous increase in log cfu/ml values of halophiles was observed in untreated rohu sauce samples throughout the storage period (Figure 3B). The same trend was noticed in papain treated sauce with slight decrease in count at day-60 and day-90 but, the count was not detected day-30 onwards in LAB fermented samples. The study revealed that the total plate count and halophile count of the papain treated and untreated samples were almost equivalent range towards the end of storage. Overall, the bacteriologically quality of sauce produced using *P. pentosaceus* FSBP4-40 was superior to enzymatically produced sauce.

Table 3. Antibacterial activity of papain and LAB treated rohu sauce after 180 d of fermentation

	Rohu sauce		
Pathogens	C	PT	LT
Micrococcus luteus	11.5±2.1[a]	14.0±1.4[a]	14.5±0.7[a]
Staphylococcus aureus FR1722	11.5±0.7[a]	15.0±0.0[a,b]	14.5±2.1[a]
Escherichia coli MTCC118	12.0±1.4[a]	14.0±0.0[a]	14.0±2.8[a]
Listeria monocytogenes Scott A	14.0±2.8[a]	16.0±0.0[c]	26.5±2.1[b]

Values (inhibition zone diameter in mm) 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)

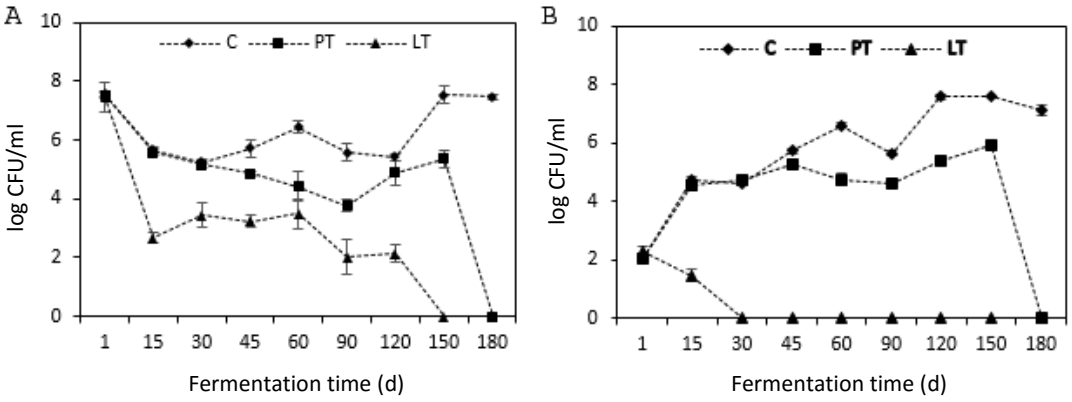

Figure 3. Total plate count (A) and Halophile count (B) of papain treated and LAB treated rohu sauce during fermentation

Dominant Bacterial Community in Fermented Rohu Sauce

The dominant bacterial colonies were randomly selected from fish sauce by their similarity in morphological characteristics from the total plate count plates. The details of biochemical characteristics of dominant bacterial groups isolated are presented in Table 4. Morphologically, the isolates were spherical in shape and Gram positive. Of the four isolates two isolates were positive for catalase activity and three isolates were oxidase positive. They exhibit potential lipolytic and proteolytic activities. The isolates grown at different pH, temperature and showed tolerance towards wide range of salt concentration. As per the Bergey`s manual of determinative bacteriology the isolates were characterized as *Pediococcus, Micrococcus, Enterococcus* and *Staphylococcus.* These were the bacterial genera associated in the fermented rohu sauce predominantly throughout the fermentation period.

Discussion

The studies have shown that uncontrolled free radicals including hydroxyl radicals, peroxyl radicals, 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical and superoxide anion radical can attack various tissue components leading to autoimmune diseases, neurodegenerative disorders and cancers (Kehrer and Klotz, 2015). Fermented fish products could be used as a potential source of nutrients and natural antioxidants (Majumdar *et al*., 2016). *In vitro* TAO activity of fermented rohu sauce is in agreement with the results of Siddegowda *et al*., (2016). They stated that the LAB fermented rohu head sauce exhibited higher TAO (ascorbic acid equivalents in µg g^{-1}) activity of 1236.25±7.07 than the enzyme treated sauce (1070.63±11.49) towards end of 120 d of storage. Whereas, the ABTS scavenging activity of both enzyme and LAB treated rohu head sauce samples were almost identical at the end of fermentation. Peralta *et al*. (2008) reported that the Philippine's salt fermented shrimp paste showed 24.3-61.5% of DPPH radical scavenging activity in 80% ethanolic extract. Aoshima and Ooshima, (2009) showed that the DPPH radical-scavenging activity of Japanese fish sauce Gyoshoyu (87.7±0.1%) was greater than the soy sauce Shoyu (32.3±3.7%). Gyoshoyu is also useful for reducing level of H_2O_2 in foods, when it is added as a liquid condiment. This anti hydrogen peroxide activity of Gyoshoyu is due to the presence of a thermostable catalase. The peptides from fermented fish products have been reported to act as antioxidants (Majumdar *et al*., 2015). Antioxidative peptides were isolated and characterized from fish sauce by-product, a solid waste generated in fish sauce industry contains natural protein hydrolysates produced from digestion of fish proteins using various protease and halophiles in the fermentation (Choksawangkarn *et al*., 2018). The study evidenced that the fish sauce by-product contained a

Table 4. Characteristics of dominant bacterial community in fermented rohu sauce

Characteristics	Isolate-1	Isolate-2	Isolate-3	Isolate-4
Colour	White	Yellow	White	White
Cell morphology	Cocci	Cocci	Cocci	Cocci
Gram staining	+	+	+	+
Catalase	-	+	+	-
Oxidase	+	+	-	+
Lipase	-	+	+	+
Protease	+	-	+	+
Growth at pH 4.2	+	-	+	-
7.5	+	+	+	+
8.5	+	+	+	+
Growth at 10º C	-	+	-	-
30º C	+	+	+	+
50º C	+	-	+	-
Growth at 0 % Salt	+	+	+	+
3.0 % Salt	+	+	+	+
6.5 % Salt	+	+	+	+
9.0 % Salt	-	+	+	-
Sugar utilization				
Maltose	+	+	+	+
Lactose	-	+	-	+
Mannitol	-	+	-	+
Sucrose	+	+	-	+
Sorbitol	-	+	+	-
Glucose	+	+	-	+
Dextrose	+	+	+	+
Species of genus	*Pediococcus*	*Micrococcus*	*Staphylococcus*	*Enterococcus*

high amount of low molecular weight proteins/peptides and had most potent antioxidant activity. The proteins present in the raw material (rohu) are hydrolysed into peptides and amino acids during fermentation. These protein hydrolysates in the sauce might be responsible for antioxidant activity. Some of these bioactive peptides have demonstrated multifunctional activities such as immunomodulatory, anticancer and antimicrobial activities along with the antioxidant properties based on their structure and other factors, including hydrophobicity, charge, or microelement binding properties (Siddegowda *et al*., 2016).

The shelf-life of the LAB fermented food products was enhanced by maintaining the acidic condition due to lactic acid produced by LAB and their antagonistic nature towards food spoilage and food poisoning bacteria (Hwanhlem *et al*., 2011). The antibacterial activity exhibited by the fish sauce samples could be due to the presence of antimicrobial peptides (bacteriocin) as well as protein hydrolysates produced during fermentation (Amit *et al*., 2011). The hydrolysates of food proteins by intestinal proteases have also been shown to be antibacterial as well as immunostimulatory in nature (Gediminas *et al*., 2006). Selected strains of lactic acid bacteria isolated and screened from Thai traditional fermented fish (pla-som) showed excellent antagonistic activity against *Escherichia coli*, *Staphylococcus aureus*, and *Salmonell*a sp. (Hwanhlem *et al*., 2011).

Jiang *et al.*, (2007) reported that, the higher microbial counts in the early stages of the fermentation were due to incomplete dissolution and improper penetration of salt into the fish and survival of non-halotolerant bacteria on fish flesh. Kilinc *et al*., (2006) testified that, the initial and final total viable counts of sardine fermented fish sauce produced at different concentrations of sodium chloride and glucose at 37ºC for 57 d were in the range of 3.95±0.05 to 4.47±0.08 log cfu/g and 3.93±0.05 to 5.64±0.09 log cfu/g, respectively. In mahyaveh, a traditional Iranian fish sauce the mean value (log cfu/g) for aerobic mesophilic count was 4.71±3.65 in the sauce samples collected from five different locations in the Southern part of Iran (Zarei *et al*., 2012). A little decrease in the bacterial counts was reported during fermentation of low salt fish sauces produced from squid processing byproducts (Xu *et al*., 2008). Majumdar *et al*., (2015) revealed that, the total plate count of Ngari and Hentaak- traditional fermented fish products of India were 6.65±1.00 log cfu/g and 7.81±0.09 log cfu/g, respectively.

Decreased counts of halophilic bacteria within 30 d of fermentation were reported in fish sauce samples prepared from anchovy hydrolysates inoculated with bacterial starter cultures (Yongsawatdigul *et al*., 2007). The bacterial counts on plate count agar containing 18% NaCl were not detected after 60 d and 90 d in the fish sauce samples inoculated with *Staphylococcus* sp. CMS5-7-5 and CMC5-3-1, respectively (Udomsil *et al*., 2015). Ibrahim, (2010) evaluated the microbial properties of fish sauce produced from Gambusia (*Affinis affinis*) with salt content of 25% (w/w) for 5 months and stated that, the log cfu/ml values for total

plate count, halophilic count were 2.0±0.06 and 2.30±0.11, respectively. Faisal *et al*., (2015) discussed that, in the initial stage of fish sauce fermentation *Micrococcus*, *Lactobacillus*, *Corynebacterium, Escherichia coli, Streptococcus* and *Pseudomonas* were detected. As the fermentation progresses *Corynebacterium*, *E.coli*, *Streptococcus* failed to survive due to the high salt concentration and at the end of 9 months, the dominant salt tolerant bacteria survived and multiplied are *Bacillus*, *Micrococcus*, *Lactobacillus* and *Pseudomonas*. 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.

The dominant bacterial genera in fermented rohu sauce are in correlation with the findings of Majumdar *et al*., (2015). The study reported that *Micrococcus* and *Staphylococcus* were the dominant bacterial genera associated with Ngari and Hentaak, the traditional fermented fish products of India. Some of the species of these genera effectively hydrolyse the proteins with their proteolytic potentiality and utilize lipids by their lipolytic property (Jini *et al*., 2011). *Lactobacillus plantarum* and *Enterococcus faecium* were the dominant lactic acid bacteria isolated from a traditional fermented fish sauce mahyaveh. These bacteria play a significant role in promoting the quality and safety and also develop organoleptic properties of fermented foods (Karparvar *et al*., 2019). Previous studies have also shown that species of *Staphylococcus*, *Micrococcus*, *Lactobacillus* and *Tetragenococcus* were the dominant bacterial community associated during the fish sauce fermentation (Lee *et al*., 2015; Faisal *et al*., 2015). Few of the species of *Staphylococcus Virgibacillus* and *Tetragenococcus* were employed as starter cultures in the acceleration of fish sauce production and improvement of sauce quality (Yongsawatdigul *et al*., 2007: Udomsil *et al*., 2011). According to Lopetcharat and Park, (2002), *Micrococcus*, *Staphylococcus* and *Bacillus* were the dominant bacterial community during the fermentation of fish sauce. Bacterial community dynamics of myeolchi-aekjeot, a Korean traditional fermented fish sauce initially dominated with the bacterial genera *Phychrobacter*, *Photobacterium* and *Vibrio*, disappear rapidly and *Salinivibrio*, *Staphylococcus* and *Tetragenococcus* appeared as major populations in the later stages of fermentation (Lee *et al*., 2015). The fermentative properties of *Pediococcus acidilactici* K7 and *Enterococcus faecium* HAB01 were exploited for the recovery of oil from freshwater fish viscera (Amit *et al*., 2010). Siddegowda *et al*., (2016) reported that, the *Pediococcus* strains with potential proteolytic and fermentative properties were the predominant lactic acid bacteria in salt fermented fish hydrolysate prepared using freshwater fish rohu (*Labeo rohita*). Based on the biochemical criteria, nutritional value and microbial aspects Ibrahim, (2010) suggested that the fish sauce is safe for human consumption as it is rich in protein and essential amino acids.

Conclusion

The study suggested that an accelerated fermentation of rohu, a freshwater fish into fish sauce with acceptable biofunctional quality in terms of nutrition using papain and native halotolerant, proteolytic lactic acid bacteria. *In vitro* antioxidant activities of *P. pentosaceus* FSBP4-40 fermented sauce exhibited superiority over enzyme treated sauce samples. LAB treated sauce showed higher antibacterial activity against pathogen *L*. *monocytogenes* Scott A. The microbiological quality of the sauce produced from fermentative method appeared to be better than the enzymatically produced sauce. *Pediococcus, Micrococcus, Enterococcus* and *Staphylococcus* were found to be the dominant bacterial genera in fermented rohu sauce. In summary, the fermentative conversion of the rohu into sauce is one of the effective methods for preservation and utilization of fish. The developed product should also applied as flavouring condiment in wide variety of sea-foods.

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