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Comparative Analysis of Bioactive Components of Enzymatically and Fermentatively Produced Fish Sauce from Sardine

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Abstract

Fish sauce, a flavored condiment produced by traditional fermentation of low-value fish. The time of fermentation and biochemical safety of sauce are the major concern in traditional fermentation. The enzymatic and fermentative methods were employed to produce Sardine (*Sardinella longiceps*) sauce under optimized conditions using response surface method. Salt (20%, w/w) and papain (3%, w/w) were used for enzymatic production; while, salt (25%, w/w), sugar (7.5%, w/w) and lactic culture (10%, w/v; *Pediococcus pentosaceus* FSBP4-40) were employed in fermentative production. Significant change in bioactive components such as pH, non-enzymatic browning, non-protein nitrogen, degree of hydrolysis, titratable acidity, total soluble nitrogen, and total volatile base nitrogen was observed in treated samples compared to the control. Total antioxidant activity (as ascorbic acid, µg/ml), percent DPPH, ABTS, and superoxide anion scavenging activities of fermented sauce were higher at 3976±90, 53.11±0.5, 99.6±0.3, 67.7±0.5 respectively; as compared to enzyme-treated samples which showed 952±70, 31.46±0.5, 76.5±1.0, 61.8±1.4 for the respective parameters, after 180 days storage. Reducing potential was found significantly higher ($P<0.05$) in treated sauces compared to the control and fatty acid profile showed different changes. The study emphasizes the role of accelerating fermentation, either enzymatically or fermentatively, to produce biochemically and biofunctional superior Sardine sauce.

Introduction

Seafood are rich in numerous useful components with high nutritional value. A diversified group of fish and shellfish from varied marine environments are responsible for the development of different flavored, highly nutritive products with great consumer acceptance (Menon & Lele, 2015). These are highly prone to chemical and microbiological deterioration due to their perishable nature. The loss of nutritional quality soon after capture reduces the self-life and allowing the growth of foodborne pathogens and spoilage (Baptista *et al.,* 2020). More than half of the aquatic product exports originate from developing countries, in terms of value and it was estimated that 78% of seafood competes in international trade as one of the major food commodities (Chen *et al*., 2020). A group of small pelagic fish is largely produced globally and significant proportion of this nutrient-rich food is processed and lost to livestock feed, fish oil and omega-rich fatty acids and vitamins. Small pelagic fish contain health-related biochemical nutritional components for humans and are an important contributor to the food and nutritional security of many poor, low-income households in developing countries (Isaacs, 2016). Sardine is a kind of marine pelagic fish that has a high content of omega-3

fatty acids compared to other marine fish and it is used as the main food for human health (Aung *et al*., 2018).

Fermentation of fish is a safe, less energy-consuming, and environment-friendly 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 sources of interesting microbes and are important industries in many countries (Zang *et al*., 2020). Fish sauce is a traditional condiment in Southeast Asia, normally containing a high concentration of salt. There is a 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 (Siddegowda *et al*., 2016). The leading fish sauce producer in the world is Thailand, with the annual production estimated to be >400 million liters with 20 out of 100 fish sauce producers contributing more than 80% of the global production (Vidanarachchi *et al*., 2014). The traditional method of fish sauce production required the fermentation period for about 9–12 months to hydrolyse fish proteins into soluble peptides and amino acids using NaCl. Focusing on effective inhibition of toxic biochemical components in fish sauces, apart from effectively deciphering their formation and toxicity is one of the major concerns in the production of fish sauce using novel bacterial cultures and commercial enzymes (Siddegowda *et al*., 2016).

In the process of fermentation of fish sauce, the diversity of microflora and their complex metabolic reactions, especially protein degradation, carbohydrate fermentation, and lipid oxidation are accompanied by the formation of flavor substances (Han *et al.,* 2022). Various novel halotolerant bacterial starter cultures have been used to accelerate the fermentation process, increase the α-amino content, enhance the sensory characteristics, and improve the microbiological quality of the fish sauce. The proteolytic enzymes from halotolerant bacteria not only shorten the fermentation period in fish sauce production, but also in turn, will help to reduce the formation of biogenic amines (Siddegowda *et al*. 2016). These halotolerant organisms are the source of exogenous proteolytic enzymes, which hydrolyze the fish tissue in the fish sauce fermentation (Jung *et al*. 2013). Several proteases from plant, animal and microbial sources have been used for the hydrolysis of fish proteins. Papain is the most common commercial protease from plant sources used for the hydrolysis of fish protein (Hoyle & Merritt, 1994; Siddegowda *et al*., 2020).

Marine-derived bioactive compounds have numerous applications in nutraceuticals and pharmaceuticals due to their health benefits and therapeutic values. Many studies have reviewed the antihypertensive, antagonist, antithrombotic, antioxidant, anticancer, and antimicrobial properties of bioactive compounds in functional foods (Atef & Ojagh, 2017). Najafian and Babji, (2019) studied the potential application of novel antioxidant peptides purified from the fermented fish sauce (*Budu*). The *in vitro* antioxidant properties of sauce prepared from rohu by enzymatic and fermentation methods were stated in Siddegowda *et al*., (2020). Against this background, the objective of the present study was to compare biochemical and bioactive compounds in sardine sauce produced from the enzymatic and fermentative method using optimized conditions.

Materials and Methods

Materials

Sardine (*Sardinella longiceps*) was collected from the local fish market (Mysuru, 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 the salt fermented fish hydrolysate. The protease used for the enzymatic hydrolysis is papain (Loba Chemie Pvt. Ltd.), 2,2`-diphenyl-1-picrylhydrazyl (DPPH), 2,2`- azinobis-3-ethyl-benzothizoline-6sulphonate (ABTS), peroxidase was purchased from Sigma-Aldrich Chemie (Steinheim, Germany). All the other chemicals used in the different analyses were of analytical grade unless otherwise mentioned.

Methods

Preparation of Sardine Sauce

The schematic flow of preparation of sardine sauce is presented in Fig. 1. Briefly, eviscerated sardine (*S. longiceps*) was sliced into small pieces of 2×1 cm size and washed in potable water 3 times. The sliced sardine 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 sardine sauce. This mixture was incubated for 24 h at 37 °C and the 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 every 15- 30 days till 6 months and further filtered using Whatman no. 1 filter paper. The resulted liquid was considered sardine sauce. The fish sauce was lyophilized and used for *in vitro* antioxidant activity.

Biochemical Parameters

The biochemical parameters of the fermented sardine sauce samples were measured using the following methods. pH measurements were accomplished by directly immersing the combined glass calomel electrode into the sample using a pH meter (Cyberscan 1000, Eutech, Singapore). Sauce samples were analyzed for non-enzymatic browning by measuring melanoidin pigment formation using the method of Hendel, *et al*. (1950). 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 trichloroaceticacid (TCA), followed by analysis by the Kjeldahl method. The degree of hydrolysis (DH) of the fermented fish sauce was estimated as per the methodology described by Hoyle and Merritt (1994). Briefly, the degree of hydrolysis was computed as % DH = (10% TCA soluble N_2 in the sample) / (Total N_2 in the sample) x 100. Titratable acidity (TA) was determined as per the method described in Sachindra *et al*. (2007) by determining the ml of 0.1 M NaOH required for increasing the pH of one mL of fermented sauce to 8.0. The total volatile base nitrogen (TVB-N) content of the fermented sardine sauce samples was measured using the Conway microdiffusion assay according to the method of Conway and Byrne (1936).

Antioxidant Activities

Fish sauce samples were dissolved (50 mg/mL) in double distilled water and homogenized at 10000 × g for 2 min- using a homogenizer (Polytron PT 3100) followed by centrifugation at 7000 × g for 15 min. The supernatant was collected and filtered through Whatman No. 1 filter paper and protein content in the filtrate was estimated by the method of Lowry *et al*. (1951). This filtrate was used for assaying various antioxidant activities. The total antioxidant activity (TAO) of the fermented fish sauce sample was determined according to the method of Prieto *et al.* (1999). The absorbance of all the sample mixtures was measured at 695 nm and TAO was expressed as ascorbic acid equivalents in micrograms per gram of sample. The DPPH radical scavenging capacity of fish sauce samples was determined by the method described in Bijinu *et al*. (2011). The scavenging activity (%) was determined by measuring the absorbance of samples at 517 nm and calculated using the formula: Scavenging activity (%) = $[1-\{(A_{sample} - A_{sample\ balnk}) / A_{control}\}] \times 100$. The superoxide anion scavenging activity of the samples was determined by the method as described in Heo *et al*. (2005). The absorbance was measured at 325 nm from 0 min and 10 min. ABTS radical scavenging activity of the samples was carried out as explained in Sachindra and Bhaskar (2008). Scavenging activity was calculated as follows: Scavenging activity (%) = $[1-\{(A_{sample} - A_{sample\ balnk}) / A_{control}\}] \times 100$. Reducing potential of the fermented fish sauce samples was assayed by the method followed by Bijinu *et al*. (2011) and absorbance was measured at different concentrations (25, 50, 75, and 100 µL). The absorbance of all the samples was measured at 700 nm using distilled water as blank.

Fatty Acid Analysis

Total lipids of the fermented sardine sauce were extracted by the 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). Briefly, lipid samples were transmethylated using 2 M methanolic NaOH followed by 2 M methanolic HCl to obtain FAME. FAME was analyzed using a gas chromatography-mass spectrometer (GCMS; Shimadzu QP2010 quadruple MS, M/s Shimadzu, Kyoto, Japan) equipped with a Carbowax (30 m x 0.25 mm ID; 0.25 µm film thickness) capillary column (M/s Cromlab, USA). Helium was used as the carrier gas. The injector and detector temperature were

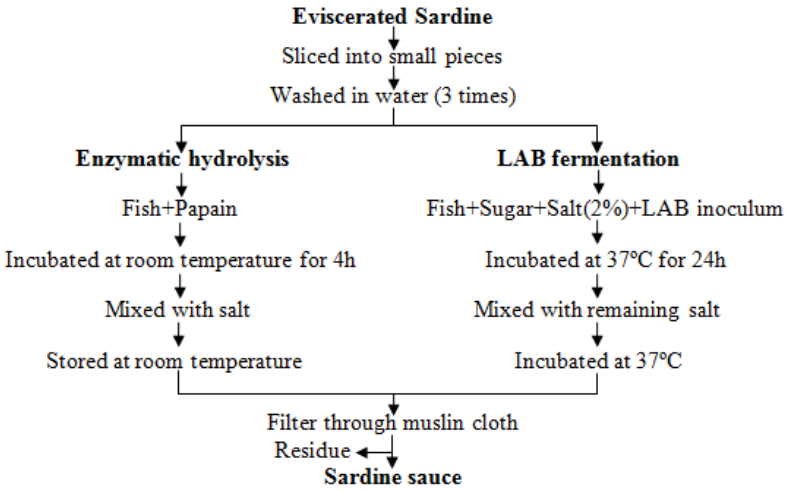

Figure 1 Schematic flow of sauce production from sardine using papain and *P. pentosaceus* FSBP4-40.

set at 250°C. The sample injection was performed in split mode (1:15). The column temperature was programmed initially at 50°C for 2 min and then ramped at a rate of 10°C per min to a final temperature of 230°C. FAME was separated at constant pressure (23.1 kpa) and peaks were identified by comparing standard mass spectra with the relative abundances of m/z ranging from 40.00 to 550. The values of fatty acids are presented in the area percentage of total identified fatty acids.

Results

Biochemical Properties of Fermented Sardine Sauce

The sauce treated with LAB showed decreased pH during fermentation, from the original 6.10±0.1 to 4.55±0.1 whereas, in the control, the pH value was increased to 5.85±0.1 from the initial 5.55±0.1. There was minor change in the pH of the enzymatically produced sauce throughout the storage period. This was insignificant in comparison with the fermentatively produced sauce (Table 1). The decrease in pH may be due to the production of acids by LAB and other autochthonous organisms. Greater browning was found in *P. pentosaceus* FSBP4-40 treated samples compared to enzyme-treated and control. It was noticed that the enzyme-treated and control samples showed almost the same non-enzymatic browning property towards the end of storage (Table 1). As shown in Table 1, the non-protein nitrogen (NPN) content of papain and LAB-treated samples increased significantly from the original 0.963±0.02 g/100 g and 1.562±0.02 g/100 g to 3.488±0.01 g/100 g and 2.282±0.01 g/100 g, respectively after 180 d of fermentation. Comparatively, a reduced amount of NPN was reported in the fermentatively produced sauce than enzyme-treated sauce.

Bioactive Components of Fermented Sardine Sauce

The degree of hydrolysis (DH) analyzed during the fermentation is shown in Figure 2A. DH of papain and LAB treated samples were found to be 12.92% and 9.98% after 180 days of storage from the original 2.21% and 5.53%, respectively. There was a 3-fold and 2-fold increase in DH of enzyme and LAB-treated sauces, respectively compared to control at the end of storage. Fig. 2B presented the changes in the total acid content of fermented sardine sauce samples. The total acid content of LAB inoculated sauce showed an almost 3-fold increase (1.40 g/100 mL) after 180 days of storage compared to the control (0.50 g/100 mL). The acid content of the papain-treated sample was 1.10 g/100 mL towards the end of storage.

The increase in TVB-N values of both the treated samples was noticed till 90 days. Later, the values were become decreased towards the end of storage. TVB-N content of fermentatively produced sauce was higher (76.451 mg/100 g) than the enzymatically produced sauce (53.289 mg/100 g) and control (58.780 mg/100 g) after 180 days (Fig. 2C). The changes in total soluble nitrogen (TSN) content of the sauce samples during fermentation are presented in Fig. 2D. Higher content of TSN was observed in the papain-treated sample (3.30 g/100 g) than in the LAB-treated sample (2.14 g/100 g).

Antioxidant Properties of Fermented Sardine Sauce

In vitro antioxidant properties such as TAO activity, DPPH, superoxide anion and ABTS radical scavenging activities of lyophilized sardine sauce are presented in Figure 3A-D. Sauce samples inoculated with *P. pentosaceus* FSBP4-40 had the better antioxidant property for all the above parameters compared to the papain-treated samples. Over 10-fold and 3-fold increase in TAO activity of LAB and enzyme-treated sauce samples, respectively in comparison to control at the end storage. TAO activity (Eq to ascorbic acid, µg/ml) for enzyme and LAB-treated sauce samples were increased to 952±70 and 3976±90 from their initial values 633±03 and 1975±12, respectively towards the end of 180 days (Fig. 3A).

The sauce produced by the fermentative method exhibited higher DPPH radical scavenging activity as compared to the enzyme-treated and control after 180 days of storage (Fig. 3B). There was significant increase in DPPH radicals scavenging activity of LAB and papain treated sauce samples from 17.93±0.8% and 18.99±0.9% to 31.46±0.5% and 53.11±0.5%,

Table 1. Changes in pH, non-enzymatic browning and NPN content of papain and LAB treated sardine sauce during fermentation

	pH			Non-enzymatic browning			NPN (g/100g)		
Day	C	PT	LT	C	PT	LT	C	PT	LT
1	5.55±0.1^{c}	5.60±0.0^{d}	6.10±0.1^{c}	0.050±0.00^{bc}	0.049±0.00^{a}	0.042±0.00^{a}	0.597±0.01^{a}	0.963±0.02^{a}	1.562±0.02^{a}
15	5.40±0.1^{bc}	5.70±0.0^{e}	4.70±0.0^{b}	0.051±0.00^{a}	0.058±0.00^{b}	0.062±0.00^{b}	0.918±0.01^{b}	1.676±0.01^{b}	1.814±0.01^{b}
30	5.35±0.1^{ab}	5.55±0.1^{cd}	4.60±0.1^{ab}	0.051±0.00^{bc}	0.073±0.00^{c}	0.072±0.00^{a}	0.983±0.02^{c}	1.903±0.01^{c}	1.901±0.03^{c}
45	5.30±0.1^{ab}	5.40±0.0^{a}	4.60±0.0^{ab}	0.049±0.00^{b}	0.091±0.00^{d}	0.087±0.00^{c}	1.148±0.01^{d}	2.220±0.03^{d}	1.915±0.01^{cd}
60	5.40±0.0^{bc}	5.50±0.0^{bc}	4.55±0.1^{ab}	0.055±0.00^{c}	0.076±0.00^{c}	0.115±0.00^{d}	1.230±0.02^{e}	2.653±0.01^{e}	1.959±0.01^{d}
90	5.20±0.1^{a}	5.40±0.0^{ab}	4.45±0.1^{ab}	0.073±0.00^{d}	0.094±0.00^{de}	0.181±0.00^{e}	1.370±0.01^{f}	2.241±0.02^{d}	1.887±0.02^{c}
120	5.35±0.1^{ab}	5.50±0.0^{bc}	4.50±0.0^{ab}	0.084±0.00^{e}	0.100±0.00^{e}	0.249±0.01^{f}	1.354±0.01^{f}	2.782±0.01^{f}	2.201±0.03^{e}
150	5.55±0.1^{c}	5.55±0.1^{cd}	4.40±0.1^{a}	0.087±0.00^{e}	0.125±0.00^{f}	0.293±0.00^{g}	1.369±0.01^{f}	3.230±0.02^{g}	2.215±0.03^{ef}
180	5.85±0.1^{d}	5.55±0.1^{cde}	4.55±0.1^{ab}	0.134±0.01^{f}	0.127±0.01^{f}	0.327±0.01^{h}	1.463±0.01^{g}	3.488±0.01^{h}	2.282±0.01^{f}

C-control, PT-papain treated, and LT-LAB treated. Values in column are mean±SD,
Different superscripts, column-wise, indicate statistically significant differences (P<0.05).

respectively. The superoxide radicals scavenging activity of the LAB-treated (67.7±0.5%) sample was higher than the papain-treated (61.8±1.4%) sample towards the end of the fermentation period (Fig. 3C).

Almost maximum ABTS radical scavenging activity was exhibited by LAB-treated sample towards the end of 180 d of fermentation (Fig. 3D). The ABTS scavenging activity of papain-treated and untreated samples were 76.5±1.0% and 62.1±2.1, respectively whereas, the bacteria fermented sauce showed 99.6±0.3%. The ferric chloride reducing power of both treated and untreated samples is given in Table 2. Higher ferric chloride reducing power was noticed in *P. pentosaceus* FSBP4-40 treated sauce sample than in the control and enzyme-treated samples. Over 6-fold increase in ferric chloride reducing power was observed in LAB fermented sauce than the control at all different concentrations of samples towards the end of 180 days. A significant difference in the ferric chloride reducing power was observed among the papain and bacteria treated samples. Overall, the antioxidant activities of LAB (*P.*

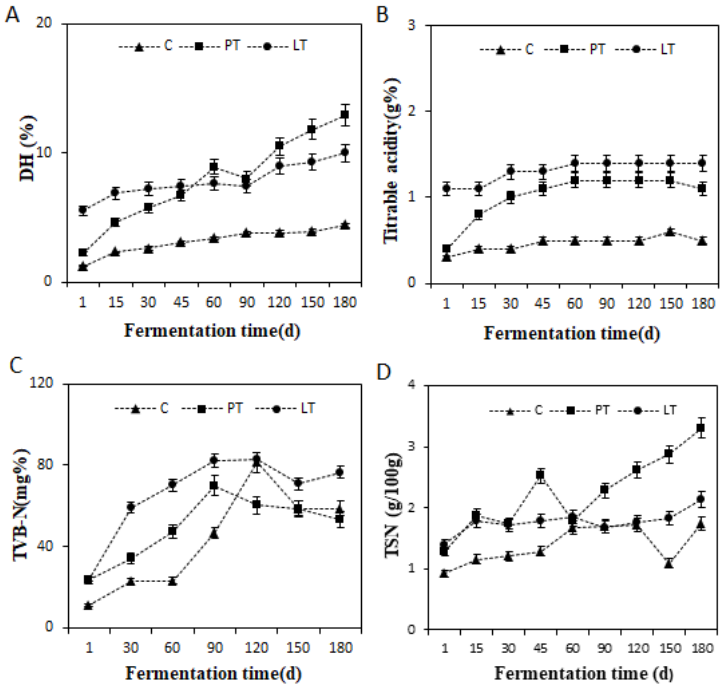

Figure 2. Degree of hydrolysis (A), Titrable acidity (B), TVB-N (C) and TSN (D) of papain and LAB treated sardine sauce during fermentation

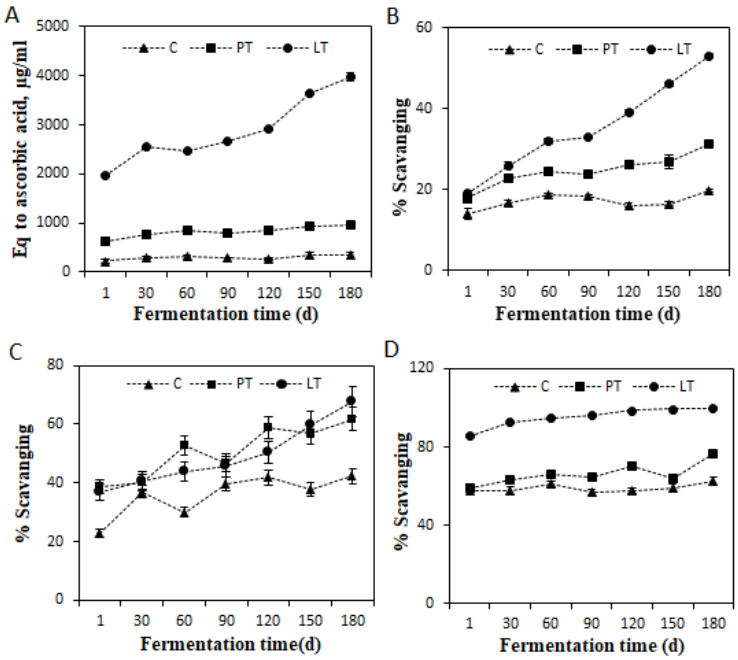

Figure 3. Total antioxidant activity (A), DPPH (B), Superoxide (C) and ABTS (D) scavenging activities of papain and LAB treated sardine sauce during fermentation

pentosaceus FSBP4-40) treated samples were higher compared to papain treated samples.

Fatty Acid Profile of Fermented Sardine Sauce

The fatty acid profile of papain-treated and LAB fermented fish sauces presented in Table 3. During the storage for 180 days, the concentration of saturated fatty acids (SFA) in the enzymatically produced sauce sample was enhanced close to 8% whereas, in fermentatively produced sauce the concentration was reduced more than 11%. There was slight decline in the unsaturated fatty acid (USFA) content of treated samples towards the end of storage. Amongst saturated fatty acids, palmitic acid (C16:0) was found to be dominant in both papain and LAB treated samples. No significant difference was noticed in the concentration of myristic acid (C14:0) and palmitoleic acid (C16:1), though they are next to the concentration of palmitic acid among the treated samples. Evident increase in arachidonic acid (C20:4n6) content of the treated samples towards the end of storage. Among the monoenoic fatty acid, oleic acid (C18:1n-9c) content of enzyme and bacteria-treated samples were 7.07±0.03% and 5.41±0.00%, respectively. Eicosapentanoic acid (C20:5n3) content of papain-treated sauce was gradually increased to 8.07±0.02% from the initial concentration of 6.65±0.01%. In contrast to that the EPA concentration of LAB-treated sauce was decreased to 0.30±0.00% from 4.98±0.01% during fermentation.

Discussion

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). Udomsil *et al*. (2015) reported that the pH values for the fish sauce prepared by adding *Staphylococcus* sp. CMC5-3-1 and CMS5-7-5 were 5.38 and 5.36, respectively. The pH values were well suitable for the sourness of the fish sauce. The result of the study is almost the same as previous studies reported by the researchers (Mohamed *et al*., 2012: Choi *et al*., 2018). Mohamed *et al*. (2012) reported that Budu, the most popular fish sauce in Malaysia has a pH range from 4.50 to 4.92, and Myeolchi Aekjeot, a fermented anchovy sauce with a pH of 4.47 to 4.89 (Choi *et al*. 2018) during fermentation. Le *et al*. (2015) stated that the amino acids accumulated during the hydrolysis of proteins are responsible for the decrease in pH of the fermented sauce. The result of the present study was in correlated with the findings of Klomklao *et al*., (2006) and Yongsawatdigul *et al*. (2007). An increase in non-enzymatic browning was observed in the samples throughout the storage period. The browning of sauce is effected by certain factors such as salt concentration, storage time, and hydrolysis of fish proteins. The Maillard browning reaction contributes brown color of fish sauce yu-lu (Lopetcharat *et al*., 2001). Peptides and amino acid release during proteolysis served as substrates for Maillard browning reaction (Yongsawatdigul *et al*., 2007). According to Klomklao *et al*. (2006), the increase in browning depends on the concentration of salt, the highest browning was observed in fish sauce produced with a low salt concentration. NPN level increased after 12 h of fermentation from the original 0.06 g/100 g to 0.69 g/100 g after 48 h of fermentation in fermented surimi with *Actinomucor elegans* XH-22 as a starter (Zhou *et al*., 2014).

The greater DH in papain-treated samples is due to the potential proteolytic activity of commercial papain. The increase in DH of the LAB fermented sample compared to the control may be due to the acid-producing ability and proteolytic activity of *P. pentosaceus* FSBP4-40 employed in the fermentation. DH was following the findings of Siddegowda *et al*. (2020). The researchers reported that, the enzyme-treated and untreated rohu (*Labeo rohita*) sauce were 24.01% and 4.87% respectively whereas, the fermentatively produced sauce showed 8.47%, towards the end of storage. An increase in the total acid content of LAB inoculated sauce is probably due to the hydrolyzed proteins in the form of amino acids and the acidic metabolites such as lactic acid produced during the fermentation. Xu *et al*. (2008) found 1.21 g/100 mL to 1.39 g/100 mL of total titratable acid content in the fast fermented, low salt fish sauce prepared from squid processing by-products. Higher TVB-N values were noticed in the sauces produced fermentatively than the enzymatic method towards the end of storage. This might be due to the difference in the production process and the frequency of lipolysis and proteolysis during fermentation. The measurement of TVB-N indicates the degree of protein degradation in samples by spoilage

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

	C				PT				LT			
DAY	25µl	50µl	75µl	100µl	25µl	50µl	75µl	100µl	25µl	50µl	75µl	100µl
1	0.05±0.0^{a}	0.10±0.0^{abc}	0.15±0.0^{ab}	0.20±0.0^{b}	0.06±0.0^{a}	0.11±0.0^{a}	0.18±0.0^{a}	0.21±0.0^{a}	0.33±0.0^{a}	0.58±0.0^{a}	0.90±0.0^{a}	1.18±0.0^{a}
30	0.06±0.0^{a}	0.12±0.0^{cd}	0.15±0.0^{ab}	0.22±0.0^{b}	0.12±0.0^{b}	0.15±0.0^{b}	0.23±0.0^{b}	0.31±0.0^{b}	0.44±0.0^{b}	0.69±0.0^{b}	1.03±0.0^{b}	1.39±0.0^{c}
60	0.07±0.0^{a}	0.13±0.0^{d}	0.17±0.0^{c}	0.22±0.0^{b}	0.12±0.0^{b}	0.19±0.0^{c}	0.28±0.0^{c}	0.37±0.0^{c}	0.52±0.0^{c}	0.74±0.0^{c}	1.16±0.0^{d}	1.49±0.0^{e}
90	0.06±0.0^{a}	0.09±0.0^{a}	0.15±0.0^{b}	0.21±0.0^{b}	0.05±0.0^{a}	0.18±0.0^{c}	0.25±0.0^{b}	0.33±0.0^{bc}	0.62±0.0^{d}	0.86±0.0^{d}	1.08±0.0^{c}	1.28±0.0^{b}
120	0.05±0.0^{a}	0.11±0.0^{bc}	0.14±0.0^{ab}	0.17±0.0^{a}	0.11±0.0^{b}	0.23±0.0^{d}	0.31±0.0^{d}	0.45±0.0^{de}	0.78±0.0^{f}	0.92±0.0^{e}	1.16±0.0^{d}	1.45±0.0^{d}
150	0.05±0.0^{a}	0.10±0.0^{ab}	0.13±0.0^{a}	0.17±0.0^{a}	0.12±0.0^{b}	0.23±0.0^{d}	0.34±0.0^{e}	0.42±0.0^{d}	0.91±0.0^{f}	1.17±0.0^{f}	1.38±0.0^{e}	1.58±0.0^{f}
180	0.07±0.0^{a}	0.14±0.0^{d}	0.18±0.0^{c}	0.25±0.0^{b}	0.15±0.0^{c}	0.35±0.0^{e}	0.39±0.0^{f}	0.47±0.0^{e}	0.98±0.0^{g}	1.24±0.0^{g}	1.44±0.0^{f}	1.67±0.0^{g}

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

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

bacteria, autolytic enzymes, deamination, and nucleotide catabolites (FDA 2004). The TVB-N values were within the acceptable range (14.1-338.6 mg/100 ml) which were found in most Southeast Asian fish sauces (Cho, *et al*., 2000). The fish sauce produced from Gambusia (*Affinis affinis*) using 25% (w/w) at room temperature for five months showed a TVB-N value of 107.80±3.95 mg/100 mL (Ibrahim, 2010). The TVB-N content of the fish sauce samples prepared from squid processing by-products was in the range of 100- 160 mg/100 g (Xu *et al*., 2008). In contrast, a high level (309.8 mg/100 g) of TVB-N was reported in mahyaveh, a traditional Iranian fish sauce (Zarei *et al*. 2012).

The increase of TSN content during the processing of fish sauce could be attributed to the combined effect of autolysis, enzyme activity, and microbial degradation of the fish muscle. Xu *et al*. (2008) stated that, the increase in TSN content in the fish sauce is due to the hydrolysis of fish proteins during fermentation. The study revealed that, the TSN of fish sauce prepared from squid processing by-products by three different manufacturing techniques is in the range of 1.958 to 2.135 g/100 ml. Udomsil *et al*. (2015) reported that the total nitrogen content (1.89 g/100 g**)** in the fish sauce inoculated with *Staphylococcus* sp. CMS5-7-5 and incubated at 35 °C for 180 days. 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 days. Total nitrogen content was in the range of 1.176 to 1.316 g/100 g in the fish sauce prepared using low salt, solid state fermentation with anchovy by-products (Yu *et al*., 2014).

In comparison with enzymatically produced sauces, the LAB-fermented sauces were exhibited dominant antioxidant activity. This could be due to the biopeptides produced during the hydrolysis of fish proteins as well as the bioactive metabolites secreted by bacteria in the fermentation. According to Siddegowda *et al*. (2020), the fermented rohu sauce exhibited higher TAO (ascorbic acid equivalents in μg g^{-1}) activity of 3441±60 than the enzyme-treated sauce (803±13) towards the end of storage. 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%). Peralta *et al*. (2008) reported that the Philippine salt fermented shrimp paste showed 24.3- 61.5% of DPPH radical scavenging activity in 80% ethanolic extract. 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-products, a solid waste generated in the fish sauce industry contains natural protein hydrolysates produced from the digestion of fish proteins using various proteases and halophiles in the fermentation (Choksawangkarn *et al*., 2018). The study evidenced that the fish sauce by-product contained a high amount of low molecular weight proteins/peptides and had the highest DPPH radical scavenging activity.

The studies have shown that uncontrolled free radicals including hydroxyl radicals, peroxyl radicals, 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals and

Table 3. Changes in fatty acid profile of sardine sauce during fermentation

	C			PT			LT		
Name of fatty acid methyl ester	DAY-1	DAY-90	DAY-180	DAY-1	DAY-90	DAY-180	DAY-1	DAY-90	DAY-180
Lauric acid (C12:0)	1.32±0.00^{b}	2.18±0.00^{c}	1.21±0.00^{a}	0.26±0.00^{a}	0.81±0.00^{c}	0.37±0.00^{b}	2.49±0.00^{a}	2.21±0.00^{b}	1.41±0.00^{a}
Tridecanoic acid (C13:0)	0.81±0.01^{b}	0.23±0.01^{a}	0.85±0.00^{c}	0.36±0.01^{b}	0.15±0.00^{a}	0.39±0.01^{c}	0.38±0.00^{a}	0.95±0.01^{b}	0.97±0.01^{c}
Myristic acid (C14:0)	11.59±0.01^{b}	12.94±0.01^{c}	10.28±0.00^{a}	11.51±0.01^{b}	14.22±0.00^{c}	10.49±0.01^{a}	15.99±0.00^{c}	11.44±0.01^{a}	11.81±0.01^{b}
Myristoleic acid (C14:1)	2.12±0.01^{b}	1.41±0.01^{a}	2.66±0.00^{c}	0.87±0.01^{c}	0.82±0.01^{b}	0.66±0.01^{a}	1.57±0.01^{a}	2.22±0.01^{b}	2.37±0.00^{c}
Pentadecanoic acid (C15:0)	0.59±0.01^{b}	0.66±0.01^{c}	0.54±0.00^{a}	0.69±0.00^{b}	0.69±0.01^{b}	0.65±0.01^{a}	1.06±0.01^{b}	0.65±0.01^{a}	0.64±0.00^{a}
Palmitic acid (C16:0)	22.12±0.02^{b}	25.83±0.01^{c}	19.89±0.01^{a}	25.31±0.01^{c}	24.46±0.01^{b}	22.11±0.01^{a}	28.20±0.01^{c}	20.32±0.02^{a}	20.45±0.01^{b}
Palmitoleic acid (C16:1)	10.71±0.01^{b}	12.41±0.01^{c}	10.59±0.01^{a}	12.46±0.01^{c}	12.02±0.02^{b}	11.13±0.00^{a}	14.28±0.01^{c}	10.93±0.01^{a}	11.68±0.01^{b}
Hepta decanoic acid (C17:0)	2.42±0.01^{c}	1.06±0.01^{a}	1.34±0.01^{b}	1.66±0.01^{a}	2.06±0.01^{b}	2.06±0.01^{b}	1.60±0.01^{c}	1.06±0.01^{a}	1.18±0.00^{b}
Cis-10 Heptadecanoic acid (C17:1)	0.64±0.01^{c}	0.26±0.01^{b}	0.15±0.00^{a}	0.36±0.01^{c}	0.26±0.00^{b}	0.14±0.01^{a}	1.51±0.01^{c}	0.36±0.01^{b}	0.28±0.00^{a}
Stearic acid (C18:0)	4.37±0.01^{a}	5.93±0.01^{b}	4.37±0.01^{a}	0.38±0.01^{a}	5.61±0.01^{c}	4.96±0.01^{b}	5.62±0.01^{c}	5.20±0.01^{b}	4.89±0.01^{a}
Oleic acid (C18:1n-9c)	6.11±0.01^{a}	8.57±0.01^{c}	6.44±0.01^{b}	8.73±0.01^{c}	8.26±0.01^{b}	7.07±0.03^{a}	7.76±0.01^{c}	5.67±0.00^{b}	5.41±0.00^{a}
Elaidic acid (C18:1n9t)	3.67±0.01^{a}	4.45±0.01^{c}	4.08±0.00^{b}	4.74±0.00^{c}	4.07±0.00^{b}	3.63±0.01^{a}	4.68±0.00^{c}	4.50±0.01^{b}	3.70±0.01^{a}
Linoleic acid (C18:2n-6c)	0.23±0.01^{a}	0.63±0.01^{b}	0.65±0.00^{c}	0.85±0.01^{b}	1.06±0.00^{c}	0.80±0.01^{a}	0.16±0.00^{a}	0.85±0.01^{b}	0.85±0.01^{b}
Lenolelaidic acid (C18:2n-6t)	0.16±0.01^{a}	0.31±0.01^{b}	0.33±0.01^{b}	0.25±0.01^{a}	0.49±0.00^{c}	0.35±0.01^{b}	0.30±0.01^{b}	0.16±0.00^{a}	0.30±0.01^{b}
γ- Linolenic acid (C18:3n6)	0.25±0.01^{a}	0.33±0.01^{b}	0.25±0.01^{a}	0.18±0.00^{a}	0.47±0.00^{c}	0.41±0.01^{b}	0.25±0.01^{a}	0.58±0.00^{c}	0.30±0.00^{b}
α- Linolenic acid (C18:3n3)	0.29±0.01^{b}	0.16±0.00^{a}	0.70±0.01^{c}	0.79±0.01^{a}	0.99±0.01^{b}	0.98±0.01^{b}	0.39±0.01^{a}	0.65±0.01^{c}	0.54±0.01^{b}
Arachidic acid (C20:0)	0.24±0.01^{a}	0.63±0.00^{b}	0.89±0.01^{c}	0.76±0.01^{c}	0.55±0.01^{b}	0.48±0.01^{a}	0.21±0.00^{a}	0.72±0.01^{c}	0.38±0.01^{b}
Eicosatrienoic acid (C20:3n6)	0.25±0.01^{a}	0.73±0.01^{b}	1.17±0.00^{c}	0.16±0.00^{a}	1.50±0.00^{b}	1.57±0.01^{c}	0.29±0.01^{a}	1.51±0.01^{c}	1.27±0.01^{b}
Arachidonic acid (C20:4n6)	0.76±0.01^{c}	0.26±0.01^{b}	0.19±0.00^{a}	0.26±0.01^{b}	0.16±0.00^{a}	2.31±0.01^{c}	0.21±0.01^{a}	0.24±0.01^{a}	5.21±0.01^{b}
Eicosapentanoic acid (C20:5n3)	1.69±0.01^{a}	3.06±0.01^{b}	5.67±0.01^{c}	6.65±0.01^{a}	7.41±0.01^{b}	8.07±0.02^{c}	4.98±0.01^{b}	5.21±0.01^{c}	0.30±0.00^{a}
Behenic acid (C22:0)	0.35±0.01^{b}	0.12±0.00^{a}	0.66±0.01^{c}	0.17±0.00^{a}	0.31±0.01^{c}	0.25±0.02^{b}	0.18±0.00^{a}	0.77±0.01^{c}	0.26±0.00^{b}
Trichosonoic acid (C23:0)	1.50±0.01^{a}	1.53±0.01^{a}	2.59±0.01^{b}	0.93±0.01^{a}	3.52±0.01^{b}	5.15±0.01^{c}	0.26±0.00^{a}	4.27±0.01^{c}	2.59±0.01^{b}
Lignoceric acid (C24:0)	0.23±0.01^{c}	0.18±0.00^{b}	0.14±0.00^{a}	0.34±0.01^{b}	0.23±0.01^{a}	3.43±0.01^{c}	0.38±0.01^{c}	0.26±0.01^{b}	0.18±0.00^{a}
Nervonic acid (C24:1n9)	0.85±0.01^{c}	0.26±0.00^{a}	0.31±0.01^{b}	3.40±0.02^{c}	0.25±0.00^{a}	0.37±0.01^{b}	0.15±0.00^{a}	0.29±0.01^{b}	0.15±0.00^{a}
SFA	45.49±0.08^{b}	51.28±0.04^{c}	42.74±0.01^{a}	42.34±0.06^{a}	52.59±0.02^{c}	50.31±0.04^{b}	56.35±0.01^{c}	47.81±0.06^{b}	44.72±0.04^{a}
USFA	27.68±0.10^{a}	32.80±0.02^{b}	33.17±0.01^{c}	39.66±0.02^{c}	37.72±0.06^{b}	37.45±0.01^{a}	36.49±0.00^{c}	33.13±0.04^{b}	32.33±0.03^{a}

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

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

superoxide anion radicals can attack various tissue components leading to autoimmune diseases, neurodegenerative disorders and cancers (Kehrer & Klotz, 2015). In contrast, the superoxide anion radicals scavenging activity of enzyme-treated rohu sauce was slightly higher (68.40±1.0%) than the bacteria treated (66.50±0.8%) sample towards the end of the storage period of 180 days (Siddegowda *et al*., 2020). Fermented fish products could be used as a potential source of nutrients and natural antioxidants (Majumdar *et al*., 2016). The proteins present in the raw material (sardine) are hydrolyzed into peptides and amino acids during fermentation. These peptides 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 antioxidant properties based on their structure and other factors, including hydrophobicity, charge, or microelement binding properties (Siddegowda *et al*., 2016).

According to Han *et al.,* (2022), the complex metabolic reactions during the fermentation of fish sauce through the diversified group of microorganisms are accompanied by the formation of flavor substances. The presence of arachidonic acid indicates the nutritional significance of fermented fish products (Majumdar *et al*., 2015). Decrease in the level of omega-3 fatty acid namely eicosapentaenoic acid and docosahexaenoic acid in sardine sauces produced with added pineapple fruit extracts (Mahrus *et al*., 2020). The fatty acid profile of the sardine sauce samples is correlated with the results of Siddegowda *et al* (2016). The researchers observed a similar trend in the rohu head sauce produced by the enzymatic and fermentation method. The study suggested that, the lipolysis play a major role in flavour formation during ripening of the sauce. The changes in the concentration of saturated and unsaturated fatty acid depend on the raw material used and the method of treatment employed during the production of fish sauce.

Conclusion

The study emphasized that an accelerated fermentation of sardine into the fish sauce with acceptable biochemical and biofunctional qualities in terms of nutrition using papain and halotolerant, proteolytic lactic acid bacteria. The biochemical components such as degree of hydrolysis, titratable acidity and total soluble nitrogen were significantly higher in treated sauce samples compared to control. *In vitro* antioxidant activities of *P. pentosaceus* FSBP4-40 fermented sauce exhibited superiority over enzyme-treated sauce samples. Eicosapentanoic acid (C20:5n3) content of papain-treated sauce was gradually increased during storage. The study suggested that fermentative conversion of the sardine into the sauce using optimized conditions is one of the effective methods for the preservation of economically underutilized fish. The developed product should also apply as a flavoring condiment in a wide variety of sea-foods. Future research studies are needed to investigate the functionalities of different bioactive components from sardine sauce and their biological effects *in vivo*.

Ethical Statement

Not applicable

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Author Contribution

First Author: Data collation, investigation, and writing the manuscript; Second Author: Supervision, resources, designed the experiments, analysed all data of the study and Third Author: Supervision, Writing-review and editing.

Conflict of Interest

We declare that we have no conflict of interest whatsoever in any form. All the co-authors are aware of its submission to Turkish Journal of Fisheries and Aquatic Sciences.

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