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Research Article

Properties and shelf-stability of co-dried common kilka (*Clupeonella cultriventris*) protein hydrolysate with agricultural residues as a new protein feed supplement

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Abstract

Developing aqua/ animal feeds from underutilized fish and agricultural by-products using hydrolyzed technology is an excellent way to provide proteins for aquaculture/ animal farming. This study reports development of a new protein feed supplement, based on fish protein hydrolysate and agricultural residues, on a pilot scale and investigates physicochemical properties, microbial quality, and shelf-stability of the product. The production conditions were based on lab-scale experiences. Accordingly, fish protein hydrolyzed solution containing 16.58% crude protein, 63.35% moisture, and 15.52% fat was mixed with agricultural by-products, *i.e.* sesame meal, rice bran, and soybean meal as fillers (60:40 w/w) and dried at 40°C for 6 h. The dried mixture was grounded after cooling, then packed in sealed plastic bags, and stored for 180 days at ambient temperature. The protein feed supplement contained 58.23% crude protein, 16.18% fat, 8.85% moisture, 9.10% ash, 2.6% fiber, 1.489% calcium, 0.89% phosphorous, and 5.34% carbohydrates of dry weight. Total essential and non-essential amino acids were 19.41g/ 100g and 20.36 g/ 100g of dry weight respectively. The protein feed supplement contained significant essential fatty acids. Levels of pH and TVB-N of the protein feed supplement increased to 5.30 and 65.20 mg/100g at the end of the study. After 180 days, there were no yeast and mold in the samples and it was still pathogen-free.

Keywords: Caspian sprat, Fish protein hydrolysate, Amino acids, Saturated fatty acids, Protein supplement

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Introduction

Animals, including livestock, and aquatic species, need proteins and amino acids as basic elements to grow under husbandry conditions. Therefore, providing adequate proteins is an important concern for most animal feed producers and farmers. In this regard, fishmeal and plants proteins, like soybean meal are usually added to the diet of domestic animals to provide the protein needs. However, it should be noted that availability of high-quality fishmeal is scarce and very costly (FAO, 2021a). Dependence on imported fishmeal and soybean meal for animal breeding has led many feeds producer to use local protein products, including fisheries and agricultural resources (Shaviklo and Alizadeh-Ghamsari, 2021). The new protein supplement can supply all nutritional properties of fishmeal, and soybean meal, required for farm-raised animals. In addition, its production cost is low, and there is no need to use advanced machines and consume high energy like it is seen in fishmeal production (Shaviklo *et al.*, 2021).

Production of supplements based on fish protein hydrolysate and agricultural residues is an innovative way to develop functional ingredients due to presence of bioactive substances such as peptides in the product (Mahdabi and Hosseini Shekarabi, 2018; Shaviklo and Alizadeh-Ghamsari, 2021). This technology has no sewage and odor problems that occur during production of fishmeal (FAO, 2021b). On the other hand, bioactive compounds can play a promising role in ensuring animal health (Etemadian *et al.*, 2021). In this technology, an attempt was made to combine fish protein solution with agricultural residues and it converts to a dry product (humidity below 10%) with a high protein content (more than 50%), which is higher than that of soybean meal and less than that of fishmeal (Shaviklo and Alizadeh-Ghamsari, 2021).

The Caspian sprat or common kilka (*Clupeonella cultriventris*) is an underutilized small fish in Caspian Sea that can be used mostly to develop fishmeal and oil. The hydrolyzed protein of this fish may have beneficial properties due to its free amino acids and nucleoid compounds such as functional peptides (Mahdabi and Hosseini Shekarabi, 2018; Janbakhsh *et al.*, 2018), and as a protein supplement can be very effective in animal, poultry, and aquatic feeding.

Based on documents that are published about developing aqua/ animal feed from hydrolyzed fish protein, the researchers have done their work on laboratory scale, but information about scale-up and shelf-stability of the product is missing. While production on a pilot scale and study on quality and quality changes of the product during storage can provide important information for industrial production and commercialization of such products. Therefore, the objective of this research was to develop a protein feed supplement based on kilka fish protein hydrolysate using filler-assisted drying method (60%:40%) on a pilot

scale based on information obtained from lab-scale production experiments and to investigate products' properties, and shelf stability. The results of this work would be useful for development of new protein resources for animal and aquatic feed industries, especially in countries that suffer from lack of fishmeal and plant protein sources to balance and formulate practical diets.

Materials and methods

Raw materials preparation

Kilka (*C. cultriventris*) was caught by fishing vessels from southern coast of Caspian Sea (Bandar Anzali, Guilan, Iran) using lift net and light-catching method. Three batches of fish each weighing 500 kg were immediately chilled and carried to the fish processing center within 4 h. Agricultural residues, including sesame meal and rice bran, formic acid, sodium bicarbonate, and α-Tocopherol were obtained from local market (Rasht, Guilan, Iran).

Feed supplement production

The feed supplement production conditions, including optimum hydrolyzing process, level of ingredients and drying temperature and time, etc., were based on our previous lab-scale feed product development but it was scaled up in the national fish processing research center (Bandar Anzali, Guilan, Iran). Freshly caught whole kilka fish (C. *cultriventris*) were completely minced using an industrial meat grinder (Chengdu Svoboda Machinery Equipment Co., Ltd. China). Then, for every 100 kg of minced fish, compounds such as formic acid (3%), sodium metabisulfite (0.2%), and α-Tocopherol (0.02%) were added. All ingredients were completely homogenized in an industrial blender (Alexanderwerk, MSL-1301, UK). The pH of the resulting mixture was set to 3.0-3.95. The mixture was transferred to 200 kg polyethylene reactors to carry out the hydrolysis process for 4 days at 35°C. The mixture was stirred gently three times a day during storage and its pH was measured using a pH meter (AZ Instrument Corp, 86501 AZ, Taiwan R.O.C) and set to the aforementioned levels. The material was completely liquidized on day 5 of processing. Fish oil was not separated because it can increase nutritional quality of the feed. A batch of hydrolyzed fish protein solution (HFPS) was kept in polyethylene buckets and stored for 180-day at ambient temperature (27±2°C) for shelf-stability studies.

To develop protein feed supplement (PFS), for every 100 kg of HFPS, 70 kg of fillers and other ingredients, including sesame meal (28%), rice bran (1.5%), sodium bentonite (2%) were added. The ingredients were completely mixed in an industrial blender machine (Alexanderwerk, MSL-1301, UK) in order to obtain a hemogenic paste. The paste was converted into small cylindrical shape pieces (5mm×3mm) and dried at 40°C until humidity reached below 10% using an industrial dryer equipped with air circulation and humidity regulation (Atmos Lebensmitteltechnik GmbH, REM-11,

Germany). After cooling, the PFS was stored in sealed plastic bags for 180 days at ambient temperature (27±2°C). During this time, the necessary tests were performed on HFPS and the PFS.

Degree of hydrolysis (DH)

To measure DH 5 ml of the HFPS was mixed with 5 ml of 10% trichloroacetic acid. After stirring for 5 m, it was centrifuged at a speed of 3200 rpm for 10 m. Then the amount of protein in the solution phase was assessed and the amount of hydrolysis (%) was measured using the following equation (Lowry *et al.*, 1951 cited by Mahdabi and Hosseini Shekarabi, 2018):

$$\mathrm{DH} = \frac{\text{The amount of protein soluble in trichloroacetic acid 10\%}}{\text{Total Protein in sample}} \times 100$$

Physicochemical Analysis

A pycnometer was used for measuring the density of HFPS. Initially, a 50 mL volume pycnometer was dried and weighed (m_1). Then, it was filled with distilled water at 25°C and weighed (m_2). The pycnometer was filled with HFPS at the same temperature and weighed (m_3). The volume of the liquid sample is equal to the volume of distilled water, so the obtained volume is equal to the volume of the liquid sample (Lau *et al.*, 1997). The amount of density was calculated using the following formula: ρ: density (kg/m^3); m: mass (g); v: volume (mL):

$$\rho = \frac{\mathrm{m}}{\mathrm{v}}$$

Gross energy of the PFS was determined by burning feed in an automatic bomb calorimeter. The gross energy was the heat liberated from burned feed and expressed as kilocalories per gram sample (McGill *et al.*, 2004). For measuring the pH of PFS, 5 g of sample was added to 50 ml of distilled water (Fagbenro and Jauncey, 1994). Then, the mixture was centrifuged at 8000 rpm for 10 min. The supernatant was used for pH determination and measured by a digital pH meter (AZ Instrument Corp, 86501 AZ, Taiwan R.O.C). Total crude proteins, moisture content, crude fat, crude fiber, calcium and, phosphorus were determined by AOAC methods (2006). Total carbohydrate was determined by colorimetric method of Dubois *et al.* (1956). To measure total volatile basic nitrogen (TVB-N), method of Malle and Poumeyrol (1989) based on steam distillation followed by titration was used.

Fatty acid analysis

The fatty acid composition was determined by method of van't Land *et al.* (2017). Briefly, a 10 g sample was separated and fat was extracted using a mixture of chloroform-methanol

solvents (2:1) and stored in chloroform solvent at −20°C. Then, 2 mg/ mL of a C19:0 solution was added to the extracts as an internal standard. Methylated fatty acids were extracted using hexane solvent. After re-dissolving in hexane solvent, they were stored at −20°C. Methylated fatty acids were separated and quantified using a gas chromatography-flame ionization detector (Agilent, 7820A GC System) with a 0.2 μm HP-88 column, 60 m×0.25 mm ID.

Amino acid assessment

Amino acid compounds were determined using dithioglycolic acid and hydrogen chloride acid solutions for 30 min at 150°C. 200 mg of sample was purified by an OASIS HLB cartridge. Separation and detection was done by LC–MS2 (Nexera 8040, Shimadzu) and Kinetix Hilic-column (2.6 μm, 150 ×2.1 mm) was used; 50 mM ammonium formate solution was normal phase (solvent A); Acetonitrile was a mobile phase gradient (solvent B); The solvent gradient was 85%. The solvent B gradient was increased from 15% to 70%. Electrospray ionization of amino acids was carried out with a voltage of 4.5 kV. The multiple reactions were monitored on a triple quadruple (van't Land *et al.*, 2017).

Microbial analyses

Total bacterial load, *Escherichia coli*, *Salmonella*, and yeast-mold counts were performed according to ISO/TC34/SC9 (2003) on the PFS within 180 days of storage at ambient temperature (27±2°C). The results were presented as colony-forming units (CFU) per gram sample.

Statistical analyses

NCSS (NCSS, Statistical Software, Kaysville, UT) was used to assess the variance (ANOVA) of analytical data. Principal component analysis (PCA) was used for visualization of the results. PCA plot was organized by a statistical program (Unscrambler@ V 9.7, CAMO Software AS, Oslo, Norway). Significant difference was considered at level of 5%.

Results

Characteristics of HFPS

Proximate composition results for kilka fish which was used for the protein hydrolyzation was 14.13% protein, 9.54% fat, 1.48% ash, and 73.00% moisture. As shown in Table 1, the HFPS contained 16.58% protein, 15.52% fat, 3.08% ash, and 63.35% moisture. Calcium and phosphorous of the HFPS were 0.65% and 0.59% respectively. Within 180 days of storage, the moisture content was increased and the protein, fat, and ash contents decreased significantly (p<0.05).

The HD (Fig. 1) increased over the storage time in HFPS (p< 0.05). The HFPS density on day 1 could not be measured due to viscous nature of the mixture but it was 1081.60±0.022 kg/ m^3 after 4 days of production. After this

time, density decreased until reaching 1010.12±0.017 kg/ m^3 after 180 days of storage. No significant difference (p> 0.05) in density was found for the product stored for 90 and 180 days at ambient temperature (data not shown).

The pH of HFPS was 3.55 after production which remained stable within 180 days of storage at ambient temperature (p>0.05). TVB-N value in HFPS was 35.31 mg/100g on day 1 but increased significantly (p<0.05) within 180 days of storage (Fig. 2).

PFS properties

Gross energy of the PFS was 3217.6 Kcal/kg, which was measured only on the first day of production. Proximate analysis, minerals, and pH of the PFS developed from HFPS are presented in Table 1. Protein, moisture, fat, ash, and carbohydrate of the PFS were 58.23%, 8.85%, 16.8%, 9.10%, and 5.34%, respectively. The PFS contained 1.49% calcium, 0.89% phosphorous, and 2.3% fiber. These components were not changed significantly within 180 days of storage at ambient temperature (p>0.05).

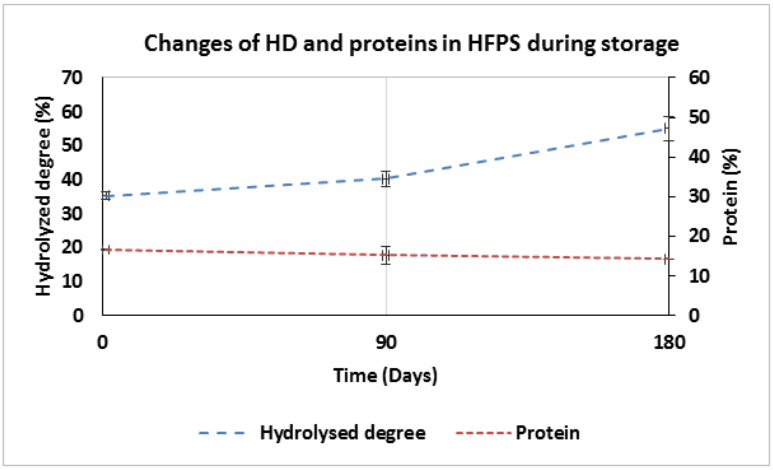

Figure 1: Changes in hydrolyzed degrees and proteins in HFPS during storage.

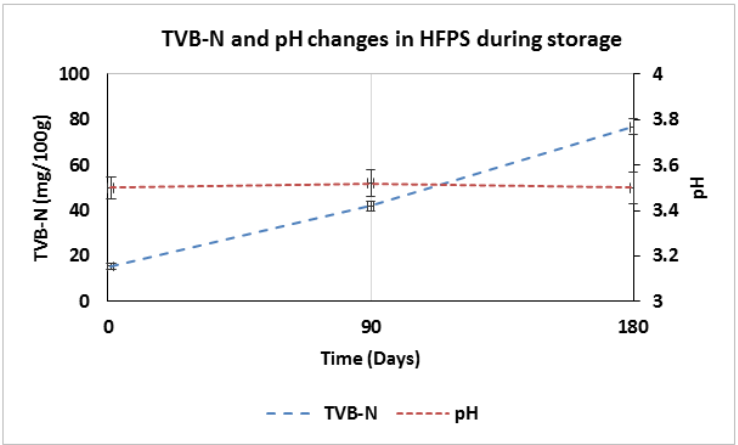

Figure 2: TVB-N and pH changes in HFPS during storage.

Table 1: Proximate compositions (%) of hydrolyzed fish protein solution (HFPS) and feed product.

Parameter	HFPS	PFS
Protein	16.58±0.01	58.23±0.03
Moisture	63.35±0.50	8.85±0.01
Ash	3.08±0.00	9.10±0.01
Fat	15.52±0.04	16.18±0.02
Fiber	-	2.30±0.01
Calcium	0.65±0.00	1.49±0.00
Phosphorus	0.59±0.00	0.89±0.00
Carbohydrate	1.47±0.02	5.34±0.04
pH	3.55±0.01	4.69±0.00

HFPS: Hydrolyzed fish protein solution; PFS: Protein feed supplement.

The pH of PFS was 4.69 which increased to 4.85 and 5.30 significantly after 90 and 180 days of storage respectively. TVB-N significantly increased in the PFS during storage time (p<0.05). TVB-N of the PFS product on days 1, 90, and 180 were respectively, 15.40±0.01, 76.30±0.21, and 95.20±0.11 mg/100g PFS (Fig. 3).

The PFS analysis results indicated significant essential fatty acids. Monounsaturated fatty acids such as oleic acid (C18:1) and polyunsaturated fatty acids such as linoleic acid (C18:2) contents were respectively 26.70±0.91 and 46.94±0.70 g/100 g fatty acid methyl ether (Table 2). Also in PFS, a set of saturated fatty acids such as myristic acid (1.83±0.01 g/100 g fatty acid methyl ether), myristoleic acid (0.31±0.00 g/100 g fatty acid methyl ether), palmitic acid (16.83±1.20 g/100 g fatty acid methyl ether), palmitoleic acid (1.50±0.01 g/100 g fatty acid methyl ether), margaric acid (0.50±0.00 g/100 g fatty acid methyl ether) and stearic acid (5.36±0.04 g/100 g fatty acid methyl ether) were found. The total content of saturated fatty acids and polyunsaturated fatty acids were 9.50% and 72.64%, respectively.

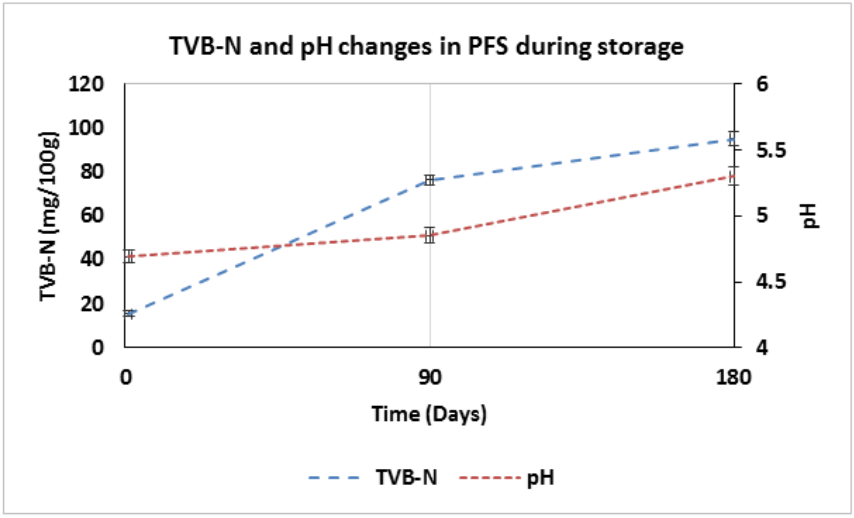

Figure 3: TVB-N and pH changes in PFS during storage.

Table 2: Fatty acid compositions of PFS.

Fatty acid compositions	(% of total fatty acids)
Myristic acid (C14:0)	1.83±0.01
Myristoleic acid (C14:1)	0.31±0.00
Palmitic acid (C16:0)	16.83±1.20
Palmitoleic acid (C16:1)	1.50±0.01
Margaric acid (C17:0)	0.50±0.00
Stearic acid (C18:0)	5.36±0.04
Oleic acid (C18:1)	26.70±0.91
Linoleic acid (C18:2)	46.94±0.70
Other	0.03±0.00
Saturated fatty acids (SFA)	9.50±1.80
Polyunsaturated fatty acids (PUFA)	72.64±2.98

PFS: Protein feed supplement.

The curve of total amino acid standard compounds is shown in Figure 4. In total 17 amino acids were detected in which glutamic acid (6.74 g/100g) was the dominant amino acid followed by aspartic acid (3.72 g/100g) and leucine (3.57g/100g). Total essential amino acids, including Histidine, Arginine, Threonine, Methionine, Valine, Phenylalanine, Isoleucine, Leucine, Lysine, and Tryptophan was 19.41 g/ 100g of dry weight, whereas total non-essential amino acids, including Aspartic acid, Glutamic acid, Serine, Glycine, Alanine, Tyrosine, and Proline was 20.39 g/ 100g of dry weight (Table 3).

Total bacterial load in - PFS increased during storage time (p<0.05). Total bacterial load increased from 0.00 log CFU/g to 3.60 log CFU/g and 4.30 log CFU/g at the day 90 and180 days storage at room temperature respectively. The PFS was free from *E. coli*, *Salmonella*, and yeast mold and remained stable (0.0 log CFU/g) within 180 days of storage at room temperature (data not shown).

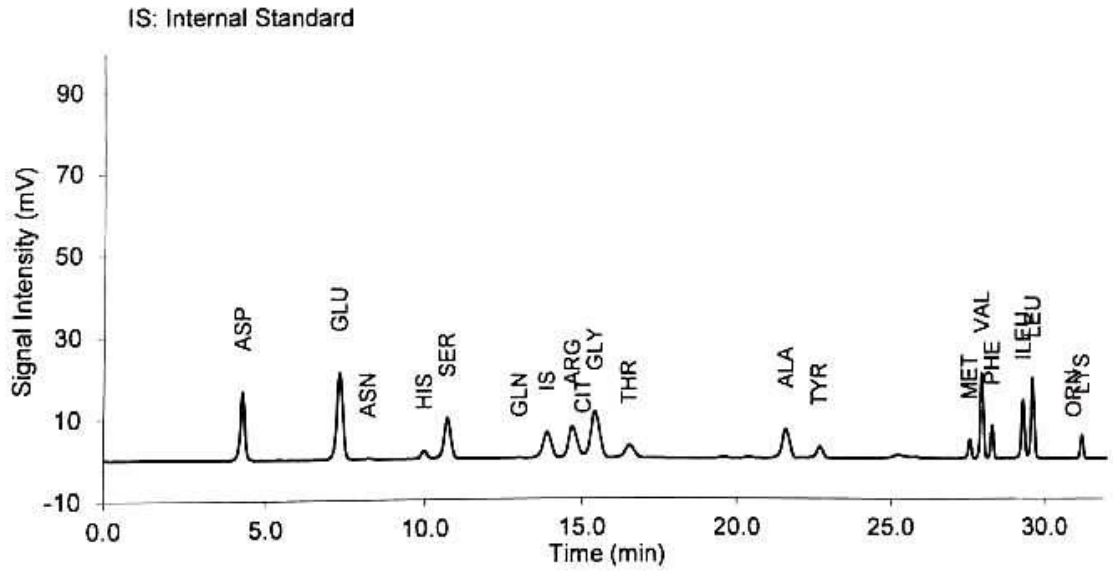

Figure 4: The curve of total standard amino acid compounds of PFS.

Table 3: Amino acid compounds contents of PFS.

Amino acid compounds	g/100g
Aspartic acid	3.72±0.001
Glutamic acid	6.74±0.001
Asparagine	0.00±0.00
Histidine	0.92±0.001
Serine	2.80±0.001
Glutamine	0.00±0.00
Arginine	3.17±0.001
Citrulline	0.00±0.00
Glycine	2.91±0.001
Threonine	1.67±0.001
Alanine	2.56±0.001
Tyrosine	1.63±0.001
Methionine	0.82±0.001
Valine	2.47±0.001
Phenylalanine	2.14±0.001
Isoleucine	1.89±0.001
Leucine	3.57±0.001
Ornithine	0.00±0.00
Lysine	2.34±0.001
Tryptophan	0.42±0.001
Proline	0.03±0.000
Total	39.80
Non-essential amino acids	20.39
Essential amino acids	19.41

PFS: Protein feed supplement.

Discussion

In acid-assisted hydrolysis, as in enzymatic hydrolysis, large peptides are broken down into smaller peptides by breaking peptide bonds around the proteins (Mahdabi and Hosseini Shekarabi, 2018; Etemadian *et al.*, 2021). The amount of formation of these small peptides in protein product to the total protein of the product is called DH (Rutherfurd, 2010). In this study, endogenous enzymes such as the enzyme-rich viscera in kilka fish along with acid are responsible for the hydrolysis process of kilka fish proteins into small peptides and small-chain amino acids (Gallardo *et al.*, 2012). DH can vary depending on temperature and time and the ratio of acid or enzyme used. It is even possible that type of fish and number of internal enzymes in its intestines increase or decrease DH. Mahdabi and Hosseini Shekarabi (2018) reported that protein source and duration of hydrolysis can affect DH. Espe and Lied (1999) reported that acid-hydrolysis degree in whole herring, whole mackerel, cod viscera, and saithe offal showed an increasing trend. They also noted that varying concentrations of the substrate or enzyme in raw materials and acids alter hydrolysis patterns.

Falch *et al.* (2006) found that the chemical composition of raw materials could be affected by several external factors, such as geographical location, gender, season, diet, fish species, age, and sexual maturity. The changes in chemical composition likely resulted in changes in DH from 35.31%-55.10%. In this study, a relatively slow increase over time was observed in DH (Fig. 1). This can be due to low temperature (Lo *et al.*, 1993; Espe and Lied, 1999) and coarseness of raw material pieces (van't Land *et al.*, 2017) at the beginning of hydrolysis. A high DH (>50%) can be beneficial and used as a supplement and an essential ingredient to enhance digestibility, absorption capability, and palatability of animal feed (Li *et al.*, 2009). On the other hand, the longer hydrolysis period contains more free amino acids, but the shorter hydrolysis period can be better nutritionally (Jackson *et al.*, 1984; Stone and Hardy, 1986; Fagbenro and Jauncey, 1993) for the animal due to having more intact proteins and short peptides. In terms of density value, the results indicated that

density of the protein liquid produced from kilka fish protein hydrolyzes slowly decreased with decreasing particle sizes, and the breakdown of proteins into peptides. There was no information from other researchers on measuring protein liquid density of fish by hydrolysis technology.

The hydrolysis of kilka fish, along with other natural compounds that were added to prevent oxidation and bacterial growth, caused a series of changes in the proximate composition of the HFPS due to hydrolysis process. For example, the amount of protein increased to 16.58%, fat to 15.52%, and ash to 3.8% . The PFS had a moisture content of less than 10% and a protein content of more than 50% , which could be a complete substitute for other protein-based ingredients in animal feeds. Goddard and Perret, (2005), Majumdar *et al.* (2014), and Madage *et al.* (2015) reported that filler-assisted drying with agricultural residues could increase the value of hydrolyzed feed. But the type and quantity of fillers can affect final product prices. This is important that additional steps of processing and techniques used for drying should be considered from economic and market acceptance points of view. Ash content in the PFS was 9.1% which was lower than the limit of 13% based on dry matter for good quality animal feed. High amount of ash is not a good feature for a feed. Because it is possible to cause mineral imbalance in it (Cho *et al*., 1993). In PFS, the amount of total fat was 16.18% for animal feed (Table 1), a significant part of which is related to the fat in the fillers (agricultural residues), including sesame meal. This fat is beneficial because it contains most of the essential fatty acids. However, it is recommended that fat balance in the diet at the time of feeding the animal should be considered for each breeder.

Every animal needs a significant amount of energy in the feed. Because the ratio of energy to protein is low, protein is first used to produce energy and then only consumed during growth. Therefore, it is important to have some energy in the feed. Size, age, climate, and husbandry system is affecting use of energy in the feed brought to the animals (FAO, 2021b).

Two important factors regarding the storage of feed are pH control and TVB-N assay. Addition of 3% acid (v/w) was enough to keep pH of the HFPS lower than 4.0 for 180 days (Fig. 2). Low pH helps to protect it against bacterial infestation and pathogenic organisms. This result was consistent with Tanuja *et al.* (2014) result. van't Land *et al.* (2017) reported that coarse raw materials such as bones and reduced initial acid contact delay pH stabilization. The result is that presence of calcium in bone acts as a buffer in the hydrolysis process (Espe and Lied, 1999; Vizcarra-Magaña *et al.*, 1999; van't Land *et al.*, 2017). The PFS had a stable pH during storage. This result was consistent with result of the work of Góngora *et al.* (2018).

The amount of crude fiber and carbohydrate in PFS produced in this study was 2.30% and 5.34%,

respectively which are acceptable for ease of movement of the gastrointestinal tract of most organisms. The contents of phosphorus and calcium respectively were 0.89% and 1.49% in the dry matter (Table 1), which presence of such elements in diet of animals is very important for mainstay of the body's soft tissue structures and muscle movements (Vitti and Kebreab, 2010). The quantity of these elements in animal feed depends on the kind and quantity of raw materials and used ingredients. Therefore, our results were different from those of the work carried out by Góngora *et al.* (2018). In their work, the crude fiber, phosphorus, and calcium were 6.7%, 0.9%, and 0.9% on the dry matter respectively.

The PFS contained considerable amounts of saturated fatty acids, monounsaturated fatty acids, and polyunsaturated fatty acids, with the most dominant fatty acids being C16:0, C18:0, and C18:1 and C18:2, respectively (Table 2). Cho and Kim (2011) reported that many organisms nutritionally need fatty acids such as polyunsaturated fatty acids. The highest polyunsaturated fatty acid in the PFS was linoleic acid which is a suitable source of omega-6 fatty acids. The animal's body is not able to synthesize it. Therefore, adding this PFS to the animal diet may improve the quality of the final feed fat. Oleic acid is considered a healthy source of fat in the diet. Stearic acid is also a precursor to oleic acid (Choi *et al.*, 2010). Fatty acids' antibacterial actions are due to presence of long-chain unsaturated fatty acids, such as oleic acid, linoleic acid, and linolenic acid. The long-chain saturated fatty acids, such as stearic acid and palmitic acid are less active in this field (Sun *et al.*, 2003; Seidel and Taylor, 2004).

Amino acids are one of the most important components of animal body. Amino acids, as building blocks of proteins, control the vital functions of animal body. Every anatomical and physiological feature of living organisms is made possible by the presence of amino acids (Karau and Grayson, 2014). The role of amino acids and saturated fats in animal nutrition and health is obvious. Therefore, feed nutritionists use amino acid supplements in animal feed to ensure health of the animal. Amino acids also improve the function of immune system of animals (Ashmead, 1993). As a result, adding such PFS as supplement seems to slightly improve the animal diet protein quality (Table 3).

PCA plot showed categories of samples based on their similarity. Accordingly, PCA plot visualizes the influences of processing conditions and storage time on quality attributes of HFPS and PFS (Fig. 5). The attributes of HFPS were characterized by the first two principal components. The two sample groups were situated on different sides of the PCA plot. HFPS samples were located side by side in the upper left half of the graph, and their predominant characteristics were pH and fat content. While the PFS samples

were placed next to each other in the upper right half of the chart, and their predominant attributes were protein, fat, moisture, ash, carbohydrate, calcium, and phosphorous contents. In addition, TVB-N and total bacterial counts as quality indexes were located in that area and indicate that these parameters were affected by storage time in PFS samples.

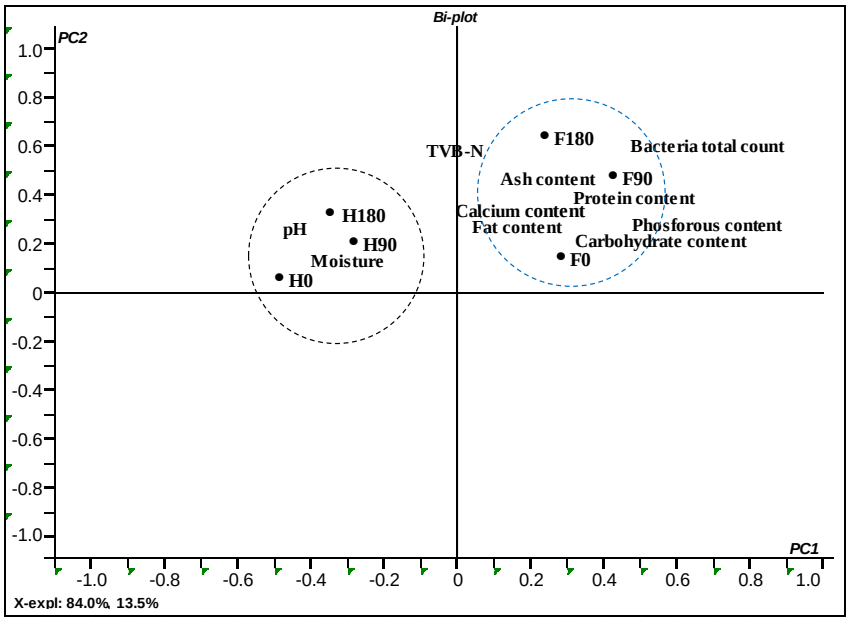

Figure 5: Principal component analysis (PCA) visualizes analytical results of HFPS (H) and PFS (F) prototypes stored for 180 days at ambient temperature (27±2ºC). Numbers show storage days.

TVB-N consists of TMA-N and NH3 and it is one of the important indicators for preserving animal feeds. Acceptable range for TVB-N index in fresh fish is between 30 and 40 mg/100g sample (Tanuja *et al.*, 2014) but fishmeal contains a higher amount of TVB-N. According to FAO (2021b), maximum values of TVB-N for grade 1, grade 2, and grade 3 fishmeal are 150, 250, and 350 mg/100g, respectively. In the present study, TVB-N levels in the feed supplement were under the maximum aforementioned limits during storage (Fig. 3). This high amount of TVB-N in fishmeal or in similar products is common and they can still be used as animal protein supplements in animal diet (Kühlmann *et al.*, 2011). It should be noted that development of TVB-N may be due to increase of ammonia due to long-term acidic protein hydrolysis as well as the type of fish (freshwater or marine fishes). However, use of fillers (agricultural residues) to dry HEPS has a decreasing role in the amount of TVB-N of the produced final feed. Ali and Sahu (2002) reported that TVB-N value for acid-assisted hydrolyzed product obtained from marine fishes was 79.8 mg/100g. But there is little information about TVB-N of acid-dried hydrolysis feed which makes it difficult to compare our study results with other works.

The PFS showed a total microbial load of 4.30±0.30 log CFU/g during 180 days of storage. Acceptable limit for total bacterial plate count of a feed product is 7 logs CFU/g on the wet weight and this is a good criterion for quality assessment of fresh products (ICMSF, 2002). The animal feed supplement should not be contaminated with *Salmonella* due to clinical illness or subclinical infections. The current increase in global feed trade is important because feed trade has led to spread of some *Salmonella* serotypes, including *Salmonella agona* worldwide. It should be noted that absence of *Salmonella* in animal feed is a safety criterion. In this study, there was no salmonella because of low pH and hydrolysis by acid in the hydrolyzed protein liquid. Total bacterial load in PFS reached 4.3 log CFU/g after 180 days of storage which was within acceptable limits. Ramasubburayan *et al.* (2013) reported that total bacterial load in one type of acid-hydrolyzed protein from marine fishery waste was 4 log CFU/ g during 30 days. Tanuja *et al.* (2014) also reported that bacterial load in acid-hydrolyzed protein from dressing waste of freshwater fishes was below 3 log CFU/ g up to six months during storage. When the PFS was tested for *E. coli, Salmonella*, yeast, and mold, none of these were observed and the feed supplement was found to be totally free from these microorganisms. This is due to lower pH of the PFS, and good hygiene practices during handling and storage processes. Therefore, the PFS prepared in the current study can be safely used in animal feeds.

Economic challenges and assessment are important parts of new feed product development process and play a major role in product success. Value-adding to the underutilized fish/ fish by-products and agricultural residues is highly profitable due to the need for new animal feed resources. Fish feed supplement based on hydrolyzed fish protein is an economic alternative to fishmeal and soybean meal, which has better nutritional performance than the other two substances in animal feed (Shaviklo *et al.*, 2021).

The HFPS contained about 63% water or more and is not economic to transport it in liquid form for any distance. Therefore, co-drying it with fillers (agricultural residues) and transporting it is very economic. Inexpensive equipment and a simplified method are needed for hydrolyzing proteins, which is scientifically and technically a turning point in the feed industry. On the other hand, such animal feeds can be distributed as functional feeds in the market. Therefore, new markets are being created for entry of functional feeds and it is a good opportunity for all industries related to animal feeds to produce and distribute healthy feeds.

Due to the importance of using local raw materials for aquaculture and animal husbandry, there is a demand for this product in the market, and because of its high nutritional value, the product is likely to find its true value with breeders and feed producers.

The use of hydrolysis technology to produce animal feed from underutilized fish species, as well as fish processing by-products can provide many advantages to the seafood industry and may give added value to animal feed producers. Based on the parameters tested in this study, a functional PFS can be produced from underutilized fish/ fishery by-products with agricultural residues. Production sources for this product are available in many developing countries and can use this potential to produce protein products for animal feed. This product has many advantages. Its cost price is much lower than fishmeal and soybean meal, which can reduce breeding costs. On the other hand, the PFS contains bioactive peptides that can affect livestock safety and performance positively, and improve product quality. Both can bring great economic value to feed producers and breeders.

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