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Influence of solid-state fermentation on nutritive values and enzymatic activities of AnchovyKilka (Clupeonella engrauliformisSvetovidov, 1941)meal by using different microorganisms

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ABSTRACT: The aim of this studywas to investigate the effect of solid-state fermentation on nutritional and enzymatic properties of Anchovy meal. Minced anchovy were mixed with25% wheat flour and inoculated with microorganisms (10^8 cell/ml) and kept in an incubator for 14 days at 37 ºC. Fermentation of Kilka has resulted in a significant increase in moisture,protein,free fatty acid, non protein nitrogenand pH valuesat the end of the period. Ash content of the fermented product was almost twice as that of the unfermented fish meal in all of the samples. Fiber content and crude fat levels of the fermented product was respectively reduced and increased ($P<0.05$) in comparison with the initialproduct.Decrease in thiobarbituric acid and proxide value content was observed in fungi and yeast during fermentation process in comparison with bacteria.A sharp increasein α-amylase and acidic protease activities was recorded in the fermented products within 6 days. The in vitro digestibility of dry matter and crude proteinwith different levelsof the fish meal were increased especially in 5 and10% levels which improved by the enzymes produced during the fermentation process.In conclusion, the results of this study suggest that fish meal produced with fermentation manner can be used as a modern aquafeed ingredient for a varietyof fish species.The latter, in turn, produces imprortant enzymes that influence digestibility of the nutrients and control the development of the rancidity in the fermented products.

Keywords: Solid-state fermentation, Anchovy Kilka (Clupeonella engrauliformis), Microorganism, Nutritive value, α-amylase, Acidic protease.

INTRODUCTION

Fishmeal is the main animal protein feed used in fish diet. Being known as an underutilized fish, Kilka fish are in consumption of the most countriesthat produce fish meal. Kilka fish, small pelagic fish,forms a large proportion of the fish in Caspian Sea and more than 90% of this population belongs to Anchovy Kilka (Clupeonella engrauliformis).

Fishmeal produced with fermentation techniques is a new feed source for the animals.Two processing methods used for producing fish meal mostly are drying and fermentation (Mondal et al., 2008). The fermentation procedure is a new method for producing fish meal. Fermented fish meal is produced by mixing fish or fish waste 1) with a source of carbohydrates and fermentative microorganisms (biological fermentation) 2) inorganic or organic acid (acid silage) 3) enzymes (enzymatic silage or protein hydrolysis) (Raghunath et al.,2002). Commercial fish meal is produced by heating and drying at a high temperature for storage and safety. The traditional fish meal processing, includingcooking, pressing, drying and grinding, was a costly and complicated process and the heat used for drying fishmeal could lead to decreased fishmeal digestibility (Yamamoto et al., 2005 and Faid et al., 1997). Drying in fishmeal production process is expensive and it requires high energy and advanced technology (Rahmi et al., 2008).Whereas, cheap technology, simplicity, low cost, high-protein digestion and no development of rancid smell in the products are some of the advantages of the fermentation method(Hasan, 2003). Moreover, fish

fermentation is a proper way to turn fish waste and underutilized fish to useful products and reduce environmental problems (Zynudheen and Ramachandran, 2008). Todays,this method is a convenient surrogate being in use.

Solid-state fermentation is a fermentation process which utilizes solid materials as a source of nutrition that must possess enough moisture to increment growth and metabolism of microorganisms (Rani Singhania et al., 2009).

Lactobacillus, Bacillus, Aspergillus and Saccharomycesare among of microorganisms which utilize the organic matters such as carbohydrates both as source of energy for growth and as source of carbon for synthesis of the cell biomass during fermentation (Wang et al., 2011). Organic acids produced through fermentatin by such organisms result in reduction of product acidity. The production of acid by microorganisms indicates that the process is developing successfully (Hasan, 2003). Reduction of acidity and production of organic acid prevent the growth of other spoilage microorganisms. Adding carbohydrates such as wheat bran, molasses, lactose, dextrose, corn and mixed grain and malt is required for growth of microorganisms to an amount of 10 percent of the fish'total weight or more (Yano et al. 2008). Wheat flour is the most common source for carbon in the process. Lactic acid bacteria (LAB) produce lactic acid during the process andAspergillus and Sacchoromyces strains are known to produce citric acid from carbohydrates (Papagianni, 2007).

The Anchovy Kilka fish,used to produce fishmeal, containviscera that cause an increase in the level of lipid in fermented product. Some microorganisms have antioxidant and antimicrobial properties and are able to slow down oxidation process of lipid (Zaika and Smith, 1975; Yano et al., 2008; Smith et al., 2005). Many scientistshave indicated that the microorganisms inoculated during the process, could improve nutrientive levels and chemical proximate of the fermented products (Yano et al., 2008 and Vijayan et al., 2008).The literatures on the use of different microorganisms in production of fish meal by solid- state fermentation are very limited. Therefore, in this study, an attempt has been made to use some microorganisms, namely bacteria: Lactobacillus plantarum, Bacillus subtilis; fungi: Aspergillus niger,Aspergillus awamori and yeast: Sacchoromyses cerevisiae, Sacchoromyses carlsbergensisfor evaluation of biochemical properties, nutrient digestibility and enzyme activity in Anchovy Kilka meal produced with solid- state fermentation technique.

MATERIALS AND METHODS

Microorganisms

Lactobacillus plantarum ATCC8014, Bacillus subtilis ATCC6633, Aspergillus niger ATCC9142, Aspergillus awamori ATCC16877, Sacchoromyses cerevisiae ATCC9763 and Sacchoromyses carlsbergensis ATCC 9080 were obtained from the Iranian Research Organization for Science and Technology (IROST). These microorganisms were grown in 100 ml of Nutrient broth (Merck KGaA, Germany) for 72 h at 37±1 ºCon a shaking incubator (IKA® KS 4000 ic control, Germeny). The cells were harvested by centrifugation at 3000×g for 10 min. The harvested cells were washed twice with sterile physiological saline and resuspended in physiological saline (100 ml). The biomass in the inoculums were grown on Mann, Rogosa and Sharp (MRS) agar (Merck KGaA, Germany) plates and Nutrient agar (Merck KGaA, Germany)plates in L. plantarum and B.subtilis, respectively and Yeast Mold agar plates (Laboratories Conda, , Madrid, Spain) in A. niger, A. awamori, S. cerevisiae and S. carlsbergensis. The suspension of each inoculum was adjusted to a McFarland standard of approximately 10^8CFU/ml in sterile saline.

Solid- state fermentation

The Kilka fish harvested from the Caspian Sea in northern Iran were purchased from local market. The fish was transported to the laboratory in a sample box kept at 4° C. All samples were stored in a -20° C before use. After 1 week, it was thawed in a refrigerator and was dried in a freeze dryer (ALPHA 1-2 LD plus). The substrate used for fermentation was Anchovy Kilka fish (Clupeonella engrauliformis) that homogenized in a blender (IKA® T25 digital ULTRA-TURRAX®, Germany) for 5 min and mixed with 25% wheat flour by stirring. 50 % moisture added to 50 g homogenized substrate for fermentation that adjusted with distilled water. The basal medium for fermentation was autoclaved at 121 ℃ for 15 min and inoculated with 1mlLactobacillus plantarum, Bacillus subtilis, Aspergillus niger, Aspergillus awamori, Sacchoromyses cerevisiae and Sacchoromyses carlsbergensis, separately in 10 g substrate and mixed thoroughly. Samples in 500 ml flask were incubated in an incubator at 37 ℃ for 14 days under static condition. The surface of the samples and the inner walls of the containers inoculated with bacteria were sprayed with potassium sorbat solution (1%) after each sampling in order to prevent moldiness and antibiotic solution (penicillin) was also added in samples inoculated fungi and yeast in order to prevent growth of bacteria.

Chemical analysis

The fermented product obtained after fermentation was dried to a constant moisture level in a hot air oven and proximate composition analysis such as moisture, crude protein, crude fat, crude fiber, ash and pH were carried out every 48 h (AOAC, 1990). All analyseswere performed three times.

The formation of primary products of lipid oxidation (peroxide value or POV) and free fatty acid (FFA) were evaluated according to the method of Woyewoda et al., 1986. Thiobarbituric acid assay was performed as described by Egan et al., 1997. The non- protein nitrogen (NPN) was determined according to the method of Licitra et al., 1996.

Preparation of crude enzyme extract

Fermented product mass was extracted with cold water at 1:4 (w/v) by homogenizing in a homogenizer (IKA$^®$ T25 digital ULTRA-TURRAX$^®$, Germany) for 2 min. The homogenate was allowed to stand for 10 min at 4 ºC followed by centrifugation (Centrifuge 5810 R, Eppendorf AG 22331, Hamburg) at 4 ºC for 30 min at 7600×g. The supernatant was filtered and made up to a known volume with cold water and was referred to as crude extract (Kumar Rai et al., 2010). All steps in the sample preparations described above were conducted at a low temperature by working on ice.

α-amylase and acidic protease activity assay

The α-amylase activity in fermented products was measured using a commercial kit (Ziestchem Diagnostics, Iran). Acidic protease activity was determined using Hemoglobin as a substrate, which released tyrosine was determined by spectrophotometer at 280 nm (Anson, 1938).Soluble protein concentration was determined using Lowry et al. (1951) method, with bovine serum albumin solution (BSA) as standard.

The enzyme activity was expressed as unit of α-amylase and acidic protease activities per soluble protein (U/g or U/mg soluble protein). All assays were carried out at 25 ºC in triplicate.

Determination of protein and dry matter digestibilityin vitro

Crude protein (CP) and dry matter (DM) digestibility of the control mixture (corn, 70%; soybean, 30%) and the same mixture added with the fermented fish meal was determined using pepsin-pancreatin method as described by Yamamoto et al. (2005). The fermented fish meal was added at the levels of 0.1, 1, 5, and 10% to complement different levels of enzymes. 15 ml of 0.1 mol/L HCL containing 627 U of pepsin (Sigma-Aldrich, Germany) was mixed with 250 mg of ground samples passed through a 1 mm sieve, and the mixture was gently shaken at 37 ℃ for 3 h. After neutralization with 0.5 mol/l NaOH, the digesta was mixed with 7.5 ml of porcine pancreatin solution (Sigma-Aldrich, Germany). 4 mg of pancreatin was dissolved in 7.5 ml of 0.2 mol/L phosphate buffer at pH 8, and the mixture was shaken in a water bath at 37 ℃ for 24 h. The solution was, then, centrifuged at 1200 r.p.m for 10 min, and filtered through a double layer of filter (upper layer, Watman 5; lower layer, membrane filter 0.22 µm, MS$^®$ MCE). The filtrate was used for the analysis of nitrogen content using the Kjeldahl method (AOAC, 1990), and the residue was used to measure DM digestibility. CP digestibility (%) = (nitrogen in supernatant/ total nitrogen in sample) × 100; DM digestibility (%) = ((weight of dried filter paper with residue – weight of dried filter paper)/ dried sample weight) × 100.

Statistical analysis

Data are presented as mean± standard deviation. The experiments were repeated three times. To test differences between treatments, all data were subjected to one-way analysis of variance (ANOVA) using statistical package SPSS (Statistical Package for the Social Sciences), version 17. Duncan's Multiple Range Test was used to separate the means among treatments with probability level $P<0.05$ (Duncan, 1955).

RESULTS AND DISCUSSION

The proximate composition data of Anchovy Kilka fish meal before and during 14 days of fermentation using 6 microorganisms (Lactobacillus plantarum, Bacillus subtilis, Aspergillus niger, Aspergillus awamori, Saccharomyses cerevisiae and Saccharomyses carlsbergensis) has been given in Figure 1 and 2.

Determination of Moisture

The moisture content of the unfermented fish meal ranged approximately 3%. As it is shown in Fig. 1, the moisture levels in fermented fish meal inoculated with bacteria, fungi and yeast increased ($P<0.05$) with progress of

fermentation period. Hence, there is a variation in percentage of moisture content during fermentation process using different microorganisms. In our study, moisture content of treated sample increased more than initial sample, significantly ($P<0.05$). Similar enhancement in moisture content has been reported by Vijayan et al., 2009 during the production of inoculated fermented fish meal product. The increase of moisture content during fermentation in all of microorganisms applied in this study may be due to proteolytic activity of microorganisms that release water through hydrolysis of peptides (Anihouvi et al., 2012 and Hammoumi et al. 1997). Moreover, microorganisms have metabolic activities that produce carbon dioxide and water (Chutmanop et al., 2008), which consequently increase the moisture of the content.

Determination of crude protein

The mean (±SD) compositional content of protein content in the raw fish and wheat flour analyzed were 56.49±0.63 and 10.22±0.01 percent, respectively.Variations in the value of crude protein during the fermentation are presented in Figure 1. During fermentation process, the crude protein content showed significant ($P<0.05$) variations between days as well as between microorganisms.

During the fermentation, the protein content increased ($P<0.05$)along with the time of fermentation for samples. Using L. plantarum and B. Subtilis a maximum increment of about 65% crude protein was recorded on day 14 comparisonwith the initial day and in compare with fungi and yeasts ($P<0.05$). The increase of crude protein may be due to the biotransformation of soluble carbohydrate in wheat flour to bacterial protein (Vijayan et al., 2009and Rajesh et al., 2010), increase in microbial cell mass (Antai and Obong, 1992) and could be attributed to the ability of microorganisms to secrete some extra cellular enzyme (proteins) (Oseni & Akindahunsi, 2011). In addition, the increase of protein could be attributed to repletion of a variety of enzymes and free hydrolyzed peptideswhich are proteinaceous (Bhatnagar et al., 2010). Vijayan et al. 2009 reported a crude protein content of 61.83% during 14 days of fermentation using L.plantarum with tuna waste and wheat flour as substrate that this is lower thanthe results of this research. However, Yamamoto et al.2005 observed that the protein content was not changed by the fermentation on sardine fish meal inoculated with A.awamorri. It has been previously shown that the increase in protein level during solid- state fermentation could confer nutritional advantages of fermented product.

Determination of pH

As it is obvious by comparing data in Fig.1, in this study, the pH of the fermented Anchovy fish gradually reduced during 14 days of fermentation and the values of all fermented samples were below 7 at the end of the fermentation process. No significant differences were noted ($P>0.05$) in pH content of inoculated samples after 14 days fermentation between different microorganisms. This is in agreement with the earlier findings by Yamamoto et al. 2005. Decreases in pH may be correlated with the growth of these microorganisms because of producing organic acid on fermentation with utilization of carbohydrates (Kumar Rai et al., 2010).The slow decrease of pH in fermented products might be due to high proportion of unhydrolysed starch in fermented product as reported by Fagbenro and Bello-Olusoji, 1996.

Determination of ash

As shown in Figure1,there was a significant increase ($P<0.05$) in ash content from 4.61-7.34% (unfermented) to 7.36%, 7.76%, 8.87%,8.50%, 8.26% and 8.16% after 14 days of fermentation with inoculated of L. plantarum, B. subtilis, A. niger, A. awamori, S. cerevisiae and S. carlsbergensis, respectively. The maximum ash content was observed in fermented product inoculating withbacteria and yeast in this study. The enhancementof the ash content with the progress of fermentation was correlated with decrease in the dry matter content (Vijayan et al., 2009 and Adebowale & Maliki, 2011) and increase of mineral content (Antai and Obong, 1992) which could be due to contribution by fermenting microorganisms (Oseni and Akindahunsi, 2011). The increase of ash content of the fermented product may be an indicator of influencing the palatability of the fish feed. Furthermore, Ferraz de Arruda et al. (2007) and Fagbenri et al.(1994) recommended that fermented fish meal should be utilized with a more tasteful ingredient such as corn meal and soybean meal in fish diet.

Determination of crude fiber

The initial crude fiber content was about 3% which had reached the maximum value ($P<0.05$) on day 4. This result indicated that S.cervisiae was highest (8.40±0.32 percent) amount of crude fiber in comparison with other microorganismsas presented in Fig.1. The increase observed during the first 4 days of fermentation ($P<0.05$) was due to the assimilation of nutrients in substrate which probably related to synthesized fiber by microorganisms, and later loss of crude fiber after 4 day may be due to break down of non-starch polysaccharide

by microorganism to single cell protein (Rajesh et al., 2010). Moreover, the fiber might be digested by microorganisms to obtain energy and other cellular activities (Yamamoto et al., 2004; Oseni and Akindahunsi, 2011 and Sabah Elkheir and Abd-ALRaheem, 2011). Similar results were reported by Rajesh et al., 2010. In general, reduction of crude fiber content in fermented fish meal may be an indication from highly digestible in fish diet (Nwanna, 2003).

Determination of Non protein nitrogen (NPN)
In the present research, the NPN content was increased ($P < 0.05$) bythe fungi and yeast during fermentationperiod (Fig. 1). Lysosomal enzymes such as catapsin D as a major muscle protease in fish (Viana et al., 1996),that might accelerate autolysis process as well assecretion of microbial acidic protease.The high levels of NPN indicate that during fermentation, non protein nitrogen such as free amino acid, ammonia, amines and peptides were produced from autolysis of protein by proteolytic enzymes activities (Vijayan et al., 2009, Ezeama and Udoh, 2012). In L.plantarum and B. subtilis, NPN content gradually decreased except for day-2. The decrease of NPN in these bacteria indicates those enzymes were surrounded by carbohydrate (Fagbenro and Bello-Olusoji, 1996). Reduceof NPN levels may be important index for the utilization of fermentation method in processing feeds.

Determination of nitrogen-free extract (NFE)
Changes in NFE value are shown in Fig.1. The NFE content decreased during fermentation period, which loss of NFE values may be correlated with breakdown of carbohydrates by amylolytic activity of microorganisms that increased protein and fat contents during fermentation process (Rajesh et al., 2010; De Lurdes Enes Dapkevičius et al., 1997). We also obtained similar observations in our study.

Determination of crude fat
Figure 2 showed that the crude fat content of fermented product inoculated with different microorganisms slowly decreased during fermentation process and then increased in experimental bacteria and yeast ($P < 0.05$). By the day 4 of fermentation process, crude fate value increased in bacteria and yeast samples, significantly ($p < 0.05$). However, the crude fat content has decreased in these fungi until the end of fermentation period. The crude fat in the unfermented product ranged from 12.33 to 13.66 % and the highest value was recorded approximately 16% in ($P < 0.05$) in L.plantarum, S. cerevisiae and S. carlsbergensis in comparison with other microorganisms in this study. The lowest percentage crude fat belonged to A.niger. Yano et al. 2008 and Nasseri et al. (2011) believed that fermentation method by bacteria, fungi and yeast degrades fat that used for generation of single cell protein and citric acid, and moreover, obtains the energy and other metabolic activities from it during fermentation process (Oseni and Akindahunsi, 2011). These results confirmed that solid- state fermentation was able to degrade and reduce the fat content in the fermented product especially in A. niger and A. awamori. The increase of crude fat levels in the case of employingL.plantarum, B. subtilis, S.cervisiae and S.carlesbergensis in this study could enable the bacteria and yeast to synthesize the fat (Vijayan et al., 2009 and Khetarpaul and Chauhan, 1989).
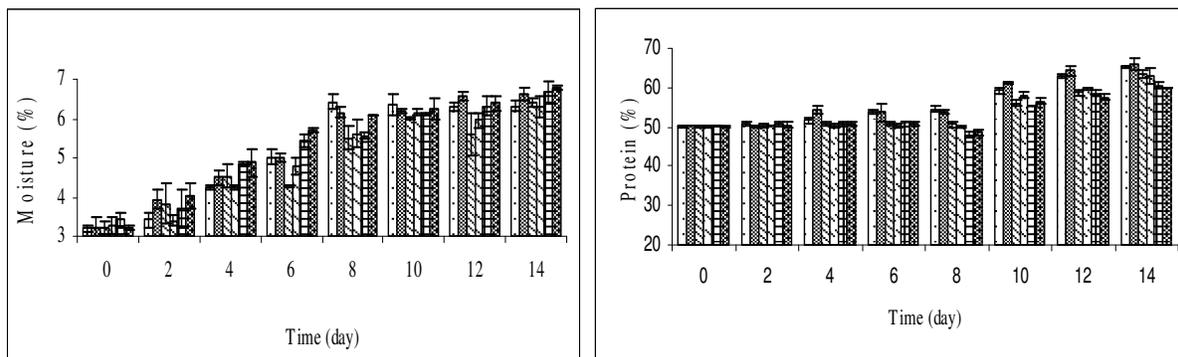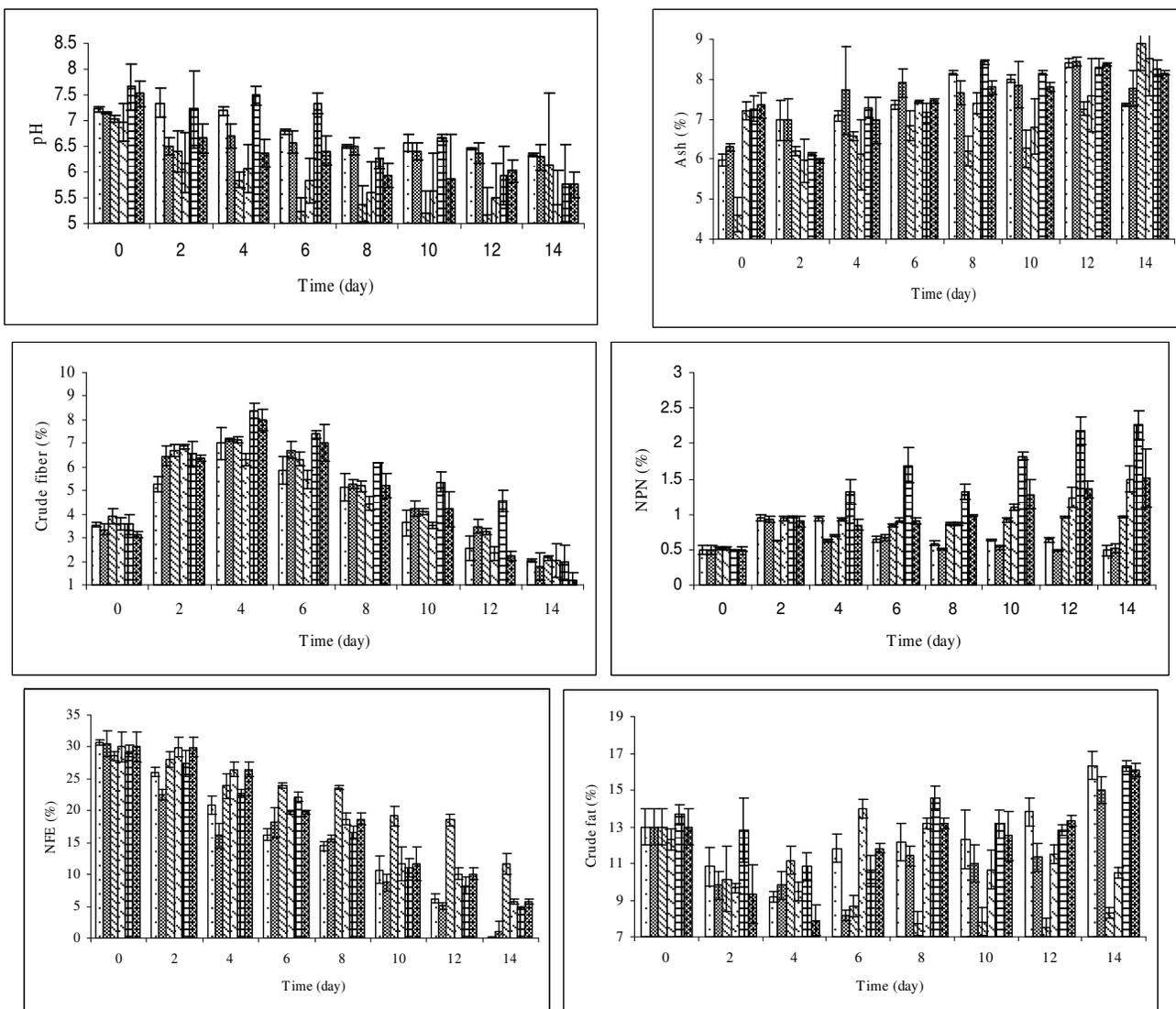
Figure 1. Proximate composition of unfermented fish meal and fermented products using Lactobacillus plantarum, Bacillus subtilis, Aspergillus niger, Aspergillus awamori, Sacchoromyses cerevisiae and Sacchoromyses carlsbergensis during 14 days solid- state fermentation method (as % dry matter)

☐ LAB ▨ B. subtillis ▧ A.niger ▨ A.awamorri ⊞ S.cervisiae ▨ S.carlesbergensis

Determination of free fatty acid (FFA)

It is well known that free fatty acids (FFA) are a result of enzymatic hydrolysis of esterified lipids. The FFA level at the beginning of the fermentation period represented approximately 5 µ mole/g fish and rapidly increased (P<0.05) by L.plantarum, B. subtilis, A. awamori, and A. niger (Fig. 2). Increasing of FFA values related toincreasing of fermentation time, on the other hand, fat lipolysis of fermented product during fermentation period with lipase enzymeby microbial origin. The increase of FFA content during fermentation indicates no toxic effect (Ahmed and Mahendrakar, 1996) for the fish diet. Thelower levels of FFA were observed in substrates inoculated with S.cervisiae and S.carlesbergensis.In yeast cells, there are β-oxidation cycles that utilize FFA content for producing other materials (Yano et al., 2008), consequently the FFA levels decreased in these yeast.

Determination of thiobarbituric acid (TBA) and peroxide value (POV)

The TBA and POV levels were used as indices to assess the level of lipid oxidation in fermented fish meal product. The changes in TBA and POV values are depicted in Fig. 2. The initial POV content was less than 1 meq peroxide/1000g oil. Results indicated that POV of the all sample increased, maximum values were reached on day 4 of fermentation, then decreased(Fig.2), so that the highest score was for inoculated samples with

A.awamorri(18.11 meq peroxide/1000g lipid).The decomposition of peroxide to secondary products (i.e.: aldehydes) (Woyewoda et al., 1986) or their interaction with proteins (Woyewoda et al., 1986), might take place to some extent and result in the lower peroxide already formed (Figure2).

TBA value of unfermented fish was approximately 2 mg malonaldehyde/ kg. The results showed that the level of TBA in inoculated samples with microorganisms in this study increased progressively (P<0.05) in the fermentation process that were steady from day 4 to 14 (P>0.05) in treated samples with fungi and yeast. This consistency of TBA content might be due to the production of peptides that have antioxidant properties during fermentation and preserved fermented products from oxidation of lipid that KumarRai et al., 2010 confirm it,although this need more study of antioxidant properties. Many authors believe that many of bacteria, fungi and yeast produce antioxidative substances in substrate (Kawai et al., 1994; Smith and Alford, 1970; Tabbene et al., 2010 and Li et al., 2012) that the amount of secretion of antioxidant materials is different in bacteria, fungi and yeast that need more study.These results showed thatTBA value of bacterial sample increased more than the others (P<0.05). This increase in fermented fish meal was probably because of organic acid (lactic acid) produced by fish meal during fermentation period which might induce the denaturation of sample proteins, leading to the release of free haem ion, a potential pre-oxidant in the muscle system of Anchovy Kilka. Therefore, it is not surprising to find a lower oxidation lipid for these bacteria.

This research offers that there is probability of repressing lipid oxidation by using fermentation method. Ndaw et al.(2008) has suggested that a maximum TBA level is 5 mg malonaldehyde / kg in fish diet, while fish meal products were consumed up to TBA value of 8 mg malonaldehyde/ kg by fish. Furthermore, among studied microorganisms, fungi and yeast (about 7-8 mg malonaldehyde/ kg) showed less TBA content. It means that fungi and yeast could decrease lipid oxidation of fermented fish meal.This phenomenon preserves the fish health because of reducingoxidation of fat (Yano et al., 2008).
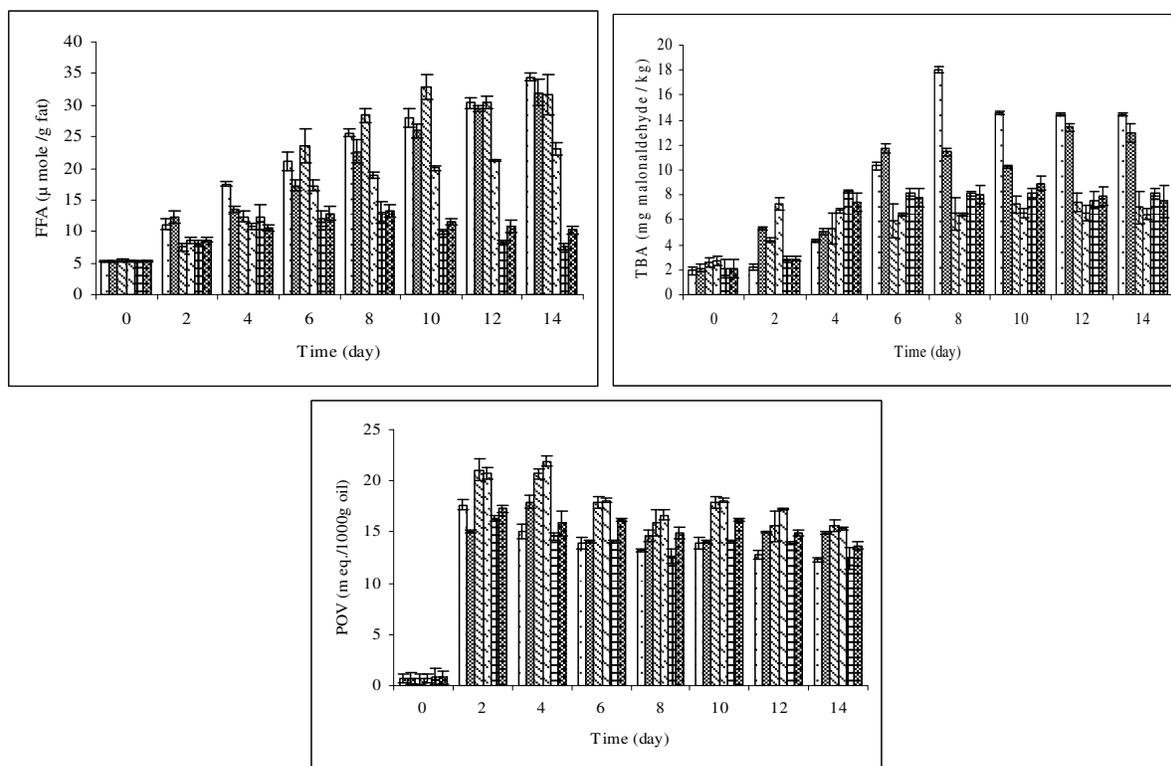

Figure 2. Changes of FFA, TBA and POV levels using Lactobacillus plantarum, Bacillus subtilis, Aspergillus niger, Aspergillus awamori, Sacchoromyses cerevisiae and Sacchoromyses carlsbergensis during 14 days of the solid- state fermentation process.

☐ LAB ▨ B. subtillis ☒ A.niger ☒ A.awamorri ⊞ S.cervisiae ▨ S.carlesbergensis

Enzymes analyses

Microbial fermentation is one of the most important sources of enzymes production that widely utilized in the industry for producing a variety of enzymes. A wide variety of extracellular enzymes are produced by

microorganisms fermentation process among them α-amylase and acidic protease are important in feed nutrition of animal such as fish (Pandey et al., 1999; Anupama et al., 2007).Microbial amylases have potential application in fermentation process because amylase can hydrolyse starch in carbohydrate sources and microbial proteolytic enzymes such as acidic protease could be used as a source of that can hydrolyse protein of fish meal and wheat flour.

Solid-state fermentation enabled α-amylase and acidic protease production by a culture of microorganisms in solid matrix, which can improve the digestibility of fish meal ingredient.

The changes in the levels of α-amylase activity in fermented product using different microorganisms are given in Figure 3. Results indicated that α-amylase activity of the all sample increased progressively, maximum values were reached on day 6 of fermentation period, then decreased on day 8 (Fig.3), so that the highest score was for 6th day of inoculated samples with A.niger (70.35 U/g).The sample preparation with microorganisms could have caused hydrolysis of the starch in wheat flour which leads to higher concentration of α-amylase activity being detected than were actually presented in the samples. The significant (P<0.05) reduction inα-amylase observed during the fermentation may be due to limitation of nutrient in substrate during fermentation process could reduceactivity of enzyme (Rani Singhania et al., 2009; Sivaramakrishnan et al., 2006) and α-amylase possibly was digested by protease (Chutmanop et al., 2008). In addition,the depression in α-amylase activity may be due to the insistence of stationary growth phase of the L. plantarum, B. subtilis, A. niger, A. awamori,S. cerevisiae andS. carlsbergensis, because the α-amylase are generated during log phase of microorganisms on substrate (Figure 4).

Changes in Acidic protease activity by these bacteria, fungi and yeast under solid- state fermentation were shown in Figure3.The amount of initial acidic protease activity was approximately 17 U/mg of proteinwhich with beginning of fermentation process by inoculated microorganisms, the acidic protease activity increased (P<0.05). Much of this activity occurred by day 6 of the fermentation, after completion of exponential microorganisms growth, the activity of this enzyme gradually reduced (P>0.05) (Fig. 4). This proposed that the production of acidic protease by these microorganisms were partially associated with their growth. Moreover, organic acids increase the secretion of acidic protease (Islam, 2012) in early days of fermentation which with decreasing of organic acid content, activity of acidic protease decreased. On the other hand, the increase of acidic protease activity in primarydays of fermentation correlated with increase of crude protein content (Bhatnagar et al., 2010) thatconfirmsthe results of this study. Moreover,acidic protease hydrolyzed protein to peptides that also produced free amino acids that acidic protease activity was reducedby free amino acids (García-Gómez et al., 2009).

A study withA. niger, it had been reported that most of the acidic protease activity was recorded on day 6 on wheat bran during fermentation period (Bhatnagar et al., 2010) that is in agreement with result of this study. The use of S. cerevisiaeand S. carlsbergensisas inoculated microorganisms in substrates during fermentation period is promising to give the product towards higher acidic protease activity, although other microorganisms had values close to those (P<0.05).
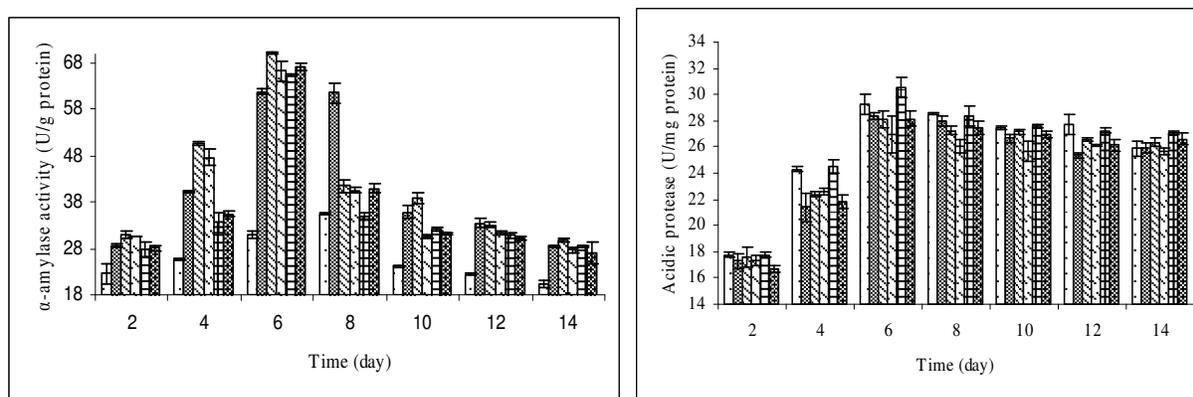

Figure 3. Specific α-amylase and acidic protease activity (mean±S.D, n=3) during solid- state fermentation period and comparison between different microorganisms.

☐LAB ▓ B. subtillis ▨A.niger ▨A.awamorri ⊞S.cervisiae ▩S.carlesbergensis
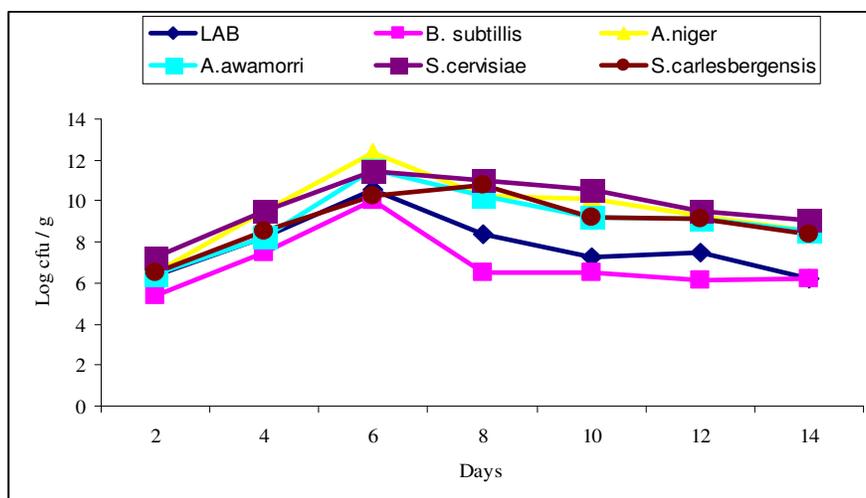
Figure 4. Changes in microorganisms count during fermentation of fish (n=3).

Crude protein (CP) and dry matter (DM) digestibility analyses in vitro

CP and DM of digestibility are the most important aspects in evaluating the suitability of fermented fish meal. The influence of fermented fish meal products as a source of providing enzymes on the in vitrodigestibility of crude protein and dry matter in combination of corn-soybean meal are shown in Table1 and 2.

In this series of experiments, the sharp increase of crude protein digestibility was observed (P<0.05) in 5 and 10 % levels of fermented product inoculated withA. niger, A. awamori,S. cerevisiae and S. carlsbergensis (Table 1). This was probably due to high levels of acidic protease of final fermented product for hydrolysis of protein in the fermented fish meal.The data clearly indicate that fermented fish meal has been shown to increase the protein digestibility that might be because of better availability of exoenzymes produced by these microorganisms.

Table 1. Influence of fermented fish meal on crude protein digestibility of corn- soybean mixture in vitro using Lactobacillus plantarum, Bacillus subtilis, Aspergillus niger, Aspergillus awamori, Sacchoromyses cerevisiae and Sacchoromyses carlsbergensis.

Group	L. plantarum	B. subtilis	A. niger	A. awamori	S. cerevisiae	S. carlsbergensis
Control	79.96 ± 0.28^b	79.36 ± 0.84^b	80.49 ± 1.76^b	79.84 ± 0.37^b	79.73 ± 0.60^b	79.90 ± 0.39^b
0.1%	80.82 ± 0.52^b	80.27 ± 0.17^b	80.77 ± 0.91^b	80.04 ± 2.20^b	79.62 ± 0.78^b	79.68 ± 1.27^b
1%	80.49 ± 0.06^b	80.95 ± 2.04^b	80.22 ± 1.47^b	80.49 ± 0.95^b	79.99 ± 0.37^b	80.06 ± 0.50^b
5%	$^B86.29\pm0.96^a$	$^B86.39\pm1.10^a$	$^A88.52\pm1.00^a$	$^A89.75\pm0.41^a$	$^A89.80\pm0.57^a$	$^A89.87\pm0.30^a$
10%	$^D86.39\pm0.15^a$	$^D86.66\pm0.85^a$	$^C88.79\pm0.45^a$	$^B89.91\pm0.33^a$	$^A90.98\pm0.85^a$	$^{BC}89.66\pm0.44^a$

Values are expressed means±S.D. of three replicates. Values within the same column with different letters (a-b) and the same raw with different letters (A-D) are significantly different (P<0.05).

Table 2. Influence of fermented fish meal on dry matter digestibility of corn- soybean mixture in vitro using Lactobacillus plantarum, Bacillus subtilis, Aspergillus niger, Aspergillus awamori, Sacchoromyses cerevisiae and Sacchoromyses carlsbergensis.

Group	L. plantarum	B. subtilis	A. niger	A. awamori	S. cerevisiae	S. carlsbergensis
Control	50.00 ± 0.38^c	50.40 ± 0.75^d	50.44 ± 0.77^c	49.99 ± 0.14^c	50.59 ± 0.57^c	50.20 ± 0.30^c
0.1%	$^A53.65\pm0.52^b$	$^{AB}52.45\pm0.48^c$	$^{AB}52.20\pm1.90^{bc}$	$^{AB}52.19\pm1.15^b$	$^B51.05\pm0.47^c$	$^B51.10\pm0.40^c$
1%	$^B54.33\pm0.27^b$	$^A55.24\pm0.56^b$	$^B54.33\pm0.25^b$	$^C53.55\pm0.11^b$	$^B54.61\pm0.49^b$	$^C53.28\pm0.11^b$
5%	$^{BC}59.69\pm1.05^a$	$^C59.31\pm0.79^a$	$^{ABC}60.82\pm1.62^a$	$^{ABC}60.35\pm1.31^a$	$^{AB}61.78\pm1.17^a$	$^A62.18\pm0.78^a$
10%	$^B60.72\pm0.61^a$	$^B60.41\pm0.96^a$	$^A62.39\pm0.23^a$	$^B60.99\pm0.67^a$	$^A63.02\pm0.69^a$	$^A63.05\pm0.71^a$

Values are expressed means±S.D. of three replicates. Values within the same column with different letters (a-b) and the same raw with different letters (A-C) are significantly different (P<0.05).

Among all of the treatments, L. plantarum and B. subtilis had the lowest (P<0.05) CP digestibilitythat could be explained partially by indigestible nitrogen compound in the fermented fish meal inoculated with these microorganisms. Furthermore, the results were showed that DM digestibility gradually increased in the different levels of fermented fish meal which was significant at 5 and 10% levels (Table 2). High content of DM digestibility might be due to the high levels of non-starch polysaccharides that hydrolyse by enzymes (Yamamoto et al., 2005).

CONCLUSIONS

In conclusion, this study indicated the efficiency of solid-state fermentation process on Kilka fish for recovery of nutritional quality of fish meal ingredient. More than 60% of crude protein in fermented product using different microorganisms was recovered that could be the best alternative ingredient in aquafeed in comparison with unfermented fish meal.Fermentation technique with inoculating microorganisms can partially reduce the lipid content. Moreover, these results indicated that fermentation had important role in improvingPOV and TBA levels in fermented fish meal and delay oxidation of fat in fish meal.From the present study, it is evident that high quality of fermented fish meal containing enzymes (α-amylase and acidic protease) can be produced using the fermentation method that increased the CP and DM digestibility of fermented product.S.cervisiae is the best producer of these enzymes, which causes increase of digestibility during fermentation. The levels of α-amylase and acidic protease enzymes obtained depended on the carbohydrate concentration in the substrate resulting in growth model of microorganismsduring fermentation process. Therefore, it can be used as an imperative ingredient in the dietary feed formulation of fish feedstuff.Careful fish feeding experiments should be carried out based on the fermented fish meal from the present investigation.The results derived from our study using microorganisms especially with A. niger, A. awamoriand S. cerevisiae, S. carlsbergensis showed that fermented fish meal can be considered as a potential substrate for the production of fermented product with solid- state fermentation which is of high industrial importance.Such an effort is in process in this laboratory.

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