EFFECT OF SALT CONTENT AND FERMENTATION TIME ON PHYSICO-CHEMICAL PROPERTIES OF FERMENTED FISH SAUCE

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A dissertation submitted to the Department of Food Technology, Central Campus of Technology, Tribhuvan University, in partial fulfillment of the requirements for the degree of B. Tech. in Food Technology

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Approval Letter

This *dissertation* entitled *Effect of Salt Content and Fermentation Time on Physico-Chemical Properties of Fermented Fish Sauce* presented by Nabin Parajuli has been accepted as the partial fulfillment of the requirements for the B.Tech. degree in Food Technology.

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Abstract

Present study was undertaken to prepare and evaluate quality of fermented fish sauce prepared from Nile tilapia fish. Nile tilapia (*Oreochromis niloticus*) was collected from Regional Agricultural Research Station, Tarahara, Sunsari. Fish sauce was prepared by fermenting the fish with varying salt concentrations. The salt concentration were varied as salt:fish = 1:1, 1:2, 1:3, 1:4 and 1:5. These samples are denoted by sample A, sample B, sample C, sample D and sample E respectively. Periodic chemical analysis of the samples were done at an interval of 15 days up to day 90 and at an interval of 30 days up to day 180. Fish sauce thus obtained was ripened in direct sunlight and caramel color was added.

Chemical analysis showed the periodical change in proximate composition of different samples undergoing fermentation. Sample of ratio 1:1 and 1:2 showed no fermentation at all due to very high salt concentration. 1:3 and 1:4 showed proper fermentation pattern whereas 1:5 was observed to be undergoing spoilage. Although sample with ratio 1:3 and 1:4 showed similar specs, from the statistical analysis, 1:3 was found to be best among all other samples. The moisture content, crude protein content, percentage acidity, pH and percentage ash content of sample with ratio 1:3 was found to be 67.28%, 23.72% (wb), 0.53%, 5.32 and 23.46% respectively whereas that of the control sample was found to be 67.23%, 22.21% (wb), 0.58%, 5.19 and 24.76% respectively. From two tail paired t-test of Sample of ratio 1:3 and control sample, similarity was observed in terms of protein content and ash whereas significant differences in terms of acidity, pH and moisture content. Also the proximate composition of sample C was in compliance with FDA, The Philippines standards. Microbiological test denoted the presence of lactose fermenting bacteria. *E. coli* was absent in fish sauce so prepared. Caramel was added in the best sample, until its color seemed to be similar to that of commercial fish sauce. Filtration method was implied but clarity could not be obtained. By using suitable clarifier there is good possibility of commercial production of fermented fish sauce from Nile tilapia in Nepal.

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List of Abbreviations

Abbreviation	Full Form
ANOVA	Analysis of Variance
AOAC	Association of Official Analytical Chemists
CCT	Central Campus of Technology
DE	Digestible Energy
EMB	Eosin Methylene Blue
EMP Pathway	Embden-Meyerhof-Parnas Pathway
FDA	Food and Drug Administration
FRD	Food Research Division
FY	Fiscal Year
GoN	Government of Nepal
LAB	Lactic Acid Bacteria
LC	Least Count
LIFDC	Low Income Food Deficient Countries
LSD	Least Significant Difference
MSG	Mono Sodium Glutamate
NARC	Nepal Agricultural Research Council
NRs.	Nepali Rupees
RARS	Regional Agricultural Research Station
ROS	Reactive Oxygen Species
TMA	Tri-methyl Amine
TVB	Total Volatile Base

Part I

Introduction

1.1 General introduction

With more than 30,000 known species, fish form the biggest group in the animal kingdom that is used for the production of animal-based foods. Only about 700 of these species are commercially fished and used for food production (Oehlenschläger and Rehbein, 2009). Fish is one of the major animal protein foods available in the tropics. This has made fishery an important aspect of study. Fish protein also provides vital protein constituents which enable the body to carry out certain functions such as growth (Omodara and Olaniyan, 2012).

The fish are jawed (Gnathostomata), aquatic (fresh water or marine), poikilothermic (cold blooded), oviparous or ovoviviparous, streamlined vertebrates with gill for respiration and fins for locomotion. They exhibit enormous diversity as far as their number, size, morphology, habitats, biology, behavior, etc., are concerned (Gupta and Gupta, 2004). Fish is generally appreciated as one of the healthiest and cheapest source of protein and it has amino acid compositions that are higher in cysteine than most other source of protein. Fish has lower cholesterol content when compared with meat and thus often recommended for consumption especially among the adult population (Olagunju *et al.*, 2012).

Majority of the nutritionists recommend that human beings should consume fish every day. Regular consumption of fish can reduce the risk of cancer, including colon, breast and prostate, lower the risk of dementia and Alzheimer's diseases and prevent the cardiovascular diseases. All of the essential amino acids needed for good protein nutrition are present in fish meat. The fish oil contain high amount of polyunsaturated fatty acid that reduce the serum cholesterol to prevent a number of coronary heart diseases. Fish is also a rich source of minerals and the most abundant micro-elements are Zinc (Zn), Iron (Fe) and Copper (Cu) (Palani *et al.*, 2011).

Fish and other seafood are very important in covering a part of the protein demand for humans. In 2000, food fish contributed 15.9% to the human diet on a worldwide basis (fish as a percentage of total animal protein intake). There are, however, great differences between continents and countries. In low-income, food-deficient countries (LIFDC) fish contributes 20.6%, in Asia 23.3%, in China 21.1%, whereas in South America the contribution amounts

only to 5.7%, in North and Central America to 7.1% and in Europe to 10.3%. The average contribution in developed countries is 12% whereas it is 18.8% in developing countries (FAO)(Oehlenschläger and Rehbein, 2009).

Fermentation is one of the oldest methods of food processing. Although modern food technology has contributed to the present day high standard of quality and hygiene of fermented foods, the principles of the age-old processes have hardly changed. In industrialized societies, a variety of fermented foods are very popular with consumers because of their attractive flavor and their nutritional value. The desirable effect of microbial activity may be caused by its biochemical activity. Microbial enzymes breaking down carbohydrates, lipids, proteins, and other food components can improve food digestion in the human gastrointestinal tract and thus increase nutrient uptake. Several bacteria excrete B vitamins into food. As a result of their growth and metabolism, substances of microbial origin are found in the fermented food, including organic acids, alcohols, aldehydes, esters, and many others. These may have a profound effect on the quality of the fermented product. Fermentation is an attractive technique because it is low cost and low technology and it can be easily carried out at the household level, often in combination with simple methods such as salting, sun drying, or heating (Robert Nout, 2001).

According to Adams (2009), fermented fish products are largely confined to east and south-east Asia, though some are produced elsewhere. As with most traditional products that are still produced principally on a cottage industry or domestic scale, there are numerous variants of some common themes and a host of local names used to describe them. Essentially, they can be divided into two categories: fish/salt products and fish/salt/carbohydrate products. Fish/salt products such as the fish pastes and sauces tend to contain relatively high levels of salt, typically in the range 15 - 25% and are used mainly as a condiment. Fish/salt/carbohydrate products range from those that resemble the fish sauces and pastes in the sense that extensive autolysis has occurred, to products analogous to other lactic fermented foods such as salami, where the bacterial production of lactic acid is a major feature.

1.2 Statement of problem

In Nepal, fish farming is growing every day. But only few species are gaining popularity while others are underutilized. Also, the fish produced are utilized only for direct

consumption by cooking. On the other hand, various processed products of fish origin are imported. The import figures are increasing day by day. Fish sauce is fermented product of fish, which is currently imported mainly from Thailand. Fish sauce is gaining popularity among the people who like Chinese, Thai or Vietnamese cuisine (Anon., 2017a). Thus producing fish sauce in Nepal can be a good alternative for utilization of underutilized fishes of Nepal and fulfill demand of Nepali market.

1.3 Objectives of study

1.3.1 General objective

1. To prepare fermented fish sauce from Nile tilapia (*Oreochromis niloticus*).

1.3.2 Specific objectives

1. To study the physico-chemical changes during fermentation.

2. To compare the proximate composition of fermented fish sauce prepared from Nile tilapia with the commercial fish sauce.

1.4 Significance of study

Fish farming is increasing day by day in context of Nepal. Being rich source of nutrients, there is the risk of deterioration too. Fermentation is a major technique of preservation of fish along with drying and brining. Fermented fish is a broad term for various products like fish sauce, fish paste, etc. Fermented fish sauce is being used currently in Nepal in the restaurants serving the cuisine of Thailand, Vietnam or other countries of Pacific Rim. The sauce used in these restaurants is imported from Thailand. According to Department of Customs, the total import of fish sauce in Nepal from Thailand alone in the FY 2073/74 is NRs. 39,298,000 (Anonymous, 2017a).

There are several freshwater fish species in Nepal that are of low commercial value. These low valued fish species can be used to prepare fermented fish sauce in Nepal. The main problem related to this study is to find out whether the fermented fish sauce prepared from commercially low valued freshwater fish is similar in nutritional composition with that commercially available in the Nepalese market, i.e.; the one prepared from Anchovy. For the selection of fish, a small talk was carried out with scientists and staffs of Regional Agricultural Research Station, Tarahara, Sunsari. It was carried out considering the cost of

production, ease of breeding, increasing consumer's demand in the world but the fish is underutilized in Nepal. The talk concluded that Nile tilapia would be suitable for study.

1.5 Limitations of study

1. Fermentation was carried out for 180 days only.

2. Microbiological analysis was not carried out during fermentation.

Part II

Literature review

2.1 Historical background

Fermented fish is a broad term for different kinds of fish products. Traditionally, preservation of fresh fish was by salting, smoking and sun-drying. Salting and drying in a tropical climate can be prolonged due to high humidity and frequent rainfall, which allows fermentation to start, and people gradually acquired a liking for the taste and the aroma of fermented fish. Another attraction of fermented fish was as a cheap process for underdeveloped countries as an alternative to heavily salted fish products. The ability of fermentation to enhance the flavor increased its production and consumption even in developed countries. Fermentation is also able to mask the taste of tainted fish products. This also increased its production and consumption (Kose and Hall, 2011b). Preservation of marine products is of great importance to the coastal poor. Preserved fish products ensure adequate protein during low fishing periods. Subsistence fishers use their abundant catch of small fish to make fermented fish paste and smoked fish with the assistance of family members (Anon. 2016a).

Fish sauce is part of what gives Southeast Asian cooking its distinctive taste. But it turns out; this cornerstone of Eastern cooking actually has a long history on another continent: Europe. And it goes all the way back to the Roman Empire. Like Asian fish sauces, the Roman version was made by layering fish and salt until it ferments. There are versions made with whole fish, and some with just the blood and guts. Some food historians argue that "*garum*" referred to one version and "*liquamen*" another, while others maintain different terms were popular in different times and places. The current convention is to use *garum* as a common term for all ancient fish sauces. Romans fermented their sauce with less salt than the modern versions, using about 15 percent salt, versus 50 percent. This creates a fermentation environment that releases more of the protein, making *garum* a good source of nutrients. It also gives it a rich, savory umami taste (Prichep, 2013).

Garum was a fermented fish sauce used as a condiment in the cuisines of ancient Greece, Rome and Byzantium. *Liquamen* was a similar preparation, and at times the two were synonymous. Although it enjoyed its greatest popularity in the Roman world, the sauce

was earlier used by the Greeks. The taste for *garum* had a social dimension that might be compared to an aversion to garlic in some modern western societies, or to the adoption of fish sauce in Vietnamese cuisine (Anon. 2016b).

In Africa salting and drying of fish for preservation is accompanied by fermentation, but the period is short (a few days) and the product is not transformed into a paste or sauce. The products are all characterized by a strong odour and, for this reason, various authors have described the product as "sink" fish. In Ghana fermented fish is called momone, an Akan word which literally means stinking. The "stink" fish of Sierra Leone has been described as fish which had developed a strong odour within 24 hours of capture and was salted for about four days and then dried (Essuman, 1992).

Watanabe (1982) described the fermented fishery products of Senegal as highly salted and semi-dried fishery products with an obnoxious odour and a cheesy but rich fishy flavour reminiscent of kusaya from Japan. The characteristic smell of fermented fish is the result of enzymatic and microbiological activity in the fish muscle. Zakhia and Cuq (1991) suggest that the organic acids produced during the fermentation of fish in Mali are mainly acetic acids, whereas it would appear that in Asia mainly lactic acid is produced. Fermented fish is, therefore, any fishery product which has undergone degradative changes through enzymatic or microbiological activity either in the presence or absence of salt (Essuman, 1992).

According to FDA , fish sauce and fish flavored sauce are clear liquid products, with a salty taste and fish flavor, obtained from fermentation of a mixture of fish and salt. Color may vary from straw-yellow to amber. Fish Sauce has a minimum of 4.0% protein while fish flavored sauce has a protein content of below 4.0% but not lower than 1.0%. Protein content shall only come from the fish material. Fish flavored sauce may also be the product of the final extraction and referred to as such due to its low protein content. Fish sauce is a translucent, non-turbid liquid product with a salty taste and fish flavor obtained from fermentation of fish and salt (Codex Alimentarius, 2016).

2.2 Varieties of fish sauce

Amano (1962) divided fermented fish products into three categories whilst Subba Rao (1967) recognized three groups according to the final appearance of the product. Several

authors have tried to classify fermented fish products according to various rules or characteristics of the ferments as reported by Hall (2002).

2.2.1 According to the mechanism of protein breakdown

(i) Traditional salted products mainly fermented by the action of enzymes normally present in fish flesh and entrails to which salt has been added.

(ii) Traditional products fermented by the combined effects of fish enzymes supplemented with microbial enzymes supplied in the form of starter cultures on fish flesh and entrails with added salt.

(iii) Non-traditional products manufactured by accelerated fermentation, acid ensilage and chemical hydrolysis (Kose and Hall, 2011a).

2.2.2 According to the substrates used in the fermentation processes

2.2.2.1 Products made from fish and salt

According to Prof. Martin Adams (2009), fish/salt products such as the fish pastes and sauces tend to contain relatively high levels of salt, typically in the range 15 - 25% and are used mainly as a condiment.

2.2.2.2 Products made from fish, salt and carbohydrate

Fish/salt/carbohydrate products range from those that resemble the fish sauces and pastes in the sense that extensive autolysis has occurred, to products analogous to other lactic fermented foods such as salami, where the bacterial production of lactic acid is a major feature (Adams, 2009).

2.2.3 According to substrate used and source of enzymes during the LAB fermentation of traditional fish products.

2.2.3.1 Group 1

This group consists of fish paste and fish sauce products from South-East Asia. They are usually prepared from whole fish, which is the only available substrate in lactic acid fermentation. The addition of salt to fish reduces the water activity to prevent microbial

spoilage. The enzymes for the fermentation process come partly from the fish digestive system and partly from the bacteria naturally present in the fish and in the salt.

2.2.3.2 Group 2

Either marine or freshwater fish can be used, prepared in different forms such as whole dressed fish, pieces and minced. Carbohydrates are usually added in the form of cooked rice (palm sugar is sometimes used). The ratio of salt to fish is approximately 1:3 or 1:4. Salting and fermenting times vary from 1 to 3 days. After the first fermenting process, more carbohydrate source is added and allowed to ferment again for another 3–4 days. The main characteristics of the products in this group are that carbohydrate is the principle substrate, and the acid thus produced is the main preservative.

2.2.3.3 Group 3

The fermented fish products in this group are similar to the products of Group 2 as far as the nature of substrates is concerned. The only difference is that the causative micro-organisms are added as a starter culture. However, the micro-organisms are not inhibited (Kose and Hall, 2011a). The list of fermented fish products of South-East Asia is given in Table 2.1.

Table.2.1 List of fermented fish products of South-East Asia

S.No.	Country	Product
1	Cambodia	Prahoc
2	Indonesia	Ketjap-Ikan, Trassi
3	Japan	Uwo-shoyu
4	Malaysia	Budu, Belachan
5	Philippines	Patis
6	Thailand	Nam-pla, Budu
7	Vietnam	Nouc-man

Source: Kose and Hall (2011a)

2.3 Manufacture

The process of fish sauce production normally involves the use of small species of fish like anchovy (*Stolephorus* sp.), sardines (*Sardinella* sp.), mackerel (*Rastrelliger* sp. and *Scomber scombrus*), gambusia (*Affinis affinis*), Pacific whiting (*Merluccius* sp.) and other low value fish species, and the methods vary according to their practices. Anchovy is used in salt-fermented fish sauce in Southeast Asian region due to its recognized high quality and it fetches good market price. There are two species of anchovy that are widely used, *Stolephorus indicus* and *S. commersonii.* The commercial manufacturing of fish sauce varies in fish species and salt ratios and this greatly affects the quality of the final product. Every country in Southeast Asia has their own formulation and the physico-chemical qualities vary from one formulation to the other (Mueda, 2015). The ancient and modern general manufacturing steps of fermented fish sauce are shown in Fig. 2.1 and in Fig. 2.2 respectively.

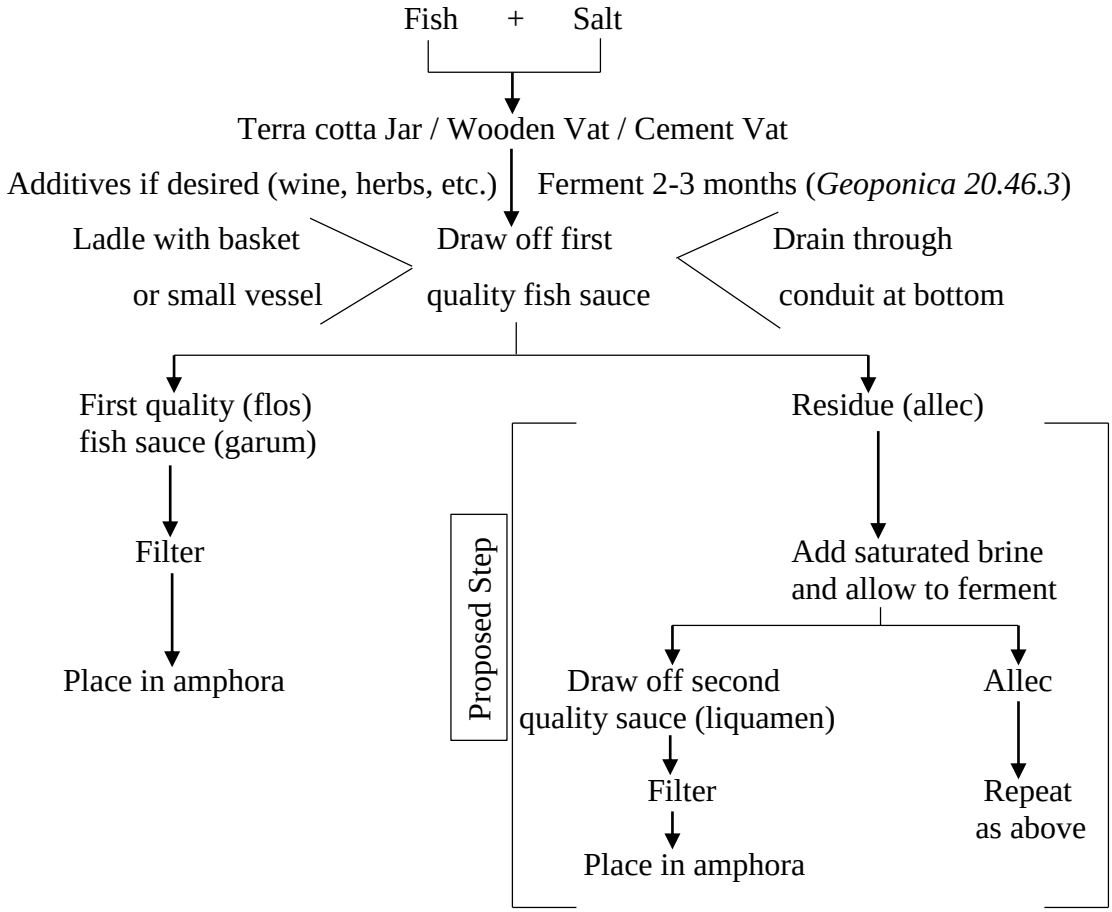

Fig. 2.1 Ancient process flow chart for manufacturing of fermented fish sauce

Source: Vansintjan (2015)

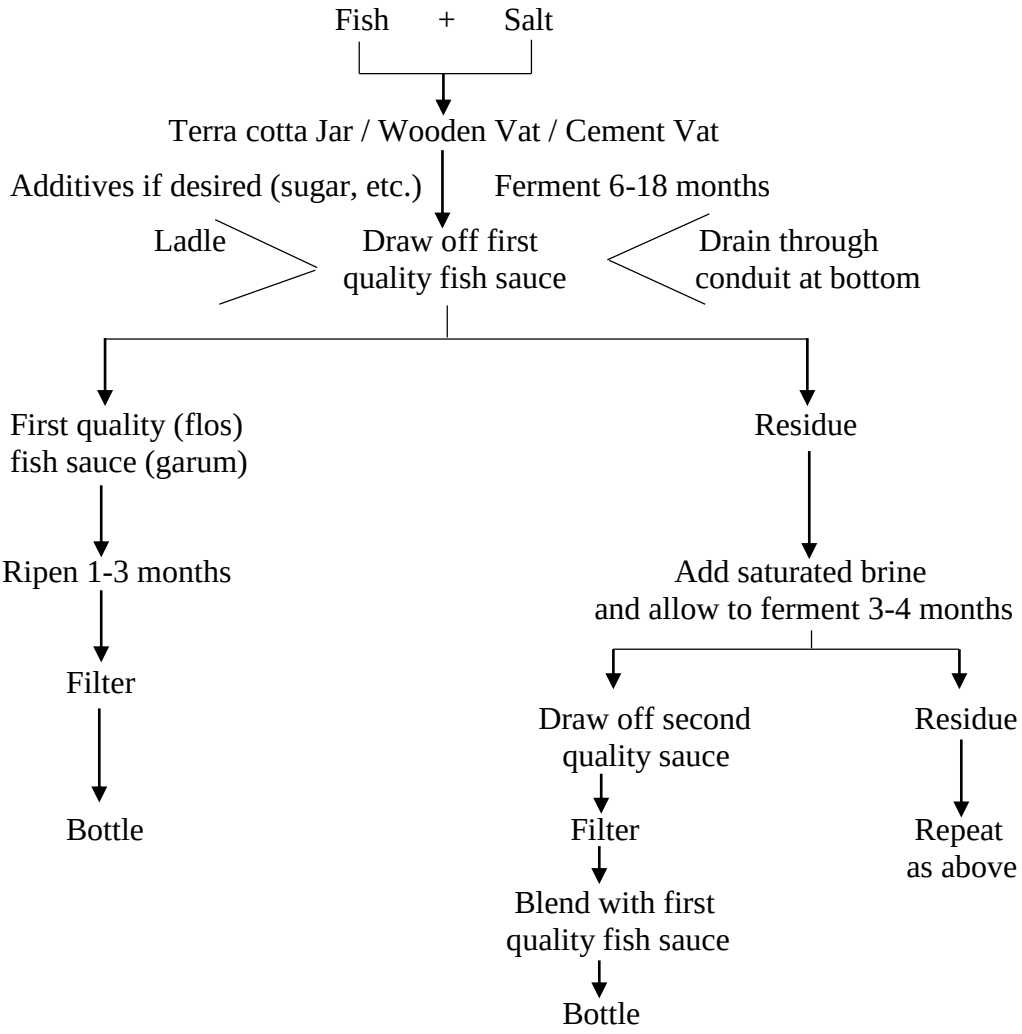

Fig.2.2 Modern process flow chart for manufacturing of fermented fish sauce

Source: Vansintjan (2015)

Major difference in physico-chemical parameters is observed in crude protein content. It is also major determinant of quality of fish sauce. Various researchers have claimed different values for crude protein content ranging from 10.85% (wb) (Ahmad *et al.*, 2015) up to 28-33% (wb) (Chaveesuk, 1991). Faithong and Benjakul (2014) have suggested that this variation in protein content is due to delay in pronounced degradation of protein during fermentation. The acidity in naturally fermented fish sauce prepared by fermentation of anchovies and sardines were found to be 0.63% as lactic acid (Orillo and Pederson, 1968).

Most of the fermented products in the local market contained more salt and the digestion process is too long and usually it takes more than six months before fish sauce can be extracted (Macachor *et al.*, 2016). Addition of histidine accelerated hydrolysis of fish

protein during fermentation in the manufacture of fish sauce and after 4 months fermentation yielded a product typical of traditional fish sauce (Sanceda *et al.*, 1996).

2.4 Flavor profile

During production of fish sauce, the flavor developed is usually described as cheesy, ammoniacal and meaty. The cheesy odor is produced by volatile fatty acids while the ammoniacal odor is due to ammonia and amines. Fish enzymes, microorganisms and fat oxidation have all been considered as possible contributors to the development of fermented fish sauce aroma (Durnfoed and Shahidi, 1998). A literature search revealed several publications on various aspects of the flavour of fish sauce. Van Veen stated that the aroma of fish sauce was due to the presence of methyl ketones and that the volatile fatty acids were relatively unimportant, while Truong (1971) considered that the aroma was due to volatile fatty acids and other workers were noncommittal. Truong (1971) identified formic acid, acetic acid, propionic acid and n-butyric acid while Saisithi *et al.* (1969) obtained similar results except that they identified iso-butyric acid instead of n-butyric acid. Saisithi *et al.* (1969) tested for carbonyl compounds but found none (Dougan and Howard, 1975).

Flavor development in fish sauce appears to be related to bacteria; activity. The aroma of fermented fish products has been attributed to the activity of various types of halophilic bacteria (Chinte-Sanchez, 2008). Beddows *et al.* (1976) proved that microbial actions are important in the development of aroma in mackerel. The action of bacteria rendered the amino acids available as substrates for the transformation of synthesis of flavor compounds in the stage of proteolysis. The conversion of leucine, isoleucine, valine and phenylalanine to aldehyde is likely derived from Strecker degradation in the fermentation process (Co and Sanderson, 1970). This refers to the degradation of amino acids in the presence of ·α-dicarbonyls or other conjugated dicarbonyl compounds that are primarily responsible for the production of flavor and odor associated with the reaction mixture (Chinte-Sanchez, 2008).

The decarboxylation and deamination of amino acids by bacteria usually occur in fish sauce fermentation. The amino acid decarboxylase is the most active in the acidic solution (pH 3 to 6) while deamination is active in an alkaline solution. The pH of fish sauce usually ranges from 6.0 to 6.5; therefore, deamination rather than decarboxylation tends to occur. Kassemsarn (1963) summarized the four general stages in the deamination process, which usually take place in fish sauce fermentation: (1) oxidative deamination, formation of a keto

acid; (2) reductive deamination, formation of a fatty acid; (3) denaturation deamination, formation of an unsaturated acid; and (4) dehydration deamination, formation of a keto acid. Reductive deamination probably occurred more than oxidative and dehydration deamination since fish sauce is produced under anaerobic conditions (Chinte-Sanchez, 2008).

2.5 Principles of fermentation

The complex ripening process consists of chemical and biochemical reactions that change the characteristics of the fish tissue and thus the sensory properties of the fish. Ripening is believed to be caused mainly by enzymatic actions which split macromolecules such as protein and fat in the fish musculature into low molecular weight compounds, e.g. peptides, amino acids and free fatty acids (Kose and Hall, 2011a). The texture of the salted fish becomes softer and tenderer during the ripening phase and a pleasant, typical taste is formed. The enzymes responsible for ripening are reported to be endogenous proteolytic enzymes from the internal organs and muscle tissue of the fish (Schubring and Oehlenschläger, 1997).

According to Anihouvi *et al.* (2007), the use of salt in fresh fish preservation as selective microbial agent has been reported by various researchers. LAB are found as the dominant micro-organisms in many fermented fish products where their primary role is to ferment available carbohydrates and thereby cause a decrease in pH. The combination of low pH and organic acids (mainly lactic acid) is the main preservation factor in fermented fish products. Generally, pH should be below 5–4.5 in order to inhibit pathogenic and spoilage bacteria (Østergaarda *et al.*, 1998). The salt concentration may range from 1% to 20% (w/w) in different forms of fermented fish having a pronounced influence on the microbial growth and the rate of fermentation, and thereby on the sensory quality and safety of the product. It is therefore of interest to identify the optimal salt concentration, which does not inhibit the growth of the fermenting micro-organisms, and in addition contributes positively to the flavor and texture of the product (Achinewhu *et al.*, 2004; Komatsuzaki N. *et al.*, 2005; Paludan-Müller *et al.*, 2002).

The other source of enzymes is known to be bacterial, mainly lactic acid bacteria (LAB) derived either from fish, salt or other ingredients used in the process. The production of lactic acid by LAB can be described as homolactic (only lactic acid produced) or heterolactic (produces carbon dioxide, lactate, acetate and sometimes ethanol). The starting point is a hexose sugar, usually glucose, and lactic acid is the end product of the well-known Embden-

Meyerhof-Parnas (EMP) glycolytic pathway and it might be necessary to promote the homolactic route if lactic acid is to be the only end product. However, lactose (a disaccharide) and pentose sugars can also be metabolized, yielding varying amounts of lactic acid (Nakano *et al.*, 2017; Santo *et al.*, 2005). The provision of fermentable sugars in a food to be preserved by the action of LAB is an essential prerequisite for a successful fermentation. Some LAB are able to catabolize amino acids, by deamination or decarboxylation, yielding carbon dioxide, ammonia and volatile fatty acids and thus contributing to the flavor of the product. Arginine is common in fish tissues and is metabolized, but any amino acid can be utilized. Decarboxylation can give rise to toxic amines and sulphur-containing amino acids can give rise to hydrogen sulphide (Hall, 2002).

According to Majumdar and Basu (2010), a series of complex biochemical processes including proteolysis, lipolysis and lipid oxidation take place during fermentation (also known as ripening stage). The ripening stage renders a product with a firm consistency having characteristic pleasant aroma and taste. The physical and chemical changes that occur during ripening determine the overall sensory qualities of this salt fermented fish product. These changes are induced by enzymes, which break down both proteins and fats.

The interaction of microorganisms during fish sauce manufacture involves mainly bacterial flora. The initial flora of the fish and salt mixture consists mainly of Gram-negative rods originating from the fish itself and from the handlers. These pathogenic microorganisms are immediately inhibited by salt primarily as a result of its ability to extract water from the fish by osmotic action (Chinte-Sanchez, 2008).

The halophilic bacteria in the fish viscera and gills, and those introduced with the salt, increase rapidly due to the availability of nutrients in the brine and the favorable salt concentration needed for their growth and multiplication. The enzymes produced by *Bacillus subtilis* and *B. coagulans* and the endogenous enzymes in the fish sauce cause protein hydrolysis. The bacterial enzymes, however, play a minimal role in the primary stage of protein hydrolysis, but they are largely responsible for the production of flavors due to deamination and decarboxylation of amino acids to form lower fatty acids and amides. Volatile fatty acids are formed due to the oxidation of fat, which could have been brought about either by microbial activities or by fish enzymes. These compounds are produced by *B. licheniformis, Micrococcus colpogenes* and *Staphylococcus epidermis* at the middle stage

of the process and by *M. roseus, M. varians* and *S. saprophyticus* at the later stage. *B. pumilus* is the dominant throughout the fermentation process (Chinte-Sanchez, 2008)

2.6 Nutritional status

Fermentation of protein-rich food (such as fish) enhances the overall protein content and bioavailability of the protein in the food. Multiple feeding studies in animals support traditional wisdom that fermented foods provide a more bioavailable and digestible form of protein. LAB present in fermented foods has been shown in numerous studies to produce vitamins, including several B vitamins and vitamin K_2. Which vitamins are produced is to a large extent dependent on the specific cultures that are present in the ferment. Fermentation of fish products may result in fewer naturally present toxins. Bioactive marine peptides are released from fish proteins during fermentation. These include a number of unique marine compounds with health benefits. It is also a good source of minerals, mainly sodium and iron. Researchers have reported that the ash content in fish sauce ranges from 19% upto 33% (Cho *et al.*, 2000; Ibrahim, 2010; Osman *et al.*, 2012; Pongsetkul *et al.*, 2014).

Some bioactive marine peptides are sold as nutraceuticals. Several other ACE inhibitor peptides have been isolated from fermented fish sauce as well, showing the potential value of fish sauce as part of a diet to maintain healthy blood pressure. Bacteriocins are anti-microbial isolates produced by salt-tolerant or halophilic species of LAB, such as those used in fermentation of fish products. These food components are currently being researched as alternative methods of food preservation. Budu, a fermented anchovy sauce of southern Thailand and Malaysia, was found to contain bacteriocins active against both gram negative and gram positive bacteria (Birks, 2015).

Analyses available are restricted to only amino acids, organic acids, or reducing sugars. Fish sauces are usually manufactured in local small factories in which different species of small fishes as well as prawns or squids are mixed with over 20% of salt, sometimes sugar or other additives, before being fermented for different periods. As a result, and because of taste differences between countries, the chemical composition of fish sauces differs among factories and among countries (Park *et al.*, 2001).

2.7 Quality of fermented fish sauce

Generally, the quality of fish sauce is determined by the following factors:

i. The level of nitrogen in the sauce. Higher the nitrogen content better is the sauce.
ii. The smell of the sauce. A good sauce will not have a strong odor to it.
iii. The transparency of the sauce. The sauce should not have any floating matters.
iv. The color of the sauce. The darker the better.
v. The ingredients of the sauce. Premium fish sauce contains only anchovies and salt. No additional water, color, MSG is added.
vi. And finally the run. The first run of the sauce, meaning the first drainage of the vats until nothing drains out anymore, is known as first run and this produces the best quality sauce (Anon. 2017b).

Cruz (1970) issued an administrative order for the first time stating the standard of fish sauce. It stated that fish sauce must possess protein not less than 6%, total solids not less than 32% and specific gravity 1.21-1.22. Various standards regarding the quality of fish sauce came after that.

Codex Alimentarius (2011) gave new standards regarding the quality parameters of fermented fish sauce that is concerned with raw materials, sensory attributes and chemical properties. The standard states that the Fish sauce shall be prepared from sound and wholesome fish or parts of fish in a condition fit to be sold fresh for human consumption. Salt used shall be of food grade quality and conform to the Standard for Food Grade Salt (CODEX STAN 150-1985). Water for preparing brine shall be potable. All other ingredients used shall be of food grade quality and conform to all applicable Codex standards. Organoleptic criteria shall be acceptable in terms of appearance, odor and taste. In terms of appearance, fish sauce must be translucent, not turbid and free from sediments except salt crystals. Fish sauce shall have an odour and taste characteristic of the product. This product shall be free from foreign matter. Total nitrogen content should not be less than 10 g/L. Competent authorities may also specify a lower level of total nitrogen if it is the preference of that country. Amino acid nitrogen content must not be less than 40% of total nitrogen content. pH should be between 5.0 - 6.5 typical for a traditional product; but not lower than 4.5 if ingredients are used to assist fermentation. Salt should not be less than 200 g/L, calculated as NaCl .

A typical composition of fish sauce as observed by Fujii *et al.* (1980) is given in Table 2.2

Table 2.2 A typical composition of fish sauce

S. No.	Parameters	Composition
1	Ash	22.50%
2	Water Content	66.20%
3	NaCl	29.1% (db)
4	Total Nitrogen	1550 mg/100 ml
5	Trimethylamine	14.9 mg/100 ml
6	Viable Cell Count	4.5×10^3 cells/ml

Source : Fujii *et al.* (1980)

2.8 Uses of fish sauce

Fermented fish sauce is generally used in the cuisine of the countries of Pacific Rim like Korea, China, The Philippines, Vietnam, Thailand, Indonesia, etc. Nowadays its use is globalized. Its use is observed on recipes like kimchi, pork curry, tom yum soup, green curry, roasted duck, etc. (Anon. ,2017c). Fish sauce and paste are widely used to flavor and enrich various dishes in the East and Southeast Asian regions. The population in these areas has rice as their staple food, and the bland taste of rice is enhanced by fish pastes and sauces. Based on their use, they face strong competition with soy sauce and other salty sauces. However, the deep roots of traditional taste preference will keep fermented fish sauces the dominant condiment in Southeast Asian countries (Chinte-Sanchez, 2008).

2.9 Health benefits of fish sauce

Researchers found protein hydrolysates were created during the fermentation process, which have significant health benefits. They improve the ability of skeletal muscle to absorb free amino acids required to build more muscle, affecting body composition, exercise performance and muscle growth (Manninen, 2009). Protein hydrolysates are also used for tissue repair, resulting in more rapid uptake of amino acids, as compared with consuming whole proteins or supplementing with free-form amino acid mixes (Thomson R.L. and J.D., 2011). Gaudel C. *et al.* (2013) showed that they promote a strong insulinotropic response, meaning they stimulate the production and action of insulin in the body. This means protein

hydrolysates found in fish sauce may reduce your insulin resistance, thereby lowering your risk for metabolic syndrome and type 2 diabetes. The effects of the protein hydrolysates are present only when you consume the fish sauce. There is no extended effect after the hydrolysates have been metabolized. During the fermentation process enzymes are also created that help to support your immune system and play a role in scavenging free radicals (Harada K. *et al.*, 2003).

Oxidative stress and free radical production are strongly linked to aging and diseases such as arthritis, cancers and neurodegenerative diseases (Harada K. *et al.*, 2003; Uttara *et al.*, 2009). Free radicals and reactive oxygen species (ROS) are generated in your body from a variety of different metabolic actions, exposure to physical or chemical conditions or during an illness or disease state. In order to reduce the negative effects of free radicals on your body, you need a steady diet of antioxidants. When free radicals overwhelm your ability to control the negative effects, oxidative stress results (Mercola, 2017). Foods that provide you with those antioxidants are functional foods as they help manage your health and reduce the effect of disease (Lobo *et al.*, 2010). Using a luminol chemiluminescence method, researchers found the antioxidants in fish sauce had strong scavenging capability (Harada K. *et al.*, 2003).

Fish sauce may be used as a partial substitute ingredient for salt as a means to reduce sodium content in food without diminishing palatability. These results may aid chefs and food manufacturers in creating foods lower in sodium content to better meet the needs and expectations of consumers, healthcare providers, governmental organizations, and consumer advocacy groups without compromising taste. Consequently, using fish sauce as a partial replacement for salt in food preparations may be a delicious and creative alternative for many low sodium diets (Huynh *et al.*, 2015).

2.10 Microbiological safety of fish sauce

The microbiological examination is important to assess the quality and the safety of budu. According to Food Act & Regulations of Malaysia (2010) , the food products ready for consumption that contaminated with pathogenic microorganisms and contains greater number bacteria than the specified numbers shall not be sold. Previous study indicated that unprocessed fish sauce were free from *E.coli, Vibrio parahaemolyticus* and *Vibrio cholera*. Among these pathogens, *E. coli* has the highest possibility of occurrence as it is the most

abundant pathogen in water (Afiza *et al.*, 2007). But none of the pathogens have been reported positive in fish sauce. Instead, other lactose fermenting gram negative bacteria have been found to be present in fish sauce (Faisal *et al.*, 2015; Fukui *et al.*, 2012).

The microbial counts and bacteria identification on processed fish sauce were important to attain safe product and the chemical composition profile will determine the characteristic and quality of fish sauce. Although some study has been done on microbiological and chemical composition on fish sauce but there has not yet been on antioxidant activity of fish sauce. Antioxidants are very important for human health, since the production of reactive oxygen species is thought to be a significant cause of aging and carcinogenesis (W. A. B. W. Ahmad, 2014).

2.11 Raw materials

The raw materials used in this study were:

1. Nile tilapia (*Oreochromis niloticus*) fish
2. Common iodized salt

2.11.1 Tilapia

Tilapia is the generic name for a group of cichlids consisting of three genera: *Oreochromis, Sarotherodon* and *Tilapia*. They are native to the Nile River and to Africa in general ranging from the upper Nile river south to the equator and west to the Atlantic coast. Today, all commercially important tilapia outside of Africa belong to the genus *Oreochromis,* and more than 90% of these farmed fish are Nile tilapia (*O. niloticus*) (Fitzsimmons, 2000; W. O. Watanabe *et al.*, 2002).

Tilapia are now regarded as the "aquatic chickens" of warm-water aquaculture as they are present in all continents except Antarctic. The fish are delicious to eat, with no fine intramuscular bones and little carcass waste, easy to breed, cheap to feed and tolerant of wide range of temperature, salinity, and water quality ranges. Also, the fish are comparatively free from parasites and diseases. The Nile tilapia (*O. niloticus*) was one of the first fish species cultured. Tilapia are the third most important cultured fish group in the world, after carps and salmonids. They are one of the most productive and internationally

traded freshwater food fish. Tilapia are omnivorous, and are capable of feeding on algae & detritus (Azim *et al.*, 2003).

They can also convert feed into high quality protein, and are one of the best fish for aquaculture, because they reproduce easily, have a short food chain, and reach a marketable size within one growing season. Farming of tilapia has increased in the last three decades as they are easy to grow and market. More than one hundred countries now farm tilapia and 98% of them are grown outside their original habitat. China, Egypt, Indonesia, Philippines and Thailand produce the most tilapia. Africa and Asia consume them as a traditional food, and they are now eaten in non-traditional countries and regions such as USA, Canada, Europe, Central and South America as well. Consumers like tilapia's firm flesh and mild flavor, so markets have expanded rapidly in the U.S. during the last 10 years, mostly based on foreign imports. In fact, tilapia sales have recently surpassed rainbow trout sales in the U.S. (Shelton, 2002). It is the freshwater fish having high protein, high ash but low lipid content. Moisture content, protein and carbohydrate content is found to decrease with an increase in size whereas fat and ash content increases with increase in size (Olopade *et al.*, 2016; Petenuci *et al.*, 2008; Salah *et al.*, 2010).

2.11.2 Common iodized salt

Common iodized salt, i.e.; the iodized salt in granular or powder form with minimum 96 percent sodium chloride, white, pale pink or light grey in color, free from visible contamination with clay, grit and other extraneous adulterant and impurities and with quality standard as fixed in (Iodized Salt (Production, Sale and Distribution) Act, 1998).

2.12 Nile tilapia farming

The Nile tilapia is feral in every country in which it has been cultured or introduced and where local conditions allow the species to establish (Costa-Pierce, 2003). Nile tilapia comprise 83% of the global production of tilapia (FAO, 2002) and are responsible for the dramatic expansion of tilapia in recent decades (Bentsen *et al.*, 1998; M. V. Gupta and Acosta, 2004). The large size at the first reproduction, rapid growth rate and versatile feeding habits with a basal position in the food chain (Costa-Pierce, 2003) justify the predominance of the Nile tilapia in tilapia production (M. V. Gupta and Acosta, 2004). As a consequence of its considerable potential for aquaculture, the species has undergone several breeding programs, which have generated different lineages. The rearing of tilapia in cages, especially

in small volumes, has increased considerably in recent decades and may become the most important aquaculture system in many countries (Vicente and Fonseca-Alves, 2013).

According to Beveridge (2004), the technique has several advantages over traditional farming, including low initial investment, utilization of available aquatic resources, enhanced production control, elimination of problems associated with excessive reproduction and ease of handling (Shinohara *et al.*, 2012). Tilapia have several favourable characteristics for aquaculture, including rapid growth rates, especially in males (Hassanien *et al.*, 2004; Toguyeni *et al.*, 2002), high feed conversion rates (Kubitza, 2000) and disease resistance (Ardjosoediro and Ramnarine, 2002) at high densities and low concentrations of dissolved oxygen (El-Sayed and Kawanna, 2004; Gall and Bakar, 1999). Roughness, ease of obtaining fingerlings and high market acceptability are additional desirable features of the species for aquaculture (Coward and Bromage, 2000; Wille *et al.*, 2002; Yi *et al.*, 1996).

2.13 Nile tilapia farming in context of Nepal

The abundant availability of water resources makes Nepal a country with potential for fish farming. However, the development of fisheries sector in Nepal has not been as expected. The result showed that there has been a steady growth in the fish production in the last 15 years, with more than 37000 mt fish produced in 2013/14. Fish yield increased by more than 2000 kilograms per hectare during the same time period. The majority of the fish productions are coming from eastern terai region, with some hilly districts developing themselves as a good hub (Parajulee Karki, 2016)

Aquaculture is the one of the fastest growing food producing sector in Nepal for the last 5-6 years with a growth rate of 8.4% per annum. Pond area has increased from 6500 ha to 9200 ha during last 6-7 years. Nile tilapia is a government recommended species for commercial aquaculture (Shrestha *et al.*, 2016). Though introduction of tilapia in Nepal has passed over a decade, its cultivation has not flourished. There is a general fear of displacement of indigenous fish species. Swar and Gurung (1988) found the reduction of 42 % in the yield of Mystus spp. and Puntius spp. after introduction of bighead carp (*Aristichthys nobilis*), silver carp (*Hypophthalmichthys molitrix*) and grass carp (*Ctenopharyngodon idella*) in the Begnas lake of Pokhara valley. But biology of tilapia favors for its cultivation in Nepal. The climatic condition in hilly regions can control to an

extent the over breeding activity of the fish. The minimum temperature of tolerance for Tilapia is 10ºC - 11ºC. It cannot survive below this temperature (Yadav, 2006).

The physiological condition can easily be exploited in Nepalese subtropical climate to control the population of tilapia. For cultivation of the fish, cold water bodies can be selected where temperature is rather favorable during summer for growth. Those water bodies can be used for stocking tilapia for 7-8 months and the fish would be harvested during winter months. Temperature below 10ºC will kill the remaining fish after the harvest and there will be no fear of wild propagation. Their population will be controlled naturally. Terai region can be utilized for brood fish stocking and seed production. In this way seed production in Terai and grow out production in hilly region will be the best combination for tilapia cultivation without the fear of displacement of the indigenous fish species of the country (Yadav, 2006).

For a large-scale production, sex reversed and YY- male techniques can be adopted to flourish the tilapia farming in Nepal especially in Terai region where the temperature does not fall usually below 10º C throughout the year. Both the technologies produce quality for that could be a good source of seed. Only male fry are produced by these techniques. The male shows better growth performance than female tilapia therefore, it is preferred. These monosex seed production techniques should be transferred and practiced in our country for its commercial production (Yadav, 2006).

The average annual fish production from fiscal year 1999/00 to 2013/14 in Nepal was 22929.1 mt. During these past 15 years, the lowest production was 14000 MT in the year 1999/00 and the maximum production was 37427 mt in the year 2013/14. The trend of annual fish production showed that fish production increased each year, except in 2008/09. In the last 15 years, there has been an increase of more than 23000 mt of fish production in Nepal compared to that of the base year 1999/00. The top ten highest fish producing districts were Bara, Saptari, Dhanusha, Rupandehi, Mahottari, Siraha, Morang, Chitwan, Parsa and Sarlahi respectively (Parajulee Karki, 2016).

Tilapia was introduced by FRD, NARC in 1999 to study the potential of their commercial production in Nepal. The success in the study has now resulted in flourished tilapia farming in Nepal along with other fishes. Agriculture and Forestry University of Rampur, Chitwan has started commercial production of all-male fry of tilapia fish for the first time in Nepal.

The university took on the endeavor to produce such type of fish to meet the demands of the farmers in the region. Professors and students of the university were successful in producing the unisexual tilapia after conducting lengthy research (Khatiwada, 2017).

2.14 Fish spoilage

After a fish dies, stiffening of the muscle called rigor mortis commences, due to the action of enzymes. Subsequently softening of the flesh occurs as self-digestion proceeds. Fish spoilage involves autolysis or self digestion, which means enzymes found in the body of the fish start breaking down the stomach walls and eventually the belly cavity. Fish that has been excessive handled undergoes quicker spoilage (Lyhs *et al.*, 2004). After death, the fish continues to secrete enzymes to digest the food in its stomach. After the enzymes digest the food, they start acting on the flesh of the fish. Proteins and carbohydrates are then broken down into simpler compounds. The pH of the fish also decreases. Lactic acid is produced as a waste product from this burning of energy. Microbes metabolize these amino acids, producing ammonia, biogenic amines such as putrescine, histamine and cadaverine, organic acids, ketones and sulfur compounds. Degradation of lipids in fatty fish produces rancid odors (Haugen and Undeland, 2003).

In addition, marine fish and some freshwater fish contain trimethylamine oxide that is degraded by several spoilage bacteria to trimethylamine (TMA), the compound responsible for fishy off odors. Iron is a limiting nutrient in fish, and this favors growth of bacteria such as *Pseudomonas* that produce siderophores that bind iron (Gram and Dalgaard, 2002). Storage and processing conditions also affect microbial growth. *Pseudomonas* and *Shewanella* are the predominant species on chilled fresh fish under aerobic conditions (Innocente *et al.*, 2007). Packing under carbon dioxide and addition of low concentrations of sodium chloride favor growth of lactic acid bacteria and *Photobacterium phosphoreum*. Heavily wet salted fish supports growth of yeasts while dried and salted fish are spoiled by molds. Addition of organic acids selects for lactic acid bacteria and yeasts (Lyhs *et al.*, 2004). Pasteurization kills vegetative bacteria but spores of *Clostridium* and *Bacillus* survive and may grow, particularly in unsalted fish (Gram and Dalgaard, 2002).

Microbial growth and metabolism is a major cause of fish spoilage which produce amines, biogenic amines such as putrescine, histamine and cadaverine, organic acids, sulphides, alcohols, aldehydes and ketones with unpleasant and unacceptable off-flavors

(Dalgaard *et al.*, 2006; Emborg *et al.*, 2005; Gram and Dalgaard, 2002). For unpreserved fish, spoilage is a result of Gram-negative, fermentative bacteria (such as Vibrionaceae), whereas psychrotolerant Gram-negative bacteria (such as Pseudomonas spp. and Shewanella spp.) tend to spoil chilled fish (Gram and Huss, 2000). It is, therefore, important to distinguish non spoilage microflora from spoilage bacteria as many of the bacteria present do not actually contribute to spoilage (Ghaly *et al.*, 2010).

2.15 Desirable characteristics of fermented fish sauce

2.15.1 Sensory characteristics

The sensory quality of fish sauce is generally determined by analyzing color, odor, taste and overall acceptance. The desirable color of fish sauce is translucent, brown to amber color. The odor of fish sauce is fishy smell along with some pungency. The taste is desired to be salty and savory along with umami (Kleekayai *et al.*, 2016). It is also desired to give sweet, caramelized, fishy after taste (Ritthiruangdej and Suwonsichon, 2006)

2.15.2 Chemical characteristics

Fish sauce is a hydrolyzed protein product produced by enzymes and microorganisms. A major change occurring during the fermentation period the conversion of proteins to small peptides and free amino acids. Generally, most of polypeptide nitrogen decreases during the fermentation period but amino nitrogen increases. The pH value drops because of released free amino acids from proteins and large polypeptides. Volatile fatty acids generally increases slowly during fermentation time (Ijoong and Ohta, 1996; Wilaipan, 1990; Yatsvinami and Takenaka, 1996).

2.16 Factors affecting fermented fish sauce quality

There are five major factors influencing fish sauce quality: fish species, salt types, the ratio of fish and salt, minor ingredients, and fermentation condition (Gildberg, 2001). Primary raw materials used in fish sauce production are fish and salt. Fish serves as a substrate for both enzymatic and microbial reactions in fish sauce fermentation. Variation in species of fish can result in variation of proximate composition of fish sauce (Kimura *et al.*, 2001; Sørensen *et al.*, 2007). Salt selects the types of microorganisms and retards or kills some pathogenic microbes during the fermentation. Also, the amount of oxygen present in the fermentation tank controls the quality. Low oxygen levels in the fermentation tank has a

synergistic effect with salt on selecting microorganisms in the process. In addition to fish, salt and oxygen level, the ratio of fish and salt is very important for fish sauce quality. Different amount of salt would have a different effect on various enzymes from fish which are playing an important role in protein degradation during fermentation. A certain aspect of fish sauce quality for a specific group of consumers can be adjusted using food additives (Lopetcharat, 1999).

Chapter III

Materials and methods

3.1 Materials

3.1.1 Raw materials

The raw materials used for this work were Nile tilapia fish collected from NARC RARS Tarahara, Sunsari and common salt. Fish sauce prepared from Anchovies available in Nepali market was used as control sample for comparison of chemical analysis of fish sauce prepared from Nile tilapia.

1.1.2 Chemicals

The chemicals required during this work were provided by Central Campus of Technology laboratory and NARC FRD laboratory. The list of chemicals required during this work are as listed appendix IV.

1.1.3 Glasswares and apparatus

The glasswares and apparatus required during this work were provided by CCT laboratory and NARC FRD laboratory. The list of glasswares and apparatus required during this work are as listed in appendix V.

3.2 Methods

3.2.1 Selection of Nile tilapia

Talk program was carried out with scientists and staffs of Regional Agricultural Research Station, Tarhara. The talk program was carried out considering the cost of production, ease of breeding, increasing consumer's demand in the world but the fish that has been kept in shadow in Nepal. Discussion was carried out on different varieties. These were: common carp (*Cyprinus carpio*), rohu (*Labeo rohita*), naini (*Cirrhinus mrigala*), bhakur (*catla catla*), silver carp (*Hypophthalmichthys molitrix*), bighead carp (*Hypophthalmichthys nobilis*), grass carp (*Ctenopharyngodon idella*), rainbow trout (*Oncorhynchus mykiss*) and Nile tilapia (*Oreochromis niloticus*). The conclusion of talk program suggested the Nile tilapia for the study.

3.2.2 Collection of raw materials

Nile tilapia fish was collected from NARC RARS Fisheries Research, Tarahara, Sunsari. The average length and weight of fish were 12-15 cm and 250-300 g. Common salt used was bought from local market of Dharan, Sunsari marketed by Salt Trading Corporation, Nepal under the brand name of "Aayo Nun". Fish sauce prepared from anchovies was bought from Bhat-Bhateni Super Store, Dharan. It was manufactured by Chuen Cheong Food Industries (Ptd) Ltd, 58 Woodlands Terrace, Singapore.

3.2.3 Preparation of fish for fermentation

3.2.3.1 Fishing

Live fish were caught by netting method by trained fishermen at NARC RARS Fisheries Research, Tarahara, Sunsari.

3.2.3.2 Cleaning

The collected fish was taken to the meat pilot plant at CCT and cleaned. Cleaning was done thoroughly with potable water in laboratory of Central Campus of Technology.

3.2.3.3 Scaling

Scaling of the fish was done by using sharp scrappers. Fish was washed with potable water after scaling was done.

3.2.3.4 Beheading

Fish was beheaded with sharp knife.

3.2.3.5 Gutting

Gut was removed by using knife.

3.2.3.6 Cutting of fins

Fins were cut using a sharp knife.

3.2.3.7 Cutting into pieces

The fish after all above processes was cut into pieces and then washed with potable water.

The flow diagram showing overall experimental detail is shown in Fig. 3.1.

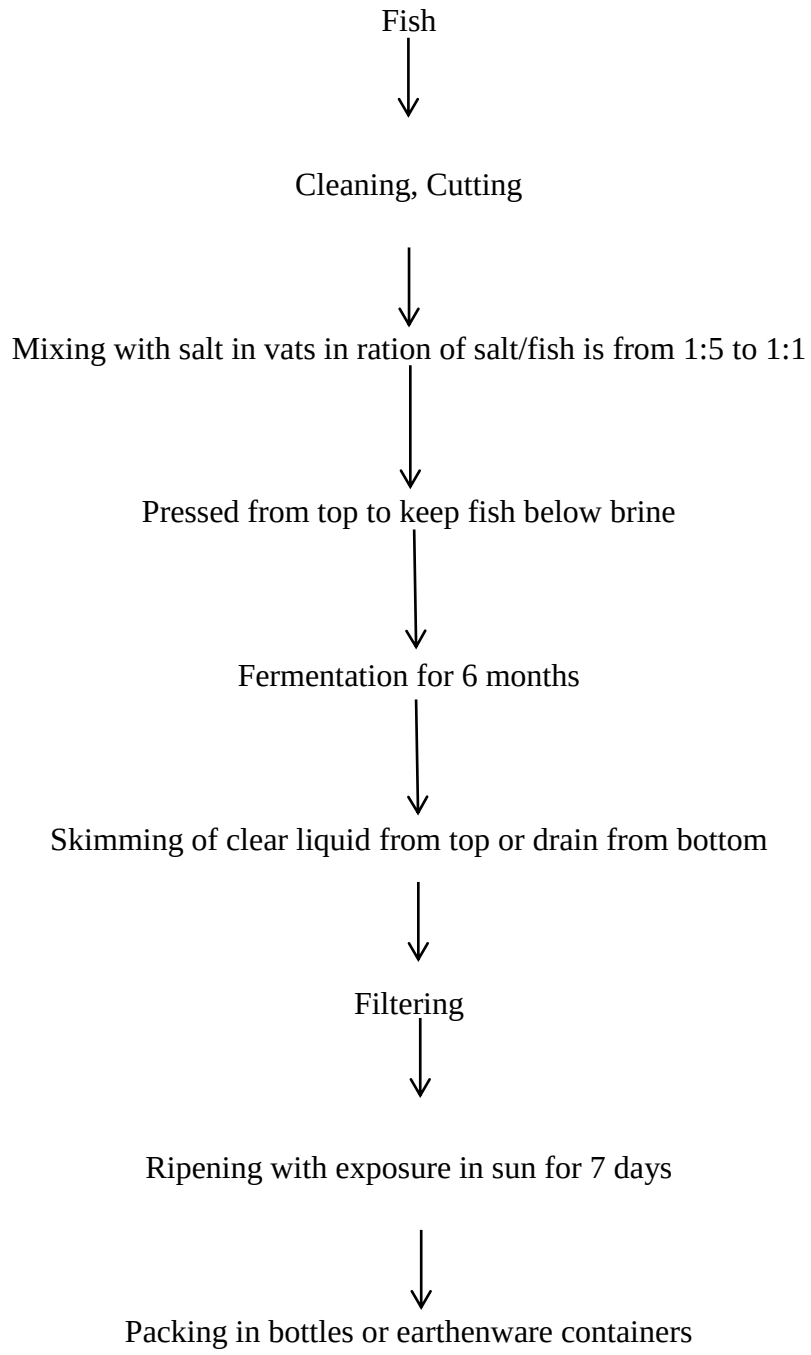

Fig. 3.1 Flow diagram showing overall experimental procedure

3.2.4 Mixing with salt

The pieces of fish was weighed and then divided into five parts of equal weight, i.e.; 400 g each. They were kept in five jars and then dry salting was done. The proportion of dry salting was salt:fish::1:1 to 1:5. The legends A, B, C, D and E were provided for the fish sauce sample of salt:fish = 1:1, 1:2, 1:3, 1:4 and 1:5 respectively.

They were then capped.

3.2.5 Fermentation

Fermentation of the fish was done for 6 months. During this period, salt extracted water from fish muscles. The fish pieces were pressed and kept under brine. Fermentation was carried out at room temperature. Proximate analysis of the extract obtained during fermentation was done regularly at an interval of 15 days upto 3 months and then at an interval of 30 days upto 6 months.

3.2.6 Filtration

After 6 months of fermentation, the liquid extract obtained was skimmed out from the remaining fish pieces. It was then filtered through muslin cloth and Whatmann filter paper No. 41.

3.2.7 Ripening

After filtration, the extract was kept on glass vessel in direct sunlight for 7 days. Ripening was done because it renders the fish sauce a pleasant odor and also helps to settle down any undigested matter present even after filtration. Color was imparted by using caramel. It was then kept in glass vessel in chilled condition.

3.3 Analytical methods

3.3.1 Proximate composition

Proximate composition is only a representation of category of compounds present in biological material (KC and Rai, 2007). The proximate composition of fish, liquid extract during fermentation, fish sauce and the fish sauce used as control were analyzed. Moisture content, crude protein, crude fat, ash content, pH and acidity were analysed.

3.3.1.1 Moisture content

Moisture content was determined by using Vitcolab hot air oven as per AOAC (2005).

Empty petri dishes were dried using air drying oven for 1 h at 105°C, transferred to the desiccators (with granular silica gel), cooled for 30 min, and were weighed. Finely ground samples were mixed thoroughly and 3 g of this sample was transferred to each of the dried and weighed dish. The dishes along with the samples were placed in the drying oven and dried for 3 h at 105°C, and then the dishes and their contents were cooled in desiccators to room temperature and reweighed. The moisture content was determined by measuring the weight of a sample before and after the water was removed by evaporation.

$$\% \text{ moisture content (wb)} = \frac{(\text{weight of wet sample} - \text{weight of dry sample})}{\text{weight of wet sample}} \times 100$$

3.3.1.2 Crude protein content

Nitrogen content was determined by micro Kjeldahl distillation method as per AOAC (2005).

2 g of sample was taken in the digestion flask followed by addition of 2 g of catalyst mixture and 25 ml of concentrated sulphuric acid. Blank was prepared in another digestion flask by adding catalyst mixture and concentrated sulphuric acid except sample. The flasks were palced in inclined position and were digested until pale blue color were obtained. The digested sample was diluted to 100 ml in a volumetric flask with distilled water. About 5 ml of digested blank was distilled for 10 min with 10 ml of 30% sodium hydroxide in a distillation apparatus and the distillate was collected in the flask containing 2% of boric acid solution and mixed indicator. The color of the boric acid was changed from bluish purple to bluish green as it came in contact with ammonia. The final nitrogen content was calculated by tirating boric acid mixture with standard 0.01N hydrochloric acid until blue color was just disappered. Similar procedure was repeated for blank. The crude protein content was then calculated by multiplying with the factor 6.25.

$$\% \text{ nitrogen (wb)} = \frac{\{\text{N of HCl} \times (\text{sample titre-blank titre}) \times 14 \times 100\}}{\text{aliquot (ml)} \times \text{weight of sample (g)} \times 1000} \times 100$$

3.3.1.3 Total ash content

Total ash was determined by ashing in Accumax India electric muffle furnace as per (AOAC, 2005).

3 g of sample was taken in the dried silica crucible. The crucible along with sample was heated in low bunsen flame until no longer fumes was produced. The crucible was then heated in muffle furnace for 3-4 h at 550°C. After complete heating, the crucible was dried in the dessicator and then final weight was taken. The difference between the initial and the final weight gave the crude ash content.

$$\% \text{ ash} = \frac{\text{weight of ash}}{\text{weight of sample}} \times 100\%$$

3.3.1.4 Fat content

Fat content was determined by semi continuous solvent extraction method (Soxhlet method) using light petroleum benzene (b.p. 60-80°C) as per AOAC (2005).

3 g of dried and ground sample was placed in a porous cellulose extraction thimble and thimble was covered with fat free cotton. The thimble was placed in an extraction chamber which is suspended above a flask containing the solvent (about 250 ml of petroleum ether) added with boiling chips and below a condenser. The flask was placed inside the heating chamber and heated at 55°C. The solvent evaporates and moves up into the condenser where it is converted into a liquid that trickles into the extraction chamber containing the sample. At the end of the extraction process, which typically lasts for 3 h, the flask containing the solvent and lipid was removed, the solvent was evaporated in drying oven at 70°C and the mass of lipid remaining was quantified gravimetrically and calculated from the difference in weight of the extraction flask before and after extraction as percentage. The crude fat in the initial sample was calculated as:

$$\% \text{ fat (wb)} = \frac{\text{weight of fat}}{\text{weight of sample}} \times 100$$

3.3.1.5 Total Acidity

Total acidity was determined by titration with sodium hydroxide as per AOAC (2005).

10 g or 10 ml of sample was measured. The solid sample was blended in mortar and pestle. Sample was then transferred quantitatively to a 100 ml volumetric flask and volume was made up with water without allowing the sample to froth. It was mixed well by shaking and inverting for a number of times. The residue was strained away in a piece of coarse linen

and the filtrate was reserved. 10 ml filtrate was pipetted out in a conical flask and titrated with 0.1N sodium hydroxide using phenolphthalein as indicator to a persistent pink end point. As lactic acid is the major acid formed during fermentation by LAB, percentage acidity is calculated as anhydrous lactic acid (Kilinc *et al.*, 2006). The acidity was calculated in terms of lactic acid anhydrous as follows:

$$\% \text{ acidity} = \frac{\text{titre} \times \text{N of NaOH} \times \text{volume made up (ml)} \times 90 \times 100}{\text{aliquot (ml)} \times \text{weight or volume of sample taken (g or ml)} \times 1000}$$

3.3.1.6 pH

pH was determined by using Labtronics LT-10 pH meter. It is measured as per AOAC (2005).

Solid sample was blended in a mortar and pestle. Liquid sample was used directly. pH meter was first calibrated with buffers of pH 4.0 and 7.0. The pH meter was then washed several times with distilled water to remove buffer. The temperature of sample was maintained. The pH meter was then dipped in the sample and reading was taken.

3.3.2 Microbiological analysis

Microbiological analysis was carried out in final product to detect the presence of *E. coli.* The test was done by spread plating of serial diluted sample in EMB agar. EMB agar performs as a selective media where gm +ve bacteria are inhibited. Lactose fermenting gm –ve bacteria gives blue core colonies whereas *E. coli.* gives colony with greenish metallic sheen (Horvath and Ropp, 1974).

3.3.3 Statistical Analysis

Statistical analysis of the data obtained were carried out in GenStat version 12 and MS-Excel 2013. The statistical tools used for analysis were ANOVA, LSD and two tail paired t-test.

Part IV

Results and discussion

Nile tilapia fish was collected from NARC RARS Fisheries Research, Tarahara, Sunsari. The proximate composition of fish was carried out. Fish sauce was prepared by fermenting in room temperature for 6 months and then ripening in direct sunlight for 7 days.

4.1 Proximate composition of Nile tilapia

Proximate analysis gives inexpensive yet very information, particularly from the nutritional and biochemical points of views. The results normally expressed in percentage and because of the fairly general nature of test employed for the determination, the term crude is usually used as a modifier; for instant, crude protein, crude fat and crude fiber etc. Therefore proximate constituent represent only a category of compounds present in biological material (KC and Rai, 2007).

The proximate composition of raw Nile tilapia are given in Table 4.1.

Table 4.1: Chemical composition of Nile tilapia (*Oreochromis niloticus*)

S.N.	Parameter	Composition
1	Moisture Content	73.70% (0.1290)
2	Crude Protein Content	13.88% (0.0355) (wb)
3	pH	6.57 (0.0058)
4	Acidity	0.31% (0.0977) (wb)
5	Ash Content	14.63% (0.0354) (db)
6	Crude Lipid	0.43% (0.0358) (wb)

*Values are the means of three determinations. Figures in the parentheses are the standard deviations.

The moisture content, crude protein content, pH, acidity, ash content and crude lipid content of the raw Nile tilapia were found to be 73.70%, 13.88%, 6.57, 0.31%, 14.63% and 0.43% respectively. Results obtained from the research carried out by Santos *et al.* (2012) can be generalised by stating that moisture, protein and carbohydrate content decreases with an increase in size whereas fat and ash content increases with an increase in size. Also the higher amount of ash is observed because fish bones along with its muscle was analysed

(Olopade *et al.*, 2016; Petenuci *et al.*, 2008). Protein content is the major quality parameter of fish sauce which remains on topmost concern during its production. **Physico-chemical changes during fermentation of fish**

Various physico-chemical changes takes place during fermentation. Fermentation is the process of conversion of complex organic compounds into simpler ones. This results in the change of physico-chemical properties of biological matter. The physico-chemical changes observed and studied during the fermentation of fish to prepare fish sauce is as follows.

4.2.1 Change in moisture content with fermentation time

As the fermentation proceeds in salty medium, the salt at first shows osmotic effect and draws out moisture from the fish. Salt then dissolves in the moisture thus drawn out. Later, the degradation products of fermentation also gets leeched out into water which reduces moisture content of the liquid extract. The change of moisture content of fish sauce with fermentation time is shown in Fig. 4.1.

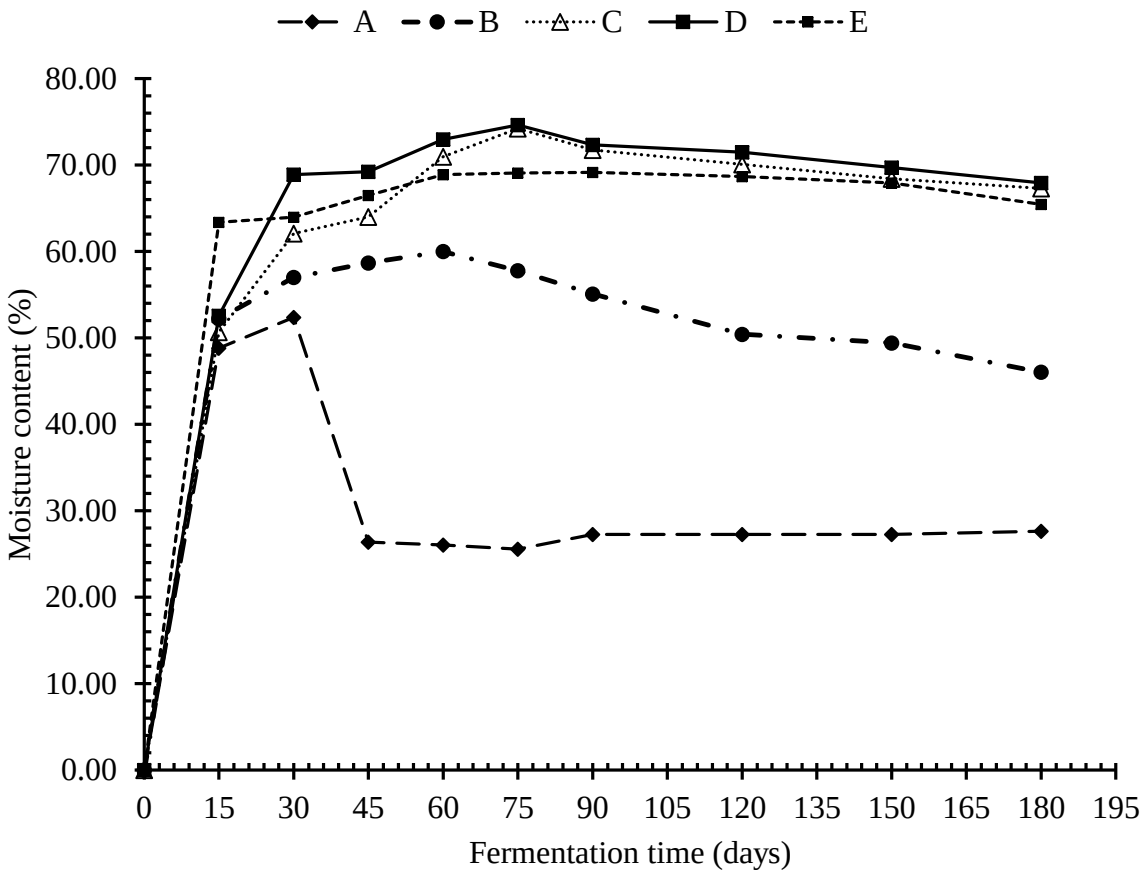

Fig. 4.1 Change in moisture content with fermentation time

The mean values of change in moisture content is tabulated in appendix I. The result was compatible as per the administrative order for standard of fish sauce (Cruz, 1970) and also with the research done by Fujii *et al.* (1980).

Statistical analysis showed that both days on fermentation and sample had significant effect on moisture content of fish sauce. It can be observed from above chart that the moisture content of all samples dropped at the initial phase of fermentation. With the increasing number of days, the moisture content of sample A further dropped and then became almost constant. On the other hand the moisture content of samples C and D increased and then remained almost constant. The moisture content of samples B and E did not change much after initial drop in moisture content. The moisture content of sample A was seen to be decreasing as the salt itself dissolved and made up supersaturated solution after drawing out water from fish. Also the decrease in moisture content in other samples are observed after about 60 to 75 days of fermentation. This might be due to increase in dissolved solute extracted from fish muscles.

The statistical analysis showed that the samples 3,4 and 5 were not significantly different with each other while they were significantly different ($p<0.05$) with samples 1 and 2. The samples 1 and 2 were also significantly different with each other. The statistical analysis using LSD among means indicated sample 4 to be best in terms of moisture content.

4.2.2 Change in crude protein content with fermentation time

In the measurement of crude protein, all the nitrogen available is the sample is taken under consideration. The change in total nitrogen for Pacific whiting fish sauce fermentation was faster (Lopetcharat, 1999). The release of water and soluble proteins from cells by osmotic pressure causes the increase in total nitrogen content during the initial phase of fermentation. Protein content is the only objective index used to classify the quality of fish sauce. The change in crude protein content of fish sauce with fermentation time is shown in Fig. 4.2.

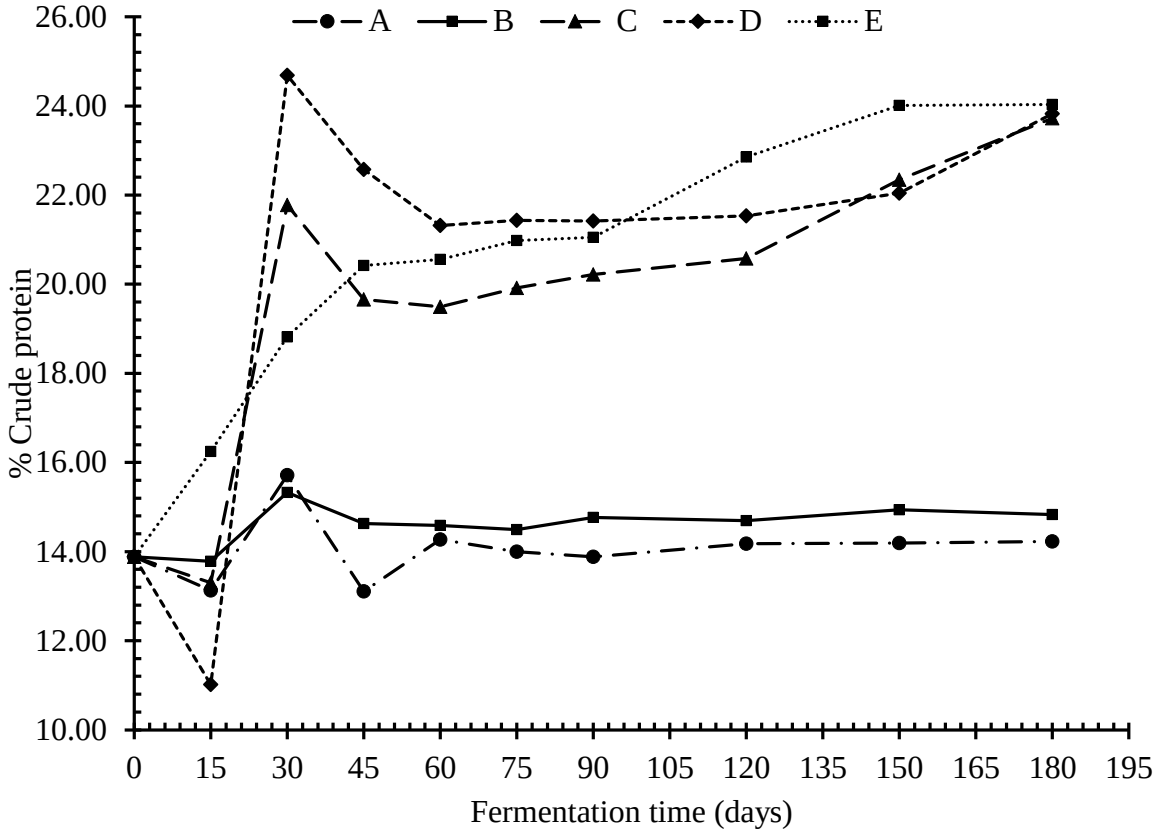

Fig. 4.2 Change in crude protein content with fermentation time

The mean values of change in crude protein content is tabulated in appendix I. Although the standard crude protein content for fish sauce is stated to be minimum 6%, there is no upper limit prescribed. But various researchers have obtained different values of crude protein content (%) ranging from minimum of 10.85±0.85 on wet basis (Ahmad *et al.*, 2015) up to maximum of 28-33% on wet basis (Chaveesuk, 1991).

There does not seem significant change in crude protein content of sample A and sample B. It might be due to absence of microbial activities as the salt concentration in those samples were very high. This might have resulted in inhibition of fermentation. Upon statistical analysis of the samples, it was observed that the samples were significantly different. There was significant difference even on the days of fermentation. The initial drop and then increase of crude protein content was also observed by Mueda (2015). Faithong and Benjakul (2014) used shrimp for study and found that only after 7-10 days of fermentation, the degradation of proteins was pronounced. Other muscle proteins were also degraded to a high extent. This resulted in increase in protein content.

The statistical analysis showed that there was no significant difference between sample A and sample B. Also there was no significant difference between sample D and sample E. Sample A and sample B were found to be significantly different from sample D and sample E. Also sample C possessed significant difference with sample A, sample B, sample C and sample D. Considering crude protein as variate, sample D was found to be best among all other samples.

4.2.3 Change in acidity with fermentation time

The fermentation in fish is carried out by halophilic LAB. This results in increase in acidity. The acidity is calculated in terms of lactic acid as it is the principle acid formed in the fermentation of fish. The change in acidity of different samples with fermentation time is shown in Fig. 4.3.

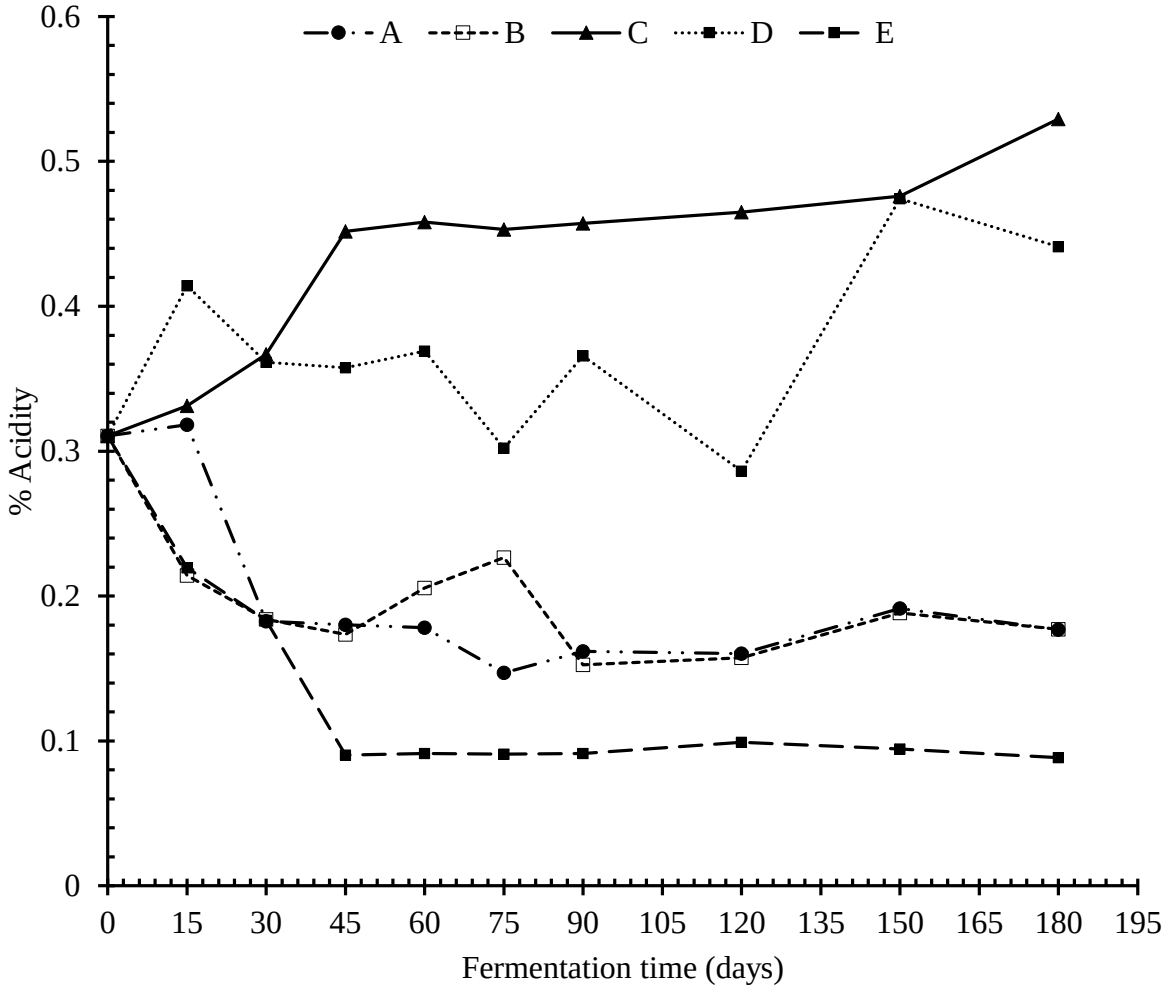

Fig. 4.3 Change in % acidity with fermentation time

The mean values of change in acidity is tabulated in appendix I. The acidity in naturally fermented fish sauce prepared by fermentation of anchovies and sardines were found to be 0.63% as lactic acid (Orillo and Pederson, 1968). Some other researchers found the acid content to be about 1.40% (Nakano *et al.*, 2017; Santo *et al.*, 2005). Upon addition of other carbohydrate source like sugar, lactose, etc. the acidity of the fish sauce was found to be as high as 27.29% as well (Nakano *et al.*, 2017). The % acidity in fish sauce is calculated as anhydrous lactic acid because the fermentation carried out in fish sauce is predominantly lactic acid bacterial fermentation (Kilinc *et al.*, 2006).

From the above figure, it can be observed that the acidity of sample A and sample B decreased slightly and then remained almost constant throughout the experimental period. This indicates that there is almost nil LAB activity in these samples. These two samples were found to be well preserved after the end of experimental period as it was in initial stage of experiment. On the other hand, the acidity of sample E has decreased significantly. Upon visual inspection it seemed to be rotting. Thus this drop in acidity might be because of increase in Total Volatile Base (TVB) content of fish. TVB content is also regarded as a major parameter to determine the spoilage of fish. Sample C and sample D both has shown increase in acidity. Among them sample C shows uniform increase in % acidity over the fermentation period whereas the change in acidity of sample D is not uniform.

Two way ANOVA showed that there is significant difference ($p<0.05$) between samples. So further LSD analysis was done to observe the difference. It showed that sample A, sample B and sample E were not significantly different with each other. Sample C and sample D were significantly different with other samples and they were significantly different with each other too. LSD also showed that sample C is the best among all the samples in terms of % acidity.

4.2.4 Change in pH with fermentation time

Change in pH is directly related with the change in percentage acidity. Higher acidity would result in lower pH values and vice-versa. As the fermentation proceeds, there is increase in acid content in liquid extract of fermented fish, which in turn results in lowering of pH. The change in pH of different samples of fish sauce with fermentation time is shown in Fig. 4.4.

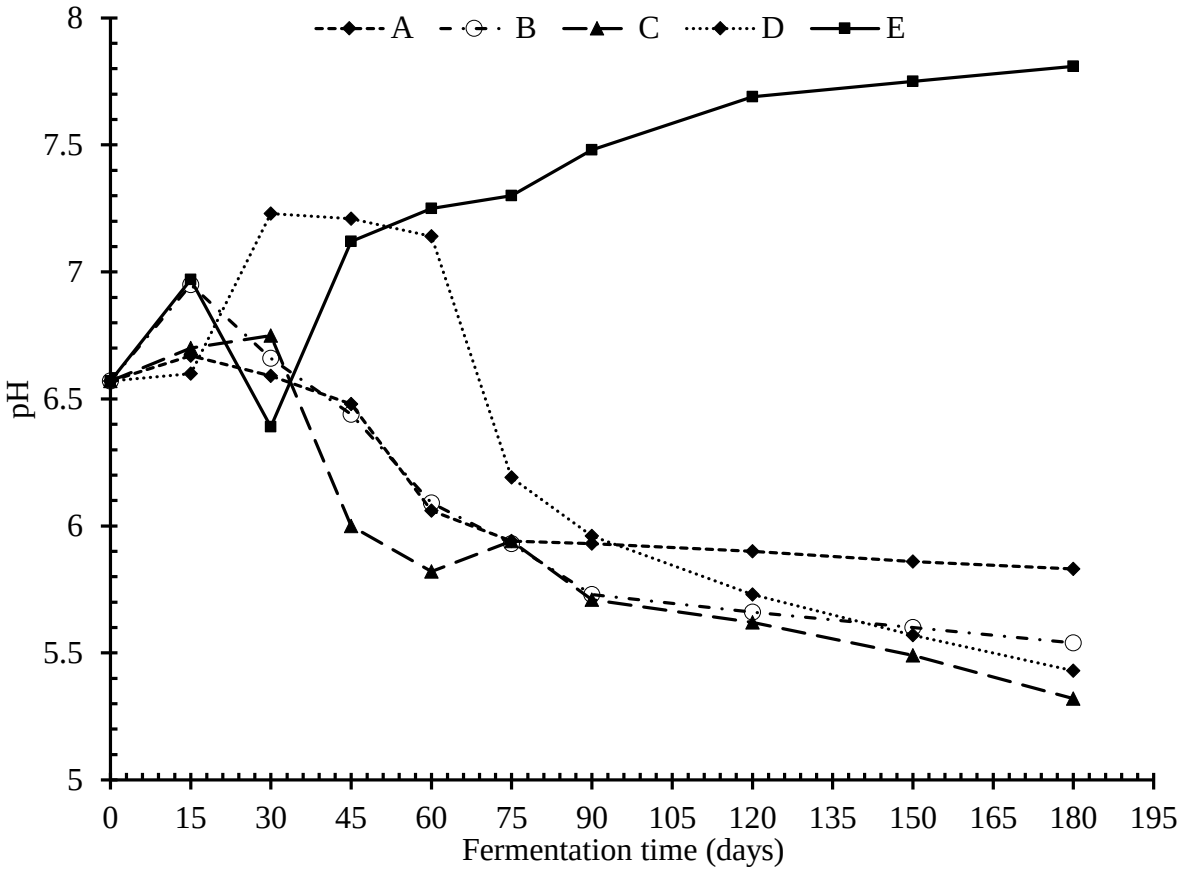

Fig. 4.4 Change in pH with fermentation time

The mean values of change in pH is tabulated in appendix I. The change in pH is observed to be varying with samples. It is because pH change is directly related with change in acidity which is resulted due to ongoing fermentation. Also pH varies with the fish used for fermentation. This is because of variation in chemical composition of different fishes. Chaveesuk (1991) used herring fish to prepare fish sauce and reported final pH to be 5.82-5.85. pH 5.96 was reported by Mueda (2015) when he used anchovy to prepare fish sauce.

Above Fig. 4.3 clearly indicates that all samples except sample E have shown a drop in pH. This lowering of pH is due to formation of lactic acid. The increment in pH of sample E might be because of ongoing spoilage. Sample A showed least lowering of pH followed by sample B. These two samples had highest proportion of salt, i.e.; salt:fish = 1:1 and 1:2 respectively. This salt proportion has inhibited microbial activity. Sample C has shown highest drop in pH followed by sample D. These two samples also showed highest increment in acidity as they are the ones in which there seemed to be proper growth of LAB.

From the statistical analysis, we can see that there is significant difference ($p<0.05$) in pH of samples. Further LSD analysis was done to observe the actual significant difference among the samples. LSD analysis showed that sample A, B and C were not significantly different with each other. Sample D was also not significantly different with sample A but it was significantly different with other samples. Sample E was significantly different with all other samples. In case of fish sauce, lower pH is desirable as it is lactic acid fermented food. Thus from LSD analysis sample C turned out to be best among other samples.

4.2.5 Change in percentage ash content with fermentation time

Change in ash content is observed as the fermentation proceeds. Fermentation results in increament of ash as the muscles breakdown releasing iron into the extract. Also sodium from bones and from salt is available in the liquid extract. The change in percentage ash content of different samples of fish sauce with fermentation time is shown in Fig. 4.5.

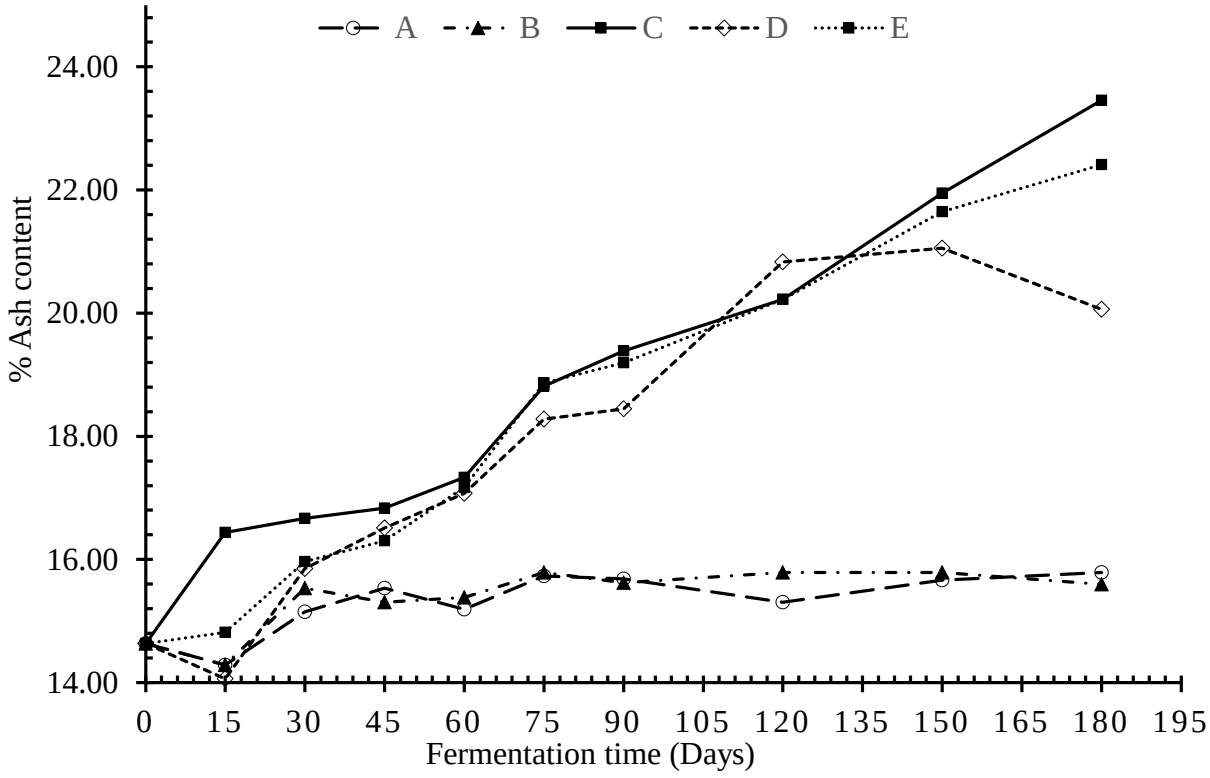

Fig. 4.5 Change in % ash content with fermentation time

The mean values of change in % ash content is tabulated in appendix I. Taking fermented samples, i.e.; sample C, sample D and sample E, we can see that the % ash content ranges from 20.06% up to 23.46%. Similar result were observed by various researchers using other

fishes like shrimp, anchovy, herring, kawala, etc. Osman *et al.* (2012) has reported the % ash content of fish sauce prepared from kawala fish to be 25.40% in fish samples in salted water and 33.41 in fish paste sample. Also Ibrahim (2010) has reported the % ash content to be 19.33% in fish sauce prepared from gambusia fish. Similarly, Pongsetkul *et al.* (2014) reported 33% ash content in shrimp paste. Cho *et al.* (2000) reported 18.2-25.8% ash content in Southeast Asian salt fermented fish sauces. All samples contained salt, which is needed for preservation. Salt content was in the range of salt:fish = 1:1 up to 1:5. This contributed to high ash content of all samples.

From the two way ANOVA test, there seemed significant difference ($p<0.05$) among the samples. It indicated that there is no significant difference between sample A and sample B. Sample D and E also do not possess any significant difference among each other. Also sample C and sample E do not differ significantly. But there is significant difference of sample A and sample B with sample C, sample D and sample E. Sample C also differ significantly from sample D. LSD analysis also showed that sample C is the best among all other samples in terms of % ash content.

4.2.6 Microbiological analysis

Microbiological analysis was done in order to detect the presence of *Escherichia coli* as it is the most prevalent pathogen present in food of fish origin. Thus EMB agar was used and spread plating was done after serial dilution of sample, i.e.; 10, 10^{-1}, 10^{-2}. The plates were incubated at 27±2ºC for 48 h. The observation showed the growth of colonies with blue core. Blue core colonies indicate the presence of lactose fermenting bacteria (Horvath and Ropp, 1974). *E. coli* was absent in the final product because no metallic green sheen was seen. Fukui *et al.* (2012) and Faisal *et al.* (2015) reported absence of any kind of pathogens. They have reported the presence of lactic acid bacteria in final product.

4.2.7 Visual appearance

Visual appearance of sample C was satisfactory. Sample D showed some precipitate. Sample E seemed rotten as the remaining fish pieces along with liquid extract had turned black. It was giving off foul smell. Sample A and sample B did not change much during the experimental period of 180 days. Caramel was used in sample C and sample D for imparting color as stated in Codex Alimentarius (2011). After addition of caramel, the color of sample C seemed similar to that of commercial fish sauce. The smell of sample C was mild and it

had strong fishy taste and smooth mouthfeel. Filtration through muslin cloth followed through Whatmann filter paper No. 1 and Whatmann filter paper No. 41 was done. But clarity was not obtained in any of the samples prepared.

4.2.8 Comparision of proximate composition of commercially available bottled fish sauce and the best fish sauce prepared in laboratory

Commercially available fish sauce in the local market of Dharan was used as control sample in order to compare the proximate composition of fish sauce prepared in the research work. The fish sauce used as control sample was manufactured by Chuen Cheong Food Industries (Ptd) Ltd, 58 Woodlands Terrace, Singapore and is available in the brand name of "Tiger Brand Premium Fish Sauce

As per the result of two way ANOVA and LSD analysis, sample C turned out to be best among other samples. Thus the proximate composition of sample C was then compared with that of control sample. The comparision of sample C with commercial fish sauce was done by using two tail t-test (Paired two sample for means). t-test was carried out in MS-Excel 2013. For the t-test, null hypothesis was set as there is no significant difference between the commercial sample and sample C. On the other hand, alternative hypothesis was set as there is significant difference between commercial sample and sample C.

For every physico-chemical parameter, separate t-test was carried out. It was carried out at 5% level of significance. The degree of freedom for each parameter was 2. For two tail t-test with 2 degree of freedom and 5% level of significance, the tabulated value, i.e.; t-critical is 4.303. From the t-test for pH, t-stat value was found to be 40. As t-stat is greater than t-critical, null hypothesis is rejected, i.e.; there is significant difference between means of pH of sample C and commercial fish sauce. Also for acidity, t-stat was found to be -18.554. Even for the acidity, null hypothesis is rejected because the absolute value, i.e.; non negative value of t-stat was greater than t-critical. Thus it can be said that there is significant difference between sample C and commercial sample.

Even in the case of moisture content, there occurred significant difference between sample C and commercial sample because t-stat value was greater than t-critical value. The t-stat value was found to be 4.380 which resulted in rejection of null hypothesis. Upon t-test of protein between two samples, t-stat value was found to be 3.164. This value was less than t-critical value. Thus null hypothesis is accepted in this case, i.e.; there is no significant

difference between sample C and commercial sample. Also in the case of percentage ash content, t-stat value was found out to be -3.652. The absolute value of t-stat was greater than t-critical, thus null hypothesis is accepted. Thus we can conclude that there is no significant difference between sample C and commercial fish sauce sample in perspective of percentage ash content. The proximate composition of control sample and sample C is shown in Table 4.3.

Table 4.2. Proximate composition of marketed fish sauce used as control sample and sample C

S.N.	Parameter	Composition	
		Commercial	Sample C
1	Moisture content	67.23% (0.011) [a]	67.31% (0.038) [b]
2	Crude protein content (wb)	22.21% (0.046) [c]	23.40% (0.608) [c]
3	pH	5.19 (0.006) [d]	5.32 (0.00) [e]
4	Acidity	0.58% (0.002) [f]	0.53% (0.006) [g]
5	Ash content (as-is basis)	24.76% (0.618) [h]	23.44% (0.018) [h]

*Values are the means of three determinations. Figures in the parentheses are the standard deviations. Figures in the row bearing the same superscripts are not significantly different (p = 0.05). The analytical results of t-test is tabulated in appendix III.

As it is observed that there is no significant difference between sample C, which turned out to be best among laboratory samples, and commercial sample of fish sauce in terms of crude protein content and percentage ash content. But there occurred significant differences between them in terms of pH, acidity and moisture content. There can be occurrence of significant differences between some parameters of fish sauce prepared from different fishes. From the results of research of Sørensen *et al.* (2007), it is observed that chemical analyses revealed differences among tropical fish sauces and experimental fish sauces made from different cold water species. Also, Gildberg (2001) has indicated in his results that even the raw materials and the fermenting conditions can result in significantly different results.

Fish sauces made from Nile tilapia was less acidic than the commercial fish sauce prepared from anchovy. This may be due to acid produced by halophilic lactic acid bacteria normally present in tropical marine fish (Kimura *et al.*, 2001). Change in pH is directly

related with change in acidity. Thus the significant difference in pH of two samples can also be related to the amount of acid produced by halophilic lactic acid bacteria. On the other hand, even though there seemed significant difference between sample C and commercial sample in terms of moisture content, both of them are within the specifications provided by Cruz (1970), FDA and Alimentarius (2011).

Chapter V

Conclusions and recommendations

5.1 Conclusions

On the basis of results and discussions, following conclusions were drawn:

1. The fish sauce prepared by fermenting Nile tilapia fish with salt:fish ratio 1:3 (Sample C) had highest amount of protein, ash and acidity. It was the best sample among others as per the statistical analysis.
2. It was found that the proximate composition of sample C was comparable with the commercial fish sauce prepared from anchovy. It also complied with the several international standards.
3. Although chemical composition were similar, clarity was not obtained.
4. Nile tilapia being cheap yet under-utilized fish in Nepal, it can be used to prepare fish sauce and reduce the import of fish sauce for consumption in Nepali market.

5.2 Recommendations

Based on the present study, following recommendations have been made:

1. Fish sauce can be prepared from Nile tilapia by fermenting for about 6 months at fish : salt = 3:1.
2. Microbiological assay of fish sauce can be done in order to find out the fermentation phenomenon and changes in microflora during fermentation.
3. Enzymes can be used and their effect can be studied.
4. Entrepreneurs can utilize Nile tilapia and can produce fish sauce without much difficulty. By using enzymes, accelerated fermentation can be done, which could prove to be helpful for commercial production.

Summary

Fish sauce is a condiment made from fermented fish and salt. It is used as a staple ingredient in various cuisines in Southeast and East Asia. Fish sauce is the clear aqueous product of prolonged salting fish fermentation. It is made from either freshwater or saltwater fish. Fish sauce is used as a flavoring ingredient. It is a translucent, clear amber yellow or brown liquid, with a salty taste and fish flavor obtained from fermentation of a mixture of fish and salt, and the fermentation takes not less than 6 month. The product is intended for direct consumption as a seasoning, or condiment, or ingredient for many Asian dishes. It is considered as high salt product due to its 20-25% sodium chloride content.

In this study, Nile tilapia fish was used to prepare fish sauce. Nile tilapia was used because it is the fish having good prospect for farming in Nepal yet it is under-utilized. On the other hand, it is also cheap. Nile tilapia fish was collected from NARC RARS Fisheries Research, Tarahara, Sunsari. The average length and weight of fish were 12-15 cm and 250-300 g. Common salt used was bought from local market of Dharan, Sunsari marketed by Salt Trading Corporation, Nepal under the brand name of "Aayo Nun". The moisture content, crude protein content, pH, acidity, ash content and crude lipid content of the raw Nile tilapia were found to be 73.70%, 13.88% (wb), 6.57, 0.31%, 14.63% and 0.43% (wb) respectively. It was kept in fermentation with varying salt concentration. The salt concentration was varied from salt:fish = 1:1 up to 1:5. The samples were kept in fermentation in air tight hard plastic containers. Periodic analysis of proximate composition was done in every 15 days up to day 90 and then every 30 days up to day 180. Percentage moisture content, percentage crude protein content, percentage acidity as anhydrous lactic acid, percentage ash content, pH and percentage lipid were analyzed in every periodic analysis.

After 180 days of fermentation, the analytical results of five different samples were obtained as follows. The moisture content, crude protein content, percentage acidity, pH and percentage ash content of sample A was found to be 27.63%, 14.23% (wb), 0.18%, 5.83 and 15.79% (as-is basis) respectively. The moisture content, crude protein content, percentage acidity, pH and percentage ash content of sample B was found to be 46.03%, 14.8.3% (wb), 0.18%, 5.54 and 15.60% (as-is basis) respectively. The moisture content, crude protein content, percentage acidity, pH and percentage ash content of sample C was found to be 67.28%, 23.72% (wb), 0.53%, 5.32 and 23.46% (as-is basis) respectively. The moisture

content, crude protein content, percentage acidity, pH and percentage ash content of sample D was found to be 67.92%, 23.83% (wb), 0.44%, 5.43 and 20.06% (as-is basis) respectively. The moisture content, crude protein content, percentage acidity, pH and percentage ash content of sample E was found to be 65.45%, 24.03% (wb), 0.09%, 7.81 and 22.41% (as-is basis) respectively. Percentage lipid content was not observed to be significant in any of the samples.

Two way ANOVA test was carried out for statistical analysis of all analytical results. Also Fisher's protected least significant difference test was carried out with 5% significance level. The statistical analysis showed that there was significant difference between samples on all proximate parameters. Among all the samples sample C was found to be best. Its chemical composition was also found to be comparable with commercial fish sauce which was used as control sample. The comparision was done by two tail paired t-test at 5% level of significance. The moisture content, crude protein content, percentage acidity, pH and percentage ash content of the control sample was found to be 67.23%, 22.21% (wb), 0.58%, 5.19 and 24.76% (as-is basis) respectively. Lipid content in control sample was nil. Also the proximate composition of sample C was in compliance with FDA, The Philippines standards. Commercially available fish sauce prepared from anchovies was used as control sample. It was bought from Bhat-Bhateni Super Store, Dharan. It was manufactured by Chuen Cheong Food Industries (Ltd) Ltd, 58 Woodlands Terrace, Singapore.

Further, microbiological test was carried out by spread plating the serial diluted sample in EMB agar and incubating it in 27±2ºC. After 48 hours of incubation, blue core colonies were seen in the plates. It denoted the presence of lactose fermenting bacteria. *E. coli* was absent in fish sauce so prepared. Caramel was added in the best sample, i.e.; sample C. Its color seemed to be similar to that of commercial fish sauce. But clarity was not obtained. Filtration method was implied but clarity could not be obtained. No chemical clarifiers were used in the study.

It is concluded that there is good possibility to prepare fish sauce from Nile tilapia in Nepal and reduce its import of fish sauce. As stated by GoN, Nepal imported fish sauce worth NRs. 39,298,000 in FY 2073/74 from Thailand alone. Nile tilapia being cheap and having good prospect of farming in Nepal, yet under-utilized, can be utilized to produce fish sauce and reduce these import figures significantly.

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Appendices

Appendix I

Table A1: Mean values of moisture content of different samples on different fermentation time

Days\Sample	A	B	C	D	E
0	73.70 (0.13)	73.70 (0.13)	73.70 (0.13)	73.70 (0.13)	73.70 (0.13)
15	48.82 (0.06)	52.22 (0.08)	50.67 (0.09)	52.55 (0.07)	63.38 (0.07)
30	52.38 (0.06)	56.99 (0.09)	62.09 (0.06)	68.89 (0.09)	63.98 (0.11)
45	26.36 (0.09)	58.67 (0.11)	64.02 (0.06)	69.23 (0.10)	66.48 (0.06)
60	26.04 (0.07)	60.00 (0.16)	70.97 (0.18)	72.96 (0.26)	68.89 (0.08)
75	25.57 (0.07)	57.77 (0.07)	74.24 (0.05)	74.65 (0.05)	69.07 (0.16)
90	27.27 (0.07)	55.09 (0.08)	71.76 (0.06)	72.33 (0.18)	69.14 (0.17)
120	27.27 (0.08)	50.43 (0.07)	70.09 (0.07)	71.49 (0.04)	68.66 (0.09)
150	27.27 (0.06)	49.42 (0.05)	68.39 (0.08)	69.72 (0.12)	67.94 (0.07)
180	27.63 (0.06)	46.03 (0.05)	67.28 (0.04)	67.92 (0.06)	65.45 (0.05)

The values in the parentheses denote standard deviation.

Table A2: Mean values of crude protein content of different samples on different fermentation time

Days\Sample	A	B	C	D	E
0	13.88 (0.04)	13.88 (0.04)	13.88 (0.04)	13.88 (0.04)	13.88 (0.04)
15	13.12 (0.02)	13.78 (0.03)	13.30 (0.04)	11.02 (0.04)	16.24 (0.03)
30	15.71 (0.03)	15.32 (0.03)	21.78 (0.03)	24.68 (0.03)	18.82 (0.01)
45	13.11 (0.08)	14.63 (0.03)	19.66 (0.04)	22.58 (0.02)	20.42 (0.03)
60	14.27 (0.01)	14.58 (0.03)	19.49 (0.03)	21.32 (0.02)	20.56 (0.03)
75	14.00 (0.08)	14.49 (0.02)	19.92 (0.03)	21.43 (0.01)	20.98 (0.03)
90	13.88 (0.04)	14.77 (0.03)	20.22 (0.12)	21.42 (0.03)	21.05 (0.04)
120	14.18 (0.04)	14.70 (0.03)	20.58 (0.02)	21.53 (0.03)	22.85 (0.02)
150	14.19 (0.04)	14.94 (0.01)	22.34 (0.02)	22.04 (0.05)	24.01 (0.03)
180	14.23 (0.02)	14.83 (0.02)	23.72 (0.61)	23.83 (0.02)	24.03 (0.03)

The values in the parentheses denote standard deviation.

Table A3: Mean values of % acidity of different samples on different fermentation time

Days\Sample	A	B	C	D	E
0	0.31 (0.10)	0.31 (0.10)	0.31 (0.10)	0.31 (0.10)	0.31 (0.10)
15	0.32 (0.01)	0.21 (0.01)	0.33 (0.01)	0.41 (0.01)	0.22 (0.01)
30	0.18 (0.01)	0.18 (0.01)	0.37 (0.01)	0.36 (0.01)	0.18 (0.01)
45	0.18 (0.01)	0.17 (0.01)	0.45 (0.01)	0.36 (0.01)	0.09 (0.01)
60	0.18 (0.00)	0.21 (0.02)	0.46 (0.01)	0.37 (0.01)	0.09 (0.01)
75	0.15 (0.01)	0.23 (0.01)	0.45 (0.01)	0.30 (0.01)	0.09 (0.01)
90	0.16 (0.01)	0.15 (0.01)	0.46 (0.01)	0.37 (0.01)	0.09 (0.01)
120	0.16 (0.01)	0.16 (0.00)	0.47 (0.01)	0.29 (0.00)	0.09 (0.01)
150	0.19 (0.01)	0.19 (0.02)	0.48 (0.01)	0.47 (0.00)	0.09 (0.01)
180	0.18 (0.01)	0.18 (0.01)	0.53 (0.01)	0.44 (0.01)	0.09 (0.01)

The values in the parentheses denote standard deviation. The indicated values of % acidity are calculated as % anhydrous lactic acid.

Table A4: Mean values of pH of different samples on different fermentation time

Days\Sample	A	B	C	D	E
0	6.57 (0.01)	6.57 (0.01)	6.57 (0.01)	6.57 (0.01)	6.57 (0.01)
15	6.67 (0.01)	6.95 (0.01)	6.70 (0.01)	6.60 (0.01)	6.97 (0.01)
30	6.59 (0.00)	6.66 (0.00)	6.75 (0.01)	7.23 (0.01)	6.39 (0.00)
45	6.48 (0.01)	6.44 (0.01)	6.00 (0.01)	7.21 (0.01)	7.12 (0.01)
60	6.06 (0.02)	6.09 (0.01)	5.82 (0.01)	7.14 (0.01)	7.25 (0.01)
75	5.94 (0.01)	5.93 (0.01)	5.94 (0.01)	6.19 (0.01)	7.30 (0.01)
90	5.93 (0.00)	5.73 (0.01)	5.71 (0.01)	5.96 (0.01)	7.48 (0.01)
120	5.90 (0.02)	5.66 (0.01)	5.62 (0.01)	5.73 (0.01)	7.69 (0.01)
150	5.86 (0.01)	5.60 (0.00)	5.49 (0.01)	5.57 (0.01)	7.75 (0.01)
180	5.83 (0.01)	5.54 (0.01)	5.32 (0.00)	5.43 (0.00)	7.81 (0.01)

The values in the parentheses denote standard deviation.

Table A5: Mean values of % ash content of different samples on different fermentation time

Days\Sample	A	B	C	D	E
0	14.63 (0.04)	14.63 (0.04)	14.63 (0.04)	14.63 (0.04)	14.63 (0.04)
15	14.29 (0.03)	14.29 (0.01)	16.44 (0.02)	14.06 (0.02)	14.81 (0.02)
30	15.15 (0.02)	15.53 (0.02)	16.67 (0.02)	15.85 (0.02)	15.97 (0.01)
45	15.53 (0.02)	15.31 (0.03)	16.83 (0.02)	16.51 (0.02)	16.30 (0.03)
60	15.19 (0.05)	15.38 (0.02)	17.33 (0.03)	17.07 (0.02)	17.17 (0.02)
75	15.73 (0.03)	15.79 (0.02)	18.81 (0.02)	18.28 (0.02)	18.87 (0.01)
90	15.69 (0.03)	15.63 (0.01)	19.39 (0.02)	18.45 (0.01)	19.19 (0.01)
120	15.31 (0.03)	15.79 (0.02)	20.22 (0.02)	20.83 (0.02)	20.22 (0.02)
150	15.66 (0.02)	15.79 (0.02)	21.95 (0.01)	21.05 (0.01)	21.65 (0.02)
180	15.79 (0.03)	15.60 (0.02)	23.46 (0.02)	20.06 (0.02)	22.41 (0.02)

The values in the parentheses denote standard deviation.

Appendix II

ANOVA of proximate constituents of fermented fish sauce

Table: B1: Two way ANOVA for moisture content as variate

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.	LSD
Sample	4	25844.63	6461.16	163.22	<0.001	3.390
Days	8	748.09	93.51	2.36	0.021	
Residual	122	4829.51	39.59			
Total	134	31422.23				

Since, F pr<0.05, samples are significantly different. So, LSD testing is necessary.

LSD at 0.05=3.390

Table B2: LSD for moisture content

Sample	Mean	
A	32.12	a
B	54.11	b
C	66.60	c
E	67.00	c
D	68.84	c

Table B3: Two way ANOVA for crude protein content as variate

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.	LSD
Sample	4	1328.011	332.003	131.46	<0.001	0.856
Days	8	431.580	53.948	21.36	<0.001	
Residual	122	308.113	2.526			
Total	134	2067.704				

Since, F pr<0.05, samples are significantly different. So, LSD testing is necessary.

LSD at 0.05=0.856

Table B4: LSD for crude protein content

Sample	Mean	
A	14.08	a
B	14.67	a
C	20.08	b
E	21.00	c
D	21.10	c

Table B5: Two way ANOVA for % acidity as variate

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.	LSD
Sample	4	1.408488	0.352122	89.57	<.001	0.03378
Days	8	0.197304	0.024663	6.27	<.001	
Residual	122	0.479621	0.003931			
Total	134	2.085414				

Since, F pr<0.05, samples are significantly different. So, LSD testing is necessary.

LSD at 0.05=0.03378

Table B6: LSD for % acidity

Samples	Mean	
B	0.1825	a
E	0.1828	a
A	0.1980	a
D	0.3689	b
C	0.4181	c

Table B7: Two way ANOVA for pH as variate

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.	LSD
Sample	4	30.5533	7.6383	33.29	<.001	0.2581
Days	8	12.3803	1.5475	6.75	<.001	
Residual	122	27.9898	0.2294			
Total	134	70.9234				

Since, F pr<0.05, samples are significantly different. So, LSD testing is necessary.

LSD at 0.05=0.2581

Table B8: LSD for pH

Sample	Mean	
C	6.039	a
B	6.067	a
A	6.138	ab
D	6.341	b
E	7.306	c

Table B9: Two way ANOVA for % ash content as variate

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.	LSD
Sample	4	324.421	81.105	65.25	<.001	0.6007
Days	8	310.234	38.779	31.20	<.001	
Residual	122	151.649	1.243			
Total	134	786.304				

Since, F pr<0.05, samples are significantly different. So, LSD testing is necessary.

LSD at 0.05=0.6007

Table B10: LSD for % ash content

Sample	Mean	
A	15.37	a
B	15.45	a
D	18.01	b
E	18.52	bc
C	19.00	c

Appendix III

Two tail paired t-test for sample C and commercial fish sauce sample.

Table C1 t-Test: Paired Two Sample for Means for pH as variate

	Sample C	Commercial Sample
Mean	5.32	5.186666667
Variance	0	3.33×10^{-3}
Observations	3	3
Pearson Correlation	0.9992644	
Hypothesized Mean Difference	0	
df	2	
t Stat	40	
P(T<=t) two-tail	0.0006244	
t Critical two-tail	4.3026527	

Table C2 t-Test: Paired Two Sample for Means for percentage acidity as variate

	Sample C	Commercial Sample
Mean	0.5264	0.580866667
Variance	3.088×10^{-5}	2.25333×10^{-6}
Observations	3	3
Pearson Correlation	0.436365108	
Hypothesized Mean Difference	0	
df	2	
t Stat	-18.55381201	
P(T<=t) two-tail	0.00289232	
t Critical two-tail	4.30265273	

Table C3 t-Test: Paired Two Sample for Means for moisture content as variate

	Sample C	Commercial Sample
Mean	67.306573	67.22957738
Variance	0.001438	0.000113214
Observations	3	3
Pearson Correlation	0.773462	
Hypothesized Mean Difference	0	
df	2	
t Stat	4.3799617	
P(T<=t) two-tail	0.0483754	
t Critical two-tail	4.3026527	

Table C4 t-Test: Paired Two Sample for Means for crude protein content as variate

	Sample C	Commercial Sample
Mean	23.40074074	22.20969136
Variance	0.369112757	0.002109545
Observations	3	3
Pearson Correlation	-0.962567496	
Hypothesized Mean Difference	0	
df	2	
t Stat	3.16464842	
P(T<=t) two-tail	0.087015114	
t Critical two-tail	4.30265273	

Table C5 t-Test: Paired Two Sample for Means for ash content as variate

	Sample C	Commercial Sample
Mean	23.43893	24.75854063
Variance	0.0003392	0.382381016
Observations	3	3
Pearson Correlation	-0.3978876	
Hypothesized Mean Difference	0	
df	2	
t Stat	-3.6516006	
P(T<=t) two-tail	0.0674913	
t Critical two-tail	4.3026527	

Appendix IV

List of chemicals used

- Catalyst Mixture (Mixture of 2.5 g of powdered Selenium dioxide, 100 g Potassium Sulfate and 20 g Copper Sulfate penta-hydrate
- Mixed Indicator Solution (Mixture of 10 ml of 0.1% bromocresol green and 2 ml of 0.1% methyl red solution which is prepared separately in 95% ethanol)
- Neutral Boric Acid
- Phenolphthalein
- Conc. Sulphuric Acid (H_2SO_4)
- Conc. Nitric Acid (HNO_3)
- Conc. Hydrochloric Acid (HCl)
- Sodium Hydroxide (NaOH)
- Oxalic Acid
- Petroleum Benzine
- Trichloro Acetic Acid
- Rosalic Acid Indicator
- Rectified Spirit

Appendix V

List of apparatus used

- Hot air oven
- Muffle furnace
- Desiccator
- Kjeldahl digestion apparatus
- Micro distillation apparatus
- Soxhlet apparatus
- pH meter
- Thermometer
- Weighing balance (LC: 0.01g)
- Silica crucible

Appendix VI

Spread plated EMB agar microbiological culture plates after 48 hours incubation in 27±2ºC

Plate A1: Control plate of EMB agar

Plate A2: Spread plate with no dilution (10)

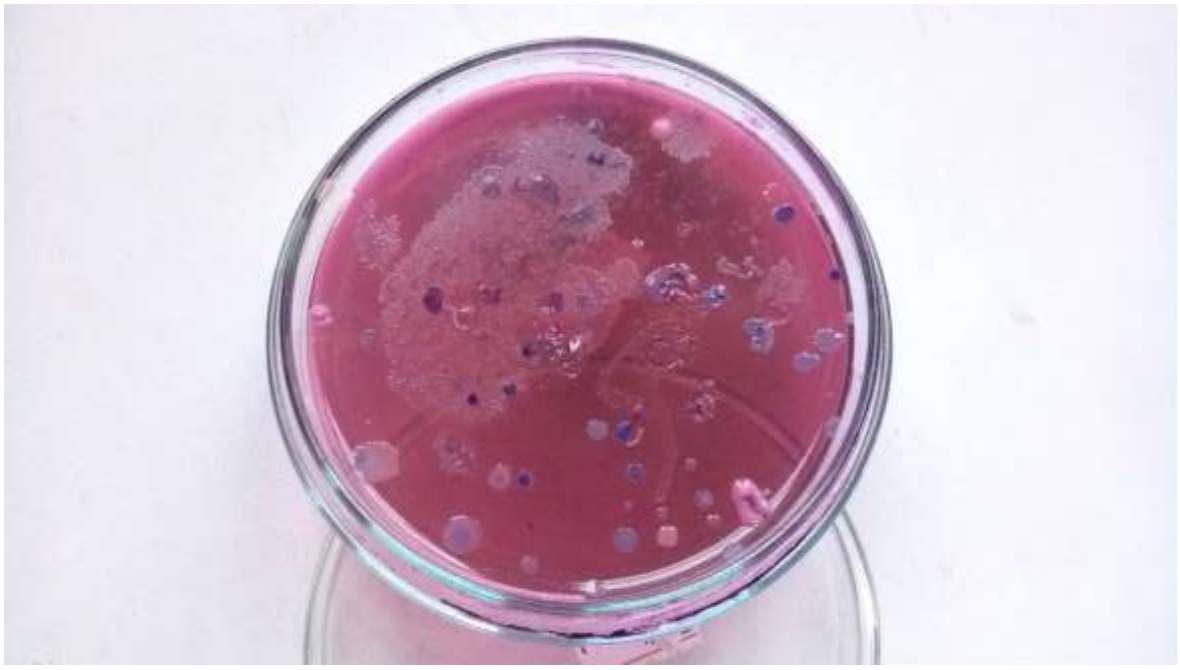

Plate A3: Spread plate with ten times dilution (10^{-1})

Plate A4: Spread plate with hundred times dilution (10^{-2})

Color Plates

Plate A5 Catching fish at RARS, Tarahara, Sunsari

Plate A6 Preparation of fish for fermentation

Plate A7 Lab work at Central Campus of Technology

Plate A8 Lab work at NARC, Khumaltar, Lalitpur

Plate A9 Preparation of EMB agar plates

Plate A10 Final product of salt:fish ratio 1:3

Plate A11 Final product of salt:fish ratio 1:4