Comparative Study of the Nutritional values of Fermented SoyaBean Meals

by

Conganige Shalitha Malshan Anthony

A capstone project report submitted in partial fulfillment of the requirements for the degree of Bachelor of Science in Engineering in
Biosystems Engineering

Examination Committee: Dr. Krishna R.Salin (Chairperson)
Dr. Anil Kumar Anal (Member)
Dr. Amararatne Yakupitiyage (Member)
Dr. Loc Thai Nguyen (Member)

Nationality: Sri Lankan

Asian Institute of Technology
School of Environment, Resources and Development
Thailand
May 2018

ACKNOWLEDGEMENTS

The success of this study would not have been possible without the guidance and support of my advisor,Dr.Krishna R.Salin. I am truly grateful for all the help, encouragement, and suggestions given as well as for putting forth just the right amount of pressure enabling me to do the best. I would like to express appreciation to my committee member Dr.Amararatne Yakupitiyage, Dr.Anil Kumar Anal and Dr. Loc T. Nguyen for their valuable comments and suggestions.

I am greatly indebted to Miss. Rasita Srikerdkruen and Miss.May Wanutsanan, two lab supervisors, Administrative secretary and Ms Chaon Pasuravong for their support and technical assistance. I would also like to appreciate Mr. Dhaval Bamaniya for the help and advices provided me in difficult situations that I faced during my project. I am also extending my appreciation to AARM family and all my classmates for their help, support and encouragement.

This degree and all my humble accomplishments are dedicated to my family for they never failed to inspire and provide me with love and support in whatever endeavor I chose to pursue.

ABSTRACT

The rapid expansion of aquaculture has increased the demand for aqua feed. As fishmeal is expensive, alternative plant based protein sources such as soybean has shown to be a good alternative for aqua feed production. Present study was done to value added to the dehulled and fullfat soya bean by fermentation and APC is added during fermentation of soyabean as ingredient to enhance the nutritional value and balance the amino acid profile of fermented product. According to results comparing crude protein content result showed that the crude protein of fermented soya was increased by probiotic bacteria with the time (FSY 24; 51.72±0.54%DW basis and AFSY 24:51.65±0.54 %DW basis of fermented soyabean.) But the fermentation time is increased to 48h the crude protein is decreased but not significantly,(FSY 48 is decreased to 48.99$\pm$0.92 and AFSY 48h is decreased up to 45.57$\pm$1.89) because the large protein molecules became too small protein molecules, long peptide bonds became short peptide bonds of protein and amino acids. After fermentation, the products pH was varied between 6.5-7.5 all the time due to $NaHCO_3$ to the fermented solution.

The physical properties (bulk density, color, pH) and anti-nutritional constituents of dehulled soya, fullfat soya, AFSY 24 and 48, FSY 24 and 48 were analyzed before and after fermentation. After fermentation, the anti-nutritional constituents like tannin, phytate present in the raw material used was reduced significantly because of fermentation from 1.48 to 0.02 mg/g, 2.98 to 1.58 mg/g respectively. It was observed that the product prepared by fermentation with *Bacillus licheniformis* and *Bacillus megaterium* was found to have high functional property and lower anti-nutritional constituents in compare to traditionally prepared product. (dehulled and fullfat soya), and the color values and bulk density will increase by the fermentation increase.

Key words: Dehulled soyabean, Fullfatted, Soyabean, Aquamimicry Fermented Soya (AFSY), Amino Peptide Concentrated (APC), Fermented Soya (FSY)

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

Abbreviation	Description
APC	Amino peptide concentrated
AFSY	Aquamimicry Fermented Soya (AFSY) Solution
FSY	Fermented Soya
ANOVA	Analysis of Variance
HPLC	High performance Liquid Chromatography
µg	Microgram
FFA	Free Fatty Acid value
AIT	Asian Institute of Technology

CHAPTER 1
INTRODUCTION

1.1 Background

Food fermentation method is widely used and one of the oldest technique for preservation of food from ancient period to enhance the nutritional quality of the product. This simple and easier technology used for food preservation with affordable cost helps to enhance the nutritional quality of food as well as to increase the food safety particularly under condition where refrigeration or other food processing facilities are not available. From the way, back of human civilization, fermentation process of milk, cereals and meat have been explained. First food fermentation was recorded in Middle East and since 6000 BC without the knowledge of microbial role in artificer way. Similarly, by nineteenth century the practice change according to the understanding the process and microorganism involvement (Blandino et.al, 2003).

Fermentations occur when microorganisms consume susceptible organic substrate as part of their own metabolic processes. Such interactions are fundamental to the decomposition of natural materials, and to the ultimate return of chemical elements to the soil and air without which life could not be sustained. Fermentation can enhance nutritional value, especially of plant materials, involves enzymatic splitting of cellulose, hemicellulose, and related Polymers that are not digestible by humans into simpler sugars and sugar derivatives. This goes on naturally in the rumen of the cow through the enzymatic action of protozoa and bacteria. It also occurs in the process of preparing silage for animal feeding. Cellulosic materials in fermented foods similarly can be nutritionally improved for humans by the action of microbial enzymes. By the way Fermentation process efficiently eliminates antinutritive compounds and improves nutritional value of SBM (Hong et al., 2004) Fermentation is one of the oldest forms of food processing and preservation (Ross et al., 2002), and also has the capacity to improve nutritional and functional properties of the original product (Frias et al., 2008). Fermentation has been widely used to increase the bioavailability of nutrients (Hotz and Gibson, 2007) and reduce the levels of antinutritional factors (Egounlety and Aworh, 2003) of SB. Several studies (Kishida et al., 2000; Frias et al., 2008; Song et al., 2008)

Preservation of food resulting from fermentation has been effective form of extending the shelf life of food for millennia. Different fermented foods like dairy, fruits, vegetables, cereals, legumes and meat products which enhance positive health benefits. Lactic acid bacteria (LAB) fermentation is low cost preservation of food, which increases food security and food safety with the reduction of toxicity, anti-nutritional factors and inhibition of spoilage and pathogenic microbes. LAB fermentation increases nutritional quality with increasing vitamins, minerals, essential amino acid and bioavailability of micronutrients which ultimately fulfil the daily nutritional requirement when consumed in daily diet. This proper nutritional supply ultimately helps in the hunger release and eradicate different nutrition related diseases which leads to death with increasing food and nutrition security because of proper utilization of underutilized crops, seasonal and highly perishable crops preserve with simple techniques of fermentation in household for future use without spoilage. Therefore, different lactic acid fermented traditional foods are

important according to health, nutritional, food safety, food security as well as economic aspects (Bourdichon et al., 2012).

Many traditionally fermented Asian foods are non-dairy prepared from the easily and locally available raw materials like cereals and legumes, fruits and vegetables, meat, fish and marine. Lactic acid bacterial like *Lactobacillus plantarum, L. brevis, L. bulgaricus, Bifidobacterium bifidus* etc enhance texture, flavors and aroma of the product along with preservation for long time due to the inhibition of growth of spoilage microorganism. Lactic acid bacteria present in fermented foods was previously reviewed and evaluated their role in many food products like yoghurts, kimchi from Korea, idli (steamed bread) of India, Philippines 'putu' etc in early 1990s (Rhee, Lee, & Lee, 2011). Similarly, there are many more traditionally fermented products in context of Nepal some of these are Gundruk, Sinki, Goyang, Khalpi, Mesu, Selroti, Kinema, Maseura etc and these products are remains within the household. There are many legumes fermented products prepared in Himalayas. People use black grams, soybean, beans which are locally grown to prepare products (Tamang, 2009).

The expansion of aquaculture production has also increased the demand for aquafeed supply. The major cost involved in aquaculture is the cost of aquafeed which comprises more than 50% of the total cost. As the demand for quality fish protein increases, the available wild resources of fish meal and oil for aquafeed production would be limited. The concerns about sustainable development of fisheries and the presence of organic and inorganic contaminants in fish meal have put pressure on using alternative feedstuffs. The major challenge faced by aquaculture industry is the development of economically viable and sustainable alternatives to fishmeal being used in aquafeed.

Both animal and plant protein sources are used in diet formulation for fish. The major problem related to use of animal protein source is inconsistent quality and chances of contamination with chemicals such as dioxin from industrial effluents. Recent researches have focused on the use of alternative protein sources for aquafeed. Many research works on feeding trials using aquafeed with partial replacement of fishmeal with different animal by-product and plant feedstuff have shown optimum growth results for various species. An ideal alternative ingredient for fishmeal should have low starch and fibre content, absence of anti-nutritional factors and high protein content with balanced amino acid profile, high nutrient digestibility and good palatability.

1.2 Problem Statement

Fermentation based feed stuff such as soybean, rice bran, punaak are considered as an alternative protein source for aqua feed production for sustainable aquaculture. Among these Soybean based fermentation give more promising results in the field trials. During fermentation, microorganisms digest the carbohydrates in soybean or soy meal and use for their own growth. The decreased dry matter and increased microorganisms weight ratio result in enhanced protein content. Fermented soy meal with *S.cerevisae* increased its

protein level from 47% to 58%, while with *L. plantarum* and *B. lactis*, protein level increased to 52.08% and 52.14%. Microorganisms used for soybean fermentation have been reported to secret protease during fermentation. 76% of In-vitro digestibility (pepsin), 38% of anti-oxidant activity.

The present study is focusing on understanding nutrition analysis of peptide based soya fermentation with *Bacillus megaterium* & *Bacillus licheniformis* (Yellow Cap & White Cap) in different time interval. This study will help in understanding overall nutrition analysis & in vitro analysis digestible energy of fermented feed stuffs.

This study enhances in better "product development with higher nutritional value. The microorganisms involved during fermentation are well understood in self-fermentation. The fermentation time is optimized so that proper conditions required to make excellent quality soyabean.

1.3 Objectives

1.1.1 Overall objective

The research overall objective is the product development of the plant based aqua feed especially from dehulled & fullfat soyabean by adding Amino Peptide Concentrated solution by the fermentation for use as a feed for fish.

1.1.2 Specific objectives

Specially, the study aims to:

- Optimize the time as the fermentation condition for AFSY & FSY from dehull & fullfat soyabean by using *Bacillus megaterium* & *Bacillus licheniformis.*
- Evaluate the proximate analysis of ingredients (APC, dehull & fullfat soya) and the fermented products.(FSY, AFSY)
- Evaluate the energy content & in-vitro digestibility of APC, AFSY, FSY in different time intervals. (24h & 48 h).
- Evaluate the anti-nutritional factors mainly tannin & phytate for FSY (24h & 48 h), AFSY (24h & 48 h), dehull & fullfat soyabean.

1.4 Scope

Since soyabean used as animal feed because of low cost and high nutritional value with good amino acid balance but raw soyabean. anti- nutritional compounds that toxic to the animals by fermentation efficiently eliminates anti-nutritive compounds and improve nutritional value of the raw material and which ultimately helps in bioavailability of nutrients and helps in food security. This research aims to optimize the fermentation condition of products using *Lactobacillus plantarum.*

CHAPTER 2
LITERATURE REVIEW

2.1 Soyabean

Botanically soybean belongs to Leguminosae family, subfamily papilionoideae, genus Glycine, L and the cultivated type is named as Glycine max (L). soybean plant is small and bushy having height 0.75- 1.25m with branching, densely or sparsely, depending on growing condition and cultivars (K. Liu, 2012). It has different color of seed coat including yellow, red, brown, black and green. These colors are due to the pigment combination especially anthocyanin and chlorophyll. Black soybean contains black coats which signifies high antioxidant bioactivity because of presence of anthocyanin in high amount (Lee, et al., 2009)

Figure 2.1 Soybean plant with green beans, green soybean, matured different color soybean

Mature soybean seed consists of basic three parts; seed coat, embryo and structures for food storage (one or more). Fully mature soybean contains approximately oil 20%, water soluble carbohydrate 10% and 40% protein. These seeds are good source of oil and protein. soybean has relatively higher protein content together with health promoting and bioactive constituents like unsaturated fatty acids, polyphenols (glycinin, saponin, isoflavones and β-sitosterol), superoxide dismutase (Kim, et al., 2011).

Soybean meal is the product obtained by extraction of oil from whole soybeans. The oil may be separated from the soybean by using solvent or expeller (Ingredients101, 2012b). Soybean meal is protein rich and has balanced amino acid profile in comparison to other plant protein sources. The protein to lysine ratio of SBM is 6.36, close to that of fishmeal (7.64). Soybean meal is abundant, competitively priced and has consistent quality compared to other plant sources (Britzman, (nd)). Soybean meal has been successfully used as source of dietary protein in several economically important fish species (Furuya et al., 2004). When Nile tilapia fingerlings (initial mean weight = 5.8g) were fed with SBM based diet supplemented with essential amino acid (lysine, methionine and threonine) and di-calcium sulphate, the total replacement of FM with SBM as protein source did not have significant effect on growth performance, carcass yield and composition of the fish (Furuya et al., 2004). The apparent digestibility coefficient(ADC) of crude protein of soybean meal was found to be 90.9% for Nile tilapia using chromic oxide as indicator (Köprücü & Özdemir, 2005). Likewise, (Dong et al., 2010) reported that ADC values of crude protein of SBM was 97.8% for juvenile hybrid tilapia. This information is useful to understand the feed utilization by fish.

Table 2.1 Nutrition Facts of Soybean

Composition	Unit	Value per 100 g	Value per cup (194 g)
Water	g	11.02	21.38
Energy	kcal	341	662
Protein	g	21.60	41.90
Total Lipid	g	1.40	2.75
Carbohydrate (by difference)	g	62.36	120.98
Fiber, total dietary	g	15.5	30.1
Sugars, total	g	2.12	4.11
Minerals			
Calcium, Ca	mg	123	239
Iron, Fe	mg	5.02	9.74
Magnesium, Mg	mg	171	332
Phosphorus, P	mg	352	683
Potassium, K	mg	1483	2877
Sodium, Na	mg	5	10
Zinc, Zn	mg	3.65	7.08
Vitamins			
Thiamin	mg	0.900	1.746
Riboflavin	mg	0.193	0.374
Niacin	mg	1.955	3.793
Vitamin B6	mg	0.286	0.555
Folate, DFE	µg	444	861
Vitamin A	IU	17	33
Vitamin E	mg	0.21	0.41
Vitamin K	µg	5.6	10.9

(Source: National Nutrient Database for standard reference, 2009)

Soybean is used as different products like soymilk, soy oil, soy flour, poultry and livestock feed, soy concentrate, tofu, soy yoghurt, protein isolates and many fermented soy products such as soy sauce, Tempeh, Natto, Sufu and Miso (Shrestha, Dahal, & Ndungutse, 2013).

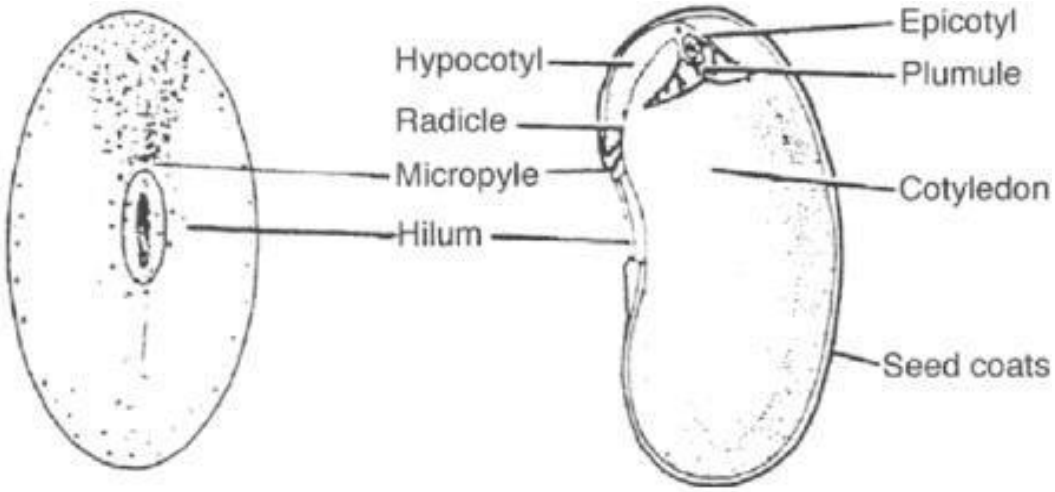


Figure 2.2: Structure of soybean seed

2.2 Plant-based source

The need for sustainable source of protein for aquaculture has shifted towards the use of plant-based proteins in aquafeed. Among leguminous crops, soybean has been the most studied for use in aquafeed as the protein source. Plant protein sources are cheap source in comparison to animal based proteins including fishmeal. Besides being cheaper, they are easily available and have consistent quality. However, they have lower palatability and vary in nutritional and biological values in comparison to fishmeal (Anderson, Lall, Anderson, & Chandrasoma, 1992; J. S. Anderson, Lall, Anderson, & McNiven, 1995). The presence of anti-nutritional factors(ANF) like protease inhibitors, lectins, phytic acid, saponins, phytoestrogens, alkaloids, tannins, cyanogens, and glucosinolates limit the use of plant based proteins in aquafeed (Murray et al., 2010) . These ANF adversely affect the digestion, absorption and physiological utilization of protein and amino acids (Kortner, 2012; Murray et al., 2010; Sandberg, 2002).

The ANF in legumes can be inactivated or reduced by heat treatment (Attia, Shehata, Aman, & Hamza, 1994; Francis, Makkar, & Becker, 2001; Singh, Gamlath, & Wakeling, 2007; Vidal-Valverde et al., 1994), dehulling (Booth, Allan, Frances, & Parkinson, 2001), germination(Alonso, Aguirre, & Marzo, 2000; Bau, Villaume, Nicolas, & Méjean, 1997) and /or fermentation (Singh, 1988).

2.3 Anti-nutritional factors in plant based sources

Numerous substances are naturally present in plants, not needed for normal growth but may have protective functions, are called secondary metabolites. These secondary plant metabolites are also referred to as anti-nutritional factors (ANFs). These ANFs adversely affect the growth performance of animals when fed on them. They may affect by reducing protein digestibility, binding to various nutrients or damaging the intestinal wall, thereby lowering digestive efficiency (Mosenthin, 2010). They can be divided into two categories: heat-labile and heat-stable ANFs. Heat-labile ANFs include trypsin inhibitors, phytates, lectins, goitrogens and antivitamins. Heat-stable ANFs include carbohydrate or soluble fiber, saponins, estrogens, allergins, and lysinoalanine.

The type and content of these ANFs varies considerably among different feedstuffs: Protease inhibitors and lectins are most significant for legume seeds (soybeans, peas, faba beans, lupins), tannins are present in rapeseed, faba beans and peas, whereas glucosinolates and sinapins dominate in rapeseed, alkaloids are important in lupins, and pyrimidine glycosides can generally be found in faba beans (Mosenthin, 2010). These ANFs exert various deleterious effects on monogastric animals, including fish.

There are number of physical and chemical means of processing methods to remove the anti-nutrients in plant proteins. These include: heat treatment (cooking, steaming), soaking, germination, decortication (dehulling), fermentation, selective extraction, irradiation, enzymatic treatment (Deshpande, 2002; Mosenthin, 2010). Heat-labile anti-nutrients such as protease inhibitors, lectins and volatile material like hydrogen cyanide can be reduced to safe level by proper thermal processing. However excessive heating can adversely affect the protein quality of feed.

Soaking the legumes in water and discarding the soaking water can also reduce water-soluble anti-nutrients such as protease inhibitors, phytates, cyanogenic glucosides and polyphenos (tannins). The extent of removal depends on soaking time and temperature, soaking medium (water, salt water or aqueous alkali) and solubility of anti-nutrients (Deshpande & Salunkhe, 2000)

Germination and fermentation helps to reduce oligosaccharides and phytates and increase the bioavailability of minerals. The use of enzymes such as β –galactosidase can remove cyanogenic glycosides. In addition to this, irradiation, extrusion cooking and protein texturisation can be used to reduce anti-nutrients. Selective breeding practices and genetic manipulation can be done to reduce or remove anti-nutrients in legumes (Deshpande, 2002).

2.3.1 Anti-nutrients in soybean

Soybean like other legumes contains a number of anti-nutritional factors or toxicant like protease inhibitors, phytic acid, lectin, saponins, tannins and oligosaccharides. The major two anti-nutritional factors are trypsin inhibitors (TIs) and lectins. There are two types of trypsin inhibitors, the Kunitz trypsin inhibitor (KTI) and the Bowman-Birk inhibitor (BBI). KTI inhibits trypsin, whereas BBI inhibits both trypsin and chymotrypsin. The inhibition of digestive enzymes reduces the digestibility of the proteins. In addition, TIs can cause excessive secretion of cholecystokinin, which in turn leads to excessive secretion of pancreatic enzymes, thus causing pancreatic hypertrophy and hyperplasia. Native lectin is resistant to digestive enzymes and binds to the small intestinal brush boarder, causing increased weight of the small intestine and pancreatic hypertrophy.

The major anti-nutritional factor of challenge in soybean is trypsin inhibitor which is a protease enzyme. To avoid any nutritional problem in animals, it is necessary to remove at least 85% of the trypsin inhibitor units. This can be easily done by heat treatment. Moist extrusion (MC>18%), can destroy up to 95% of the trypsin inhibitor with significant loss of lysine. However, dry extrusion has deleterious effect on lysine. Moist extrusion is better than dry extrusion and traditional roasting (Rokey, 2007b).

The major anti-nutritional factor of challenge in soybean is trypsin inhibitor which is a protease enzyme. To avoid any nutritional problem in animals, it is necessary to remove at

least 85% of the trypsin inhibitor units. This can be easily done by heat treatment. Moist extrusion (MC>18%), can destroy up to 95% of the trypsin inhibitor with significant loss of lysine. However, dry extrusion has deleterious effect on lysine. Moist extrusion is better than dry extrusion and traditional roasting (Rokey, 2007b).

2.3..2 Challenges and limitation of plant-based aqua feed

Plant based aqua feed face challenge in aquaculture due to the presence of anti-nutritional factors. The plant based feeds investigated in different fishes have shown lower digestibility and histological changes in intestines of fish, mainly that of carnivorous species.

2.3.3 Nutritional value of fish fed with plant-based aqua feed

Fish is regarded as nutritive source of protein and omega-3 fatty acids for human consumption. The fatty acid profile of fish is reflection of the diet consumed by the fish. Fish do not produce these fatty acid by itself, but has to be supplied by diet. In natural environment, fish get this fatty acid through consumption of zooplanktons and algae. It is reported that the muscle fatty acid composition indicated higher level of polyunsaturated (n-6) fatty acids in trout fed plant protein diet and a higher content in polyunsaturated n-3 fatty acids and n-3/n-6 ratio in trout fed fish meal diet. To increase the level of omega-3 fatty acid in fish, the fish can be supplied with diet rich in this fatty acid just few weeks before harvesting (de Francesco et al., 2004).

2.4 Anti-Nutritional constituents

Foods contains more than 50 complex substances and chemical compounds which nourish human body. The nutrients present in foods includes water, lipids, proteins, carbohydrates, vitamins and minerals which helps in proper functioning of body. Additionally, most of the plant foods composed of natural components or anti-nutrients which generally concern with the human health by reducing the nutrients availability and absorption in the body when consumed (Prathibha, Nambisan, & Leelamma, 1995). The antinutrients presents in foods adversely effects on human health by inhibiting protein digestion, iron and zinc absorption and normal growth of body leading to malnutrition (Omoruyi, Dilworth, & Asemota, 2007). Anti-nutritional factors present in plant foods includes enzyme inhibitors (amylase and trypsin inhibitor), oxalates, saponins, phytate, total polyphenols, tannins, cyanide, etc (Adane Tilahun, 2009).

2.4.1 Oxalates

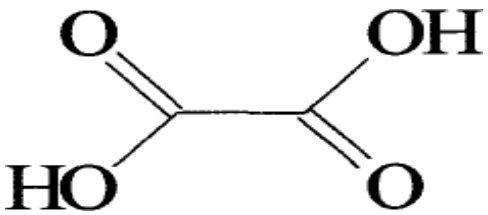


Figure 2.3 Structure of oxalic acid

Oxalis in the plant, commonly called wood sorrel form oxalic acid (HOOC-COOH) which is strong organic acid widely distributed (Liebman, 2012). The bonds formed between

minerals like calcium, sodium, potassium and magnesium and oxalic acid are strong which results in the oxalate salt formation and these salts are known as oxalates. Oxalate salt like potassium and sodium are soluble but calcium oxalate is insoluble. This insoluble calcium oxalate play vital role in kidney stone formation due to precipitation of calcium oxalates in kidneys (Noonan & Savage, 1999).

Oxalic acid is widely spread and common components available in most of plant families and if found higher in corms and leaves (Savage & Catherwood, 2007). Oxalates is one of the major limiting constituents found in taro which cause irritation and astringent taste when eaten raw and cause swelling of mouth, throat and lips. Oxalate is found both in leaves and tubers but their effect is reduced during cooking (Paull, Tang, Gross, & Uruu, 1999)

2.4.2 Phytates

Phytic acid is common in plant kingdom with hexaphosphate of myo-inositol, especially present in seeds like cereal grains, legumes, fruits and vegetables. In plant, this compound is primary storage of phosphorus accounting up to 80% total phosphorus. Strong binding occurs between anions phosphate of phytic acid and metallic cations (Ca, K, Fe, Zn, Mn and Mg) which forms mixed salt known as phytate or phytin (Raboy, 2001).

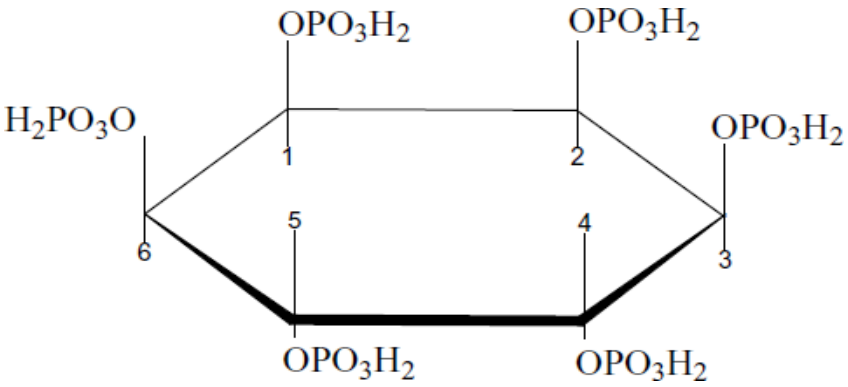


Figure 2.4 Molecular structure of phytate (John *et al.*, 2004).

Phytate is complex and cannot absorbed and digested in gastrointestinal tract because of its complex negative charge (12 negative charge with 6 phosphate groups) form which can be dissociated depending on pH condition. Deficiencies of nutritionally useful minerphosphorus is the action of cationic binding of phytate salt and also lowers the bioavailability of phosphorus (Gibson *et al.*, 2006)

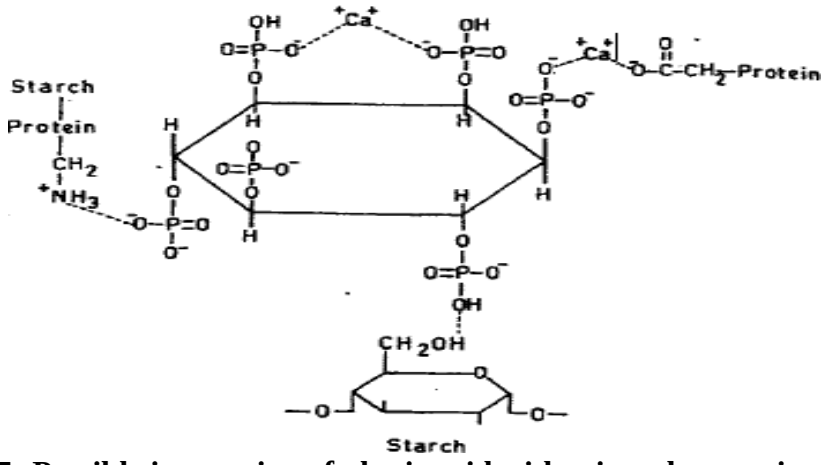


Figure 2.5: Possible interaction of phytic acid with minerals, protein and starch

2.4.3 Tannin

Tannin are phenolic compounds which is water soluble and have molecular weights of 500- 3000 which usually give phenolic reactions having special characteristics like ability of precipitating gelatin, protein and alkaloids. The dark color and astringent taste of food is often due to tannins. They can have a large influence on the nutritive value of many foods eaten by humans such as vegetables, fruits, chocolate, tea, alcoholic and non-alcoholic beverages, etc. Foods rich in tannins are considered to be of low nutritional value because they precipitate proteins, inhibiting digestive enzymes and iron absorption and ultimately effects on the utilization of minerals and vitamins from meals (Tinkílíç, 2001).

Tannin are indigestible complex with protein and affects protein digestion, poor absorption of calcium and iron as well as carbohydrate with reducing energy. Tannin are usually available in leaves, fruits, roots, barks and trees wood (Adeparusi, 2001).

2.4.4 Cyanide

Hydrocyanic acid (HCN) is volatile compounds which rapidly evaporates in air at temperature of 28°C, dissolve in water and easily lost during storage, analysis and transportation (FAO, 1990). The cyanide consumption even in low amount for long time can induce to iodine deficiency causing goiter. Cytochrome oxidase enzyme in mitochondria cell is inactivated by hydrogen cyanide, binding with Fe^{3+}/Fe^{2+} present in enzyme and this activity cause reduction of oxygen utilization in tissue and reduce energy availability in cell. Similarly, this compound inhibits tricarboxylic acid (TCA) cycle and reduce rate of glycolysis. Cyanide increase level of blood glucose as well as lactic acid with ATP/ADP ratio reduction with shifting mechanism to anaerobic to aerobic (Adane Tilahun, 2009). Food with cyanide content if taken in excessive amount increases vulnerability to cyanide toxicity (FAO, 1990).

2.4.5 Elimination and reduction of antinutrients

To eliminate and reduce the level of toxic compounds and antinutritional factors present in food, researcher are working with many methods including biotechnology, breeding and different processing methods like cooking, extrusion cooking, roasting, fermentation, etc. (Adane Tilahun, 2009).

2.4.6 Breeding as a means of reducing antinutrients

The most common method used during plant research is breeding of plant to increase nutritional value along with the decrease of antinutritional factors present which are responsible to the undesired quality. This also helps in enhancing the acceptability and palatability of plant foods (Adane Tilahun, 2009).

2.4.6 Biotechnological techniques for reduction of antinutrients

Different biotechnology like mutation, gamma radiation have been done to enhance taro utilization with developing high yield taro, resistance to pest and fungal attack during maturation and quality improvement by oxalate content reduction (Joint, 2004).

2.4.7 Processing methods for reduction of antinutrients

The most simple and common method for reducing antinutrients present in food is processing methods and these methods includes germination, extruder cooking, soaking, roasting, boiling and fermentation. These types of simple and common methods helps in reducing toxic substances with the increase of palatability, shelf life as well as post-harvest loss (Adane Tilahun, 2009).

2.4.8 Boiling

Antinutrients which are water soluble are effectively reduced during boiling root crops like taro, cassava leading to the reduction of cyanide, oxalate and some soluble phytate (Adane Tilahun, 2009).

2.4.8 Natural fermentation

Fermentation is most economical and oldest methods for food preservation especially in tropical countries where food spoilage favor due to high humidity and high temperature (Obadina, Oyewole, Sanni, & Tomlins, 2006). Lactic acid fermentation is mostly applied in household level which reduce spoilage and reduce volume with improving nutrient value, taste and appearance. Natural fermentation of food available locally, seasonally and underutilized foods helps in food security by making suitable for consumption, reducing antinutrients compounds as well as reduction of cooking time (Egbe, Brauman, Griffon, & Trche, 1995). Many food ingredients like legumes, cereals and vegetables are used for preparation of fermented products. Many microorganisms used during fermentation are the part of natural microflora available in raw materials which helps in toxicity reduction imparting health benefits. Different microorganisms as well as fermentation condition helps in the reduction of significant amount of phytate available in raw materials. During fermentation, the microflora used includes Lactic acid bacteria, yeast and mold (Greiner & Konietzny, 2006).

Spontaneous vegetable fermentation occurs because of naturally occurring surface lactic acid bacteria, i.e., *Pediococcus* spp., *Leuconostoc* spp., *Lactobacillus* spp., etc.; however for plant materials the frequently used starter is *Lactobacillus plantarum* for Lactic acid fermentation (Panda, Parmanick, & Ray, 2007). During natural fermentation process microorganisms present in raw materials acted and become unfavorable for spoilage and

pathogenic microbes present like *Bacillaceae* and *Enterobacteriaceae* (Adane Tilahun, 2009*)*.

2.5 Fermentation

There are two types of fermentation process: aerobic fermentation and anaerobic fermentation

Aerobic fermentation:

The main feature of aerobic fermentation is the provision for adequate aeration. In some cases, the amount of air needed per hour is about 60 times the medium volume; therefore, bioreactors used for aerobic fermentation have a provision for adequate supply of sterile air, which is generally sparged into the medium. In addition, these fermenters may have a mechanism for stirring and mixing of the medium and cells, Aerobic fermenters may either be of the stirred-tank type, in which mechanical motor- driven stirrers are provided, or to the air-lift type, in which no mechanical stirrers are used and the agitation is achieved by the air bubbles generated by the air supply. Generally, these bioreactors are of closed or batch type, but continuous flow reactors are also used. Such reactors provide continuous source of cells and are also suitable for product generation when the product is released into the medium. There are two types of aerobic fermentation processes: submerged culture method and semisolid or solid-state method.

Submerged culture method:
In this process, an organism is grown in a liquid medium that is vigorously aerated and agitated in large tanks (fermenters). The fermenter could be either an open tank or a closed tank, may be a batch type or a continuous type, and is generally made of a noncorrosive type of metal or glass lined or is made of wood. In batch fermentation, the organism is grown in a known amount of culture medium for a defined period of time, after which the cell mass is separated from the liquid before further processing in continuous culture. The culture medium is withdrawn depending on the rate of product formation and the inflow of fresh medium. Most fermentation industries today use the submerged process for the production of microbial products.

Semisolid / solid-state method
In this method, the culture medium is impregnated in a carrier, such as bagasse, wheat bran, and potato pulp, the organism is allowed to grow on this. This method allows a greater surface area for growth. The production of the desirable substance and its recovery is generally easier and satisfactory. In a development of fermentation process, the composition of the culture medium plays a major role and will determine to a very great extent the level of the end product. For example, a culture medium containing sucrose enables better production of citric acid by A. *nigar* than any other carbohydrate. The pH, temperature of incubation, aeration, etc. are all important factors in fermentations, and

these have to be optimized for each type of formation. Emphasis is generally placed on the use of cheap raw materials so that the cost of production is low.

2.5.1 Effect of fermentation on pathogenic organisms

The various metabolites like lactic, acetic acids produced by LAB helps in lowering the pH which inhibits the pathogenic microorganisms (Breidt & Fleming, 1997). The mechanism behind is the cytoplasm of harmful bacteria gets acidified and anions gets accumulated inside cells and destruction occurs. The wide variety of gram positive and gram negative organism were inhibited by the action of *Lactobacillus bulgaricus* and *Lactobacillus acidophilus*. Mold growth were controlled by the application of *Lactobacillus plantarum* and *Propionibacterium shermanii*. The minimum effective quantity of LAB (*Lactobacillus acidophilus, Lactobacillus salivarius, Lactobacillus delbrueckii*) filtrates required to inhibit the inoculum of 10^3 cfu/ml of pathogenic bacteria like *Staphylococcus aureus, Bacillus coagulans, E. coli, Pseudomonas aeruginosa* tested was found to be between 20 to 26% (Varadaraj, Devi, Keshava, & Manjrekar, 1993).

2.5.2 Microorganism

Lactic acid bacteria is belong to Gram-positive, grow anaerobically as a fermentative bacteria, formed in non-spore, and are traditionally used in the food development by fermentation. In addition, lactobacilli are also worked as starter cultures in industrial and traditional food fermentation since they support to the conservation, texture, and flavor of the fermented food products. De Vires et.al (2006) confirmed that L. plantarum has the ability to form amino acids.

Fermentation is a metabolic reaction in which microorganism has a main role. Microorganism result many enzymes as biocatalyst in the reaction. Because the enzyme is specific in use, so that it is important to choose the right microorganism to obtain certain product. Microorganism was used in this fermentation is Lactobacillus plantarum. This microorganism has the ability to convert glucose to the lactic acid and other acids.

L. plantarum are homofermentative, so no strain produces gas from glucose. In addition these LAB produce acetic acid that causes a pungent off-flavor. *L. plantarum* reduced the pH to below 4.3. The different temperature need the different time to reduce pH, such as reported by Essid (2009) that reducing pH in temperature 15, 25 and 37°C was occured for 72, 48 and 24 hours respectively. *L. plantarum* has the proteolytic activity that cause it could hydrolyze caseins. The enzymatic activities of *L. plantarum* can be determined by esterase, esterase-lipase, lipase, aminopeptidase and protease activities. All the strains of exhibited strong a-galactosidase activity, whereas b-glucuronidase and N-acetyl-glucosaminodase were detected by only 35% and 11% of *L. plantarum* strains, respectively. Other osidic activities (b-galactosidase; a-glucosidase; b-glucosidase; a-mannosidase; b-fucosidase) were not detected in any strain.

2.5.3 LAB bacteriocins as biopreservatives

Due to the increase in the awareness against health and food habits consumers are more focused on selecting the food no or less chemical preservatives. The long-life food without having a preservatives or modification of process or packaging is a huge challenge, therefore the solution for all these problems is a use of bio preservatives. LAB is a great source of bioactive compounds like bacteriocins which are effective against harmful organisms and finally preserves the quality of food materials. Several metabolic products like acids, hydrogen peroxides, enzymes, gases and diacetyls are antimicrobial in itself (Ouwehand, 1998). The specific proteinaceous substance like bacteriocin's inhibits the growth of pathogens like *Clostridium, Listeria, Staphylococcus, Enterococcus, Bacillus spp.* and increase the shelf of food products (Soomro, Masud, & Anwaar, 2002). There are four class of bacteriocin's produced by LAB. Nisin is produced by *Lactobacillus lactis sub spp. lactis* strain belongs to class I lantibiotics which is effective against gram positive including *Listeria spp*. (Jack, Tagg, & Ray, 1995). It prevents the growth of spores of *bacillus* and *clostridium* spores by the addition of calcium chelator. The cationic peptides which is relatively small of about 30 to 100 amino acids are called as class II bacteriocins with higher stability towards heat. *Lactobacillus genera* produce the helveticin j as a class III bacteriocins which are safe and natural inhibitors of pathogens. Similarly, class IIa pediocin produced by the *Pediococcus acidilactici* is effective against the *listeria* in vegetables, cheese and meat products (Soomro et al., 2002).

2.5.4 The reaction of lactic acid bacteria fermentation can be described by this pathway:

1. EMP Pathway

The EMP pathway is used by homofermentative lactic acid bacteria, Enterococcus faecalis, Bacillusspp., and yeasts. Ray (2004) mentioned that lactic acid bacteria fermentation resulted end products as lactate (by homofermentative lactic bacteria), and heterofermentative lactic bacteria produce lactate, acetate, ethanol, CO2, diacetyl, acetone.

Glucose phosphate □ Fructose diphosphate □ 2 Pyruvate
Lactate fermentation: Pyruvate □ Lactate
Alcohol fermentation: Pyruvate □ Acetaldehyde □ Ethanol
↓
CO2

2. HMP Pathway

The HMP pathway is also called the HMP shunt, pentose cycle, or Warburg–Dick-ens–Horecker pathway. It is used by heterofermentative lactic acid bacteria, Bacillus spp., and Pseudomonasspp.

Glucose-phosphate □ Phosphogluconate □ Ribulose phosphate
↓
CO2

□Ribose phosphate □ Acetyl phosphate + Pyruvate
Acetyl-P □ Acetate or Ethanol
Pyruvate □ Lactate"

3. Pentose Phosphoketolase Pathway

This pathway is used by Escherichia coli, Enterobacter aerogenes, Bacillus spp., and some lactic acid bacteria.
Ribose phosphate Xylulose phosphate
 Acetyl-Phosphate + Pyruvate
 Acetate or Ethanol + Lactate
Lactic acid bacteria also has ability to hydrolyse protein to the simpler amino acids and peptides. Metabolism of single amino acids is carried out through different types of deamination (producing the C-skeletone and NH_3), decarboxylation (producing amines and CO_2), and hydrolysis (producing the C-skeletone, CO2, NH_3, and H_2). The C-skeletones (fatty acids, a keto acids, unsaturated acids) are then used to supply energy and other metabolic products.

2.5.5 Growth pattern

Bacteria grow by binary fission in which there are several steps, such as DNA replication and separation, split the DNA, septum and invagination formation of wall and cell segregation. In this process, one cell asexually divides into two cells, each an essentially true replica of the original cell. Bacteria growth can be determined by increase in number or mass. Therefore, two kind of measurement, counting chamber and tubidimetry can be used to count the mass and the number of cell, respectively. Counting cell numbers, enumerating CFUs, is a methode in which the CFU values are enumerated at different times of growth and a growth curve is plotted using log 10 CFU vs. time. Tubidimetry is a methode to measure optical density in a spectrophotometer at a given wavelength (above 300 nm, usually at 600 nm) of a cell suspension.

Cell growth consist of many stages. Initially, the population does not change (lag phase) in which the cells assimilate nutrients and increase in size. Although the population remains unchanged because of change in size, both cell mass and optical density indicate some increase. Following this, the cell number begins increase, first slowly and followed by a rapid rate. The cells in the population differ initially in metabolic rate and only some multiply, and then almost all cells multiply. This is the exponential phase (also called logarithmic phase). Growth rate at the exponential phase follows first-order reaction kinetics and can be used to determine generation time. Following this, the growth rate slows down and finally the population attends the stationary phase. In this section, a few cells die and a few cells multiply, keeping the living population stable. It is caused by the nutrient shortage and accumulation of waste products. After the stationary phase, the population starts the death phase, in which the cell death rate is higher than its multiplication.

2.5.6 Temperature

Temperature is very important role in microbial growth because it influences the enzyme reactions. In a certain range, with every 10°C rise in temperature, the catalytic rate of an enzyme doubles. Similarly, the enzymatic reaction rate is reduced to half by decreasing the temperature by 10°C.Microorganisms are divided into three groups on the basis of their temperature of growth, each group having an optimum temperature and a temperature range of growth:

(1) thermophiles (grow at relatively high temperature), with optimum temperature 55°C and range 45 to 70°C;
(2) mesophiles (grow at ambient temperature), with optimum at 35°C and range 10 to 45°C; and (3) psychrophiles (grow at cold temperature), with optimum at 15°C and range –5 to 20°C.

2.5.7 pH effect

pH indicates the hydrogen ion concentrations that has a high effect to microbial activity. The lower than limitation cause the cells not only stop growing but also lose viability, the rate of which depends on the extent of pH reduction. Different microbial has different limitation of pH. The pH range of growth for Gram-negative bacteria is 4.5 to 9.0; for Gram-positive bacteria, 4.0 to 8.5; for yeasts, 2.0 to 8.5; and for molds, 1.5 to 9.0.

2.5.8 Substrate concentration

Substrate is consumed by microorganism to produce the products. This reaction rate is not constant, but changing anytime due to substrate concentration. It can be determined by Monod Equation as below:

$$r_c = \frac{kC_A C_C}{C_A + C_m}$$

r,C = reaction rate

k = reaction rate constant

C,A = concentration of substrate

C,c = concentration of cell

C,m = Monod constant

2.5.8 Time

Fermentation is greatly affected by the time. Basically, products was increase along the time. But, for microbial concentration, there is maximum product concentration showed that reaction cannot be continued anymore because the excessive product become a poison for microbe. It can be explained by this graph:

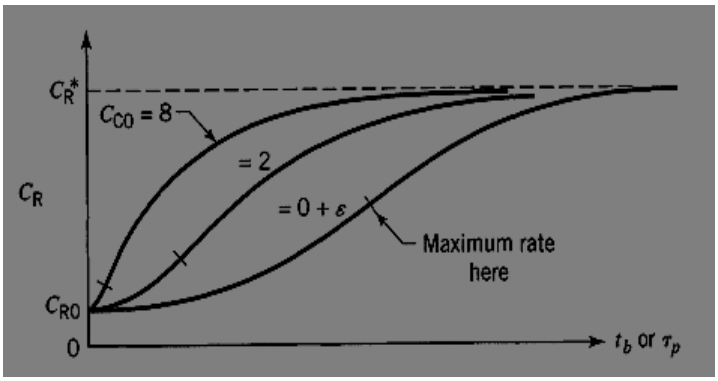


Figure 2.6 :Cell growth in a uniform friendly environment (Levenspiel, 1999)

2.6 Probiotics in Aquaculture

The term probiotic is used to indicate bacteria that support the health of other organisms. It means "for life" as it is originated from two Greek words "pro and "bios". Probiotics are considered as the counterpart of antibiotics. Elie Metchnikoff was probably the first who generated the novel idea of probiotic in 1907, and speculated that fermented milk products could be helpful for human health (Kesarcodi-Watson, 2008). Parker (1974) stated that probiotics are organisms which balance intestinal microbial flora. Usually, "Probiotics", "Probiont", "Probiotic bacteria", or "Beneficial bacteria", are the terms synonymously used for probiotic bacteria.

Generally, probiotics are cultured products or live microbial feed supplements, which beneficially affect the host by improving its intestinal balance and health of the host (Fuller, 1986). The first probiotic discovered was *Lactobacillus* sp., the lactic acid producing bacteria. They were thought to prevent colonization of the gut by other disease causing bacteria – a process known as competitive exclusion. Presently the ranges of probiotics extend well beyond the *Lactobacillus*sp. Including *Bacillus* sp., *Vibrio* sp., *Pseudomonas*, yeasts and algae (Abidi, 2003). Moreover, probiotic is a live microbial adjunct which has a beneficial effect on the host by modifying the host-associated or ambient microbial community, by ensuring improved use of the feed or enhancing its nutritional value, by enhancing the host response towards disease, or by improving the quality of its ambient environment (Verschuere et al., 2000).

In addition, probiotics are nonpathogenic and nontoxic microorganisms without undesirable side-effects when administered to aquatic organisms. They have health benefits for aquatic organisms under cultivation as a means of diseases control, supplementing or even in some cases replacing the use of antimicrobials (Iranto and Austin, 2002).

Generally, a probiotic microbe is able to colonize the gastro-intestinal tract, but the intestinal microflora in aquatic animals' changes rapidly with the constant influx of microbes coming from water and food. The microbial community of the gut can therefore be considered to be transient in nature which allows the extension of the probiotic concept to the use of live microbial preparations in ponds (Abidi, 2003). Therefore, in aquaculture it is difficult to delink probiotics from bio remediators (Karunasagar, 2001).

Probiotics have reduced the occurrence and interval of diseases by direct inhibitions of pathogens and/or enhancement of colonization resistance. There are various mechanisms through which probiotics have shown to inhibit pathogenic bacteria both in vitro and in vivo. Mohapatra et al., (2014) reported that dietary multispecies probiotic supplementation, with two bacterial species (*B. subtilis* and *L. lactis*)and one yeast (*S. cerevisiae*), resulted in enhanced growth and better hemato-immunological status of the *Labeo rohita* fingerlings reared at higher water temperature. Adding such probiotics might have reduced stresses associated with temperature, which in turn lowered the apoptosis in probiotic fed fish.

As mentioned above, probiotics is a rather new concept and relatively newer in the field of aquaculture. Most of the studies have been conducted within a decade and published. Due to methodological and ethical issues of animal studies, it has always been difficult to understand the mechanisms of action of probiotics, and only partial explanations are available.

Although competition for adhesion sites has been widely suggested as the mode of action there is little evidence in literature to demonstrate this. There are studies reporting an adhesion of certain bacteria to intestinal mucous in vitro but transferral of these two in vivo models has not produced supporting results (Hansen and Olafsen, 1999). Attachment availability of potential probiotic seen in vitro cannot be assumed to demonstrate the real effect in vivo. Additionally, while studies to date have demonstrated the ability of certain bacteria to adhere to intestinal mucous *in vitro* (Olsson et al., 1992; Garcia et al., 1996, Joborn et al., 1997), they failed to assess a competitive exclusion effect.

Vine et al. (2004) demonstrated a competitive exclusion effect with five probiotics versus two pathogens on fish intestinal mucus. They found that the presence of one of the probiotics on the mucus inhibited the attachment of one of the pathogens tested. Interestingly, pre-colonization with the other probiotics encouraged attachment of the two pathogens. However, the general trend from their study showed that post treatment with the probiotics displaced the pathogen.

2.6.1 Choice of probiotics

Whether commercial probiotic improves the survival of fish depends solely on the strains of probiotic selected. Therefore, it is essential to know the origin, safety (non-pathogenic) and ability of the strain to survive to the transit through the gastrointestinal tract of the host (e.g. resistance to bile salts, low pH, and proteases). The ability of microorganisms to colonize is often considered as one of the main selection criteria for potential probiotics, that is, the efficient adherence to intestinal epithelial cells to reduce or prevent colonization of pathogens (Vine et al. 2004b). In addition, potential probiotics must exert its beneficial effects (e.g. enhanced nutrition and increased immune response) in the host. Finally, the probiotic must be viable under normal storage conditions and technologically suitable for industrial processes.

Probiotics, live bacteria and yeasts which, when administered in adequate amounts, confer a health benefit on the host, are used with varied successes to treat both infectious and noninfectious enteritis in animals and human beings (Marteau et al., 2010). Probiotics may compete with dietary dependent pathogenic bacteria and the supplementation of putative

probiotics helps better utilize feed thereby enhance growth (Ghosh et al., 2003; Carnevali et al., 2006; Wang and Xu 2006; Sealey et al., 2009). After considering these scientific proofs and commercial application, a wide range of probiotics have been commercially produced in different parts of the world. However, not all the probiotics have been found to be effective. Factors such as type of probiotic bacteria used, species of fish, stages of life cycle, culture conditions, locations and climatic conditions seem to largely affect their efficacies. Therefore, aquaculture farmers are in need of more evidences from the real field and varying culture conditions at different stages of life cycle of cultured species. If certain probiotics clearly show that they could enhance the disease resistance, and more importantly improve growth and survival of fish, they could overcome the concerns regarding limitations and side effects of antibiotics and other drugs (Das et al., 2013; Sahu et al., 2008).

Zhang (2014) reported that the dietary *Bacillus subtilis* as probiotic with fructo oligosaccharide as pre-biotic had a significant synergic effects on enhancing the immune responses and disease resistance of juvenile ovate pompano. Very recently, it has reported that the *Bacillus* species isolated from the gut of healthy *Labeo rohita* showed a great potential for the protection of fish against *Aeromonas hydrophila* infection enhancing the immune response (Ramesh et al., 2015).Positive performance were also reported in *Oreochromis niloticus* (Lara-Flores et al., 2003; Aly et al., 2008; Hassaan et al., 2014; Osman et al., 2010; Abu-Elala et al., 2013), in *Catla* (Das et al.,2013; Krishnaveni et al., 2013; Kadhar et al., 2012), in common carp (Ramakrishnan et al., 2008; Dhanaraj et al., 2010; Bisht et al., 2012; Gupta et al., 2014), in silver carp (Sehandi et al., 2012), in Rainbow trout (Panigrahi et al., 2004 & 2005; Adineh et al., 2013; Tukmechi et al., 2011; Sealey et al., 2009; Sharifuzzaman et al.,2014).

Among the probiotic bacteria, Lactic acid bacteria (LAB), a part of the natural microflora in the gut of a fish, is well recognized in aquaculture. LAB have the ability to produce bacteriocins, which may inhibit the growth of Gram-negative fish pathogens (Brunt and Austin 2005; Nayak et al., 2007; Yin et al., 2007; Ramakrishnan et al., 2008; Wang 2011; Mohapatra et al., 2012; Nguyen et al., 2014). Other microbial groups such as *Bacillus*, *Lactobacillus, Lactococcus* (Shubhash et al., 2007; Ramakrishnan etal., 2008; Sugita et al., 2009; Nandi et al., 2009; Touraki et al., 2012) *Saccharomyces cerevisiae* (Pal et al., 2007; Pooramini et al., 2009; Duarte et al., 2012) and *Pseudomonas pseudoalcaligenes* (Chaudhary and Qazi 2007) have been reported as probiotics in aquaculture. Some reports suggested that different probiotics show different performances in species of fish and their different stages of life cycle, and their mixtures may produce better results than alone (Yanbo et al., 2006; Mohapatra et al., 2012).

Probiotics are often prepared mixing some enzymes such as amylase and phytase which help digestion of starch or complex carbohydrates, and phosphorous. A phytase is a type of phosphatase enzyme that catalyzes the hydrolysis of an organic and indigestible phytic acid, a form of phosphorus that is found in grains and oil seeds and releases a digestible form of inorganic phosphorus. Phytases are found in animals, plants, bacteria, and most commonly in certain types of fungi.

Gomez-Gil et al., (2000), concluded the methods to select probiotic bacteria for use in aquaculture include:
(i) collection of background information;
(ii) acquisition of potential probiotics;
(iii) evaluation of the ability of potential probiotics to out-compete pathogenic strains;
(iv) assessment of the pathogenicity of the potential probiotics;
(v) evaluation of the effect of the potential probiotics in the host; and
(vi) economic cost/benefit analysis. Moreover, selection criteria of probiotics are mainly determined by bio-safety considerations, methods of production, processing, administration and location in the body where the microorganisms are expected to be active. Other factors include stimulation of immune response, selective stimulation of beneficial microbes and suppression of harmful ones, beneficial systemic efforts and physiological aspects(Antony and Philip, 2008).

The application of probiotics in aquaculture shows promise, but needs considerable efforts of research. It is essential to investigate the best way of introduction and the optimal dose of probiotic to keep the organism healthy. It may be wise to carry out long- term study to make sure that the bacteria keep innocuous, without risk of apparition of potentially detrimental mutants (Gatesoupe, 1999).Generally, in vitro antagonism tests, are performed to select probiotics in which pathogens are exposed to the candidate probiotics or their extracellular products in a liquid or solid medium (Balcazar et al., 2006). Diagrammatic representation of selection of probiotics as bio-control agents in aquaculture is as follows (Balkazar et al., 2006).

2.7 soybean fermentation

Soybean foods compose with good nutritional and functional qualities, not only due to their high protein and oil content but also because of phytochemicals. Acceptance of SB protein products as animal feed has increased because of low cost and high nutritional value with a good amino acid (AA) balance (Frias et al., 2008). But raw soyabean is toxic due to their high concentration of serine protease inhibitors or trypsin inhibitors for ruminants.(TI) (Dunsford et al., 1989; Li et al., 1990). To increase acceptability of soybean ,anti-nutritional factors and those need to be eliminated. Fermented SBM (FSBM) is produced from SBM using fungal and bacterial strains. (predominantly *Aspergillus oryzae* and *Lactobacillus subtilis*, respectively) during soybean fermentation efficiently eliminates antinutritive compounds and improves nutritional value of SBM (Hong et al., 2004). FSBM based diet is also recommended for certain fish species as a replacement of fish meal in several studies (Yamamoto et al., 2010; Kader et al., 2012; Yuan et al., 2012).

SBM is not only rich in protein, but also contains almost all the essential and non-essential AAs in good ratio except the two sulfur containing AAs. Glutamic acid is present in largest amount followed by aspartic acid, arginine, alanine, glycine and serine, and proline in SBM. Among the essential AAs, leucine has the highest concentration, followed by lysine, isoleucine, valine, threonine, tyrosine, phenylalanine and histidine. In lower amounts are cysteine and methionine (Hong et al., 2004; Song et al., 2008). Plant proteins, such as SBM, are less expensive than animal protein sources but contain anti-nutritional factors that limit the application of SBM in animal feed. During fermentation process degrading anti-nutritive and allergenic compounds of SBM, thereby increasing the possibilities of utilization of various processed products of SB. The fermentation conditions and nutritional quality of the FSBM thus produced can vary depending on the type of

microorganism used. Aspergillus is the most popular species due to its capacity to produce enzymes such as hemicellulases, hydrolases, pectinases, protease, amylase, lipases, and tannases (Pinto et al., 2001; Mathivanan et al., 2006). In case of bacterial fermentation, various Lactobacillus species and Bacillus subtilis are preferred (Yang et al., 2007).

2.7.1 Microbial fermentation of soybean meal

Microbial fermentation of SBM is achieved by using a fungi or a bacterial strain. The fermentation process can be a solid state fermentation or a submerged fermentation and SBM can be subjected to both processes depending on its state, Much like submerged fermentation, the process related to solid state fermentation has been reported to upgrade the nutritional quality of SBM that can be used in aqua-feed and animal feed industries (Singh et al., 1990; Lena et al., 1997) and is increasingly being employed for SBM fermentation. The peptides content and fibrinolytic enzyme activity increased and the anti-nutrition factors reduced after fermentation. Solid state fermentation also resulted in an increase of in vitro trypsin digestibility and nitrogen solubility under alkaline conditions (Amadou et al., 2010a) and improvement of the nutritional quality of SBM. The efficiency of solid state fermentation in improving nutritional quality and reducing the anti-nutritional factors were ascertained by the works of Amadou et al. (2010a, b).

2.7.2 Comparison of fungal and bacterial fermentation

Fungi-based fermentation: Several species of *Aspergillus* genus have been used to ferment SBM like *A. oryzae* (Feng et al., 2007a, b; Liu et al., 2007), *A. usamii* (Hirabayashi et al., 1998), A. awamori (Kishida et al., 2000), *A. niger* (Mathivanan et al., 2006**)** *A. oryzae* and *Rhizopus oligoporous* established the importance of fermentation in nutritional quality improvement and also created the path for future research works on FSBM. Reduction of TI and large size protein content in FSBM has several beneficial effects when fed to non ruminants such as poultry and swine. Fermentation with *Aspergilli* almost completely eliminates phytate, resulting in a protein source for feed with highly available phosphorus (Ilyas et al., 1995) as well as zinc. Fermentation with fungi also successfully reduces the amount of stachyose and raffinose in SBM. Breakdown of carbohydrates can be attributed to the *α-galactosidase* produced by Aspergilli used in fermentation.

Apart from degrading the anti-nutritional factors, fungal fermentation increases the nutritional value of feed by increasing the crude fat, crude ash, dry matter and CP contents (Hong et al., 2004; Feng, et al., 2007a, b). The increases in protein and fat contents may partially be attributed to the decrease in carbohydrate content during fermentation. Fermentation also significantly increases small size peptides (<15 kD) in FSBM (Hirabayashi et al., 1998). Though FSBM has approximately 10% more CP than SBM, essential AA profile remains unchanged after fungal fermentation (Zamora and Veum, 1979; Hong et al., 2004). Fermentation of SBM with *A. oryzae* did not affect the essential AA concentration but increased the concentrations of glycine, glutamine, and aspartic acid.

Bacteria-based fermentation: Traditionally, Bacillus spp. has been used to produce fermented soy based foods (Hanet al., 2000). Similar to fungal fermentation, bacterial strains also degrade various anti-nutritional factors of SBM including TI. Lactobacillus plantarum is another bacterial strain which has frequently been used to ferment SBM

(Amadou et al., 2010a, b; Amadou et al., 2011). Fermentation with lactic acid bacteria like L. plantarum results in protein hydrolysis and increased liberation of free AAs, thus the resulting FSBM has significantly higher total free AA content as compared to SBM. However, histidine, threonine, methionine and phenylalanine contents do not change whereas leucine, isoleucine, aspartic acid and proline increase after fermentation (Amadou et al., 2010b). Much like fungal fermentation, bacterial fermentation also decreases the protein size, which can be attributed to the enzymes of L. plantarum and to the fermentation process itself (Hong et al., 2004). In vitro trypsin digestibility also increases after fermentation, thereby improving nutritional and functional properties compared to the SBM (Frias et al., 2008; Amadou et al., 2010a).

Table 2.2: Comparison of nutritional quality of unfermented, fungi-fermented and bacteria-fermented soybean meal

Nutritional components affected by fermentation	Unfermented soybean meal	Types of organism used for fermentation	
		Fermented soybean meal	
		Fungi	Bacteria
Crude protein content (%)	34.5	37.4	37.5
Soluble protein content (%)	20	24	33
In-vitro digestibility (pepsin)	60.5	67.4	76
Anti-oxidant activity (%)	8	27	38
Small-sized peptides (<15 kD) (%)	5	35	63

[1] Hong et al., 2004; Teng et al., 2012.

2.7 Aquamimicry

Aquamimicry was the intersection of aquatic biology and technology (symbiotic) synergistically in mimicking the nature of aquatic systems to create living organisms for the well-being development of aquatic animals. Moreover, aquamimicry was mimicking of aquatic systems for high quality (growth) of aquatic animal. Many organism in the aquatic system can receive the benefit together. Therefore, the living feed was increasing in the system.

2.8 Energy content (Bomb calorimeter)

Calorimetry method was an important way for analysis of energy content in the samples, and this method interact with heating during reaction. Melville (2014).

The bomb calorimeter typically consists of a metal bomb designed to withstand heat and pressure, a large Dewar flask to hold the bomb and a known volume of water, a means of remotely igniting the sample (typically electrically, through the used of a fuse wire), and a means of accurately measuring the temperature of the water. Because UC Berkeley is nothing if not a wealthy and generous institution, the Parr bomb used for this experiment came with a number of additional frills and features, including a pellet press to compress the sample into a compact form and the ability to fill the bomb with compressed oxygen, both of which ensure that complete and total combustion of the sample occurs. The sealed

bomb acts as a closed system, and the energy from the adiabatic combustion of a known mass of sample heated the bomb calorimeter and the water a measurable amount.

Azim and Little (2008) found energy content in biofloc between percentage of protein in commercial feed (35 and 24%) with biofloc technology on Nile tilapia by Gallenkamp Bomb Calorimeters**.** The result showed that 35% of protein in commercial feed with biofloc had lower energy (kJ/g) that 24% of protein in commercial feed with biofloc of 18.62 and 19.04 kJ/g respectively.However, Emerenciano et. al.,(2013) found and calculated gross energy content of floc according to Tacon (1990).

The basically energy content of carbohydrate, fat (palmitic acid) and protein (alanine) were 4, 9 and 4 Kcal/gram, respectively.

The method of working of it was direct calorimetry method. The heat from combustion burning of sample was measured. The sample was took in chamber and charged by oxygen gas in high pressure. After that, electricity transfer to the fuse, and there can be ignites occur. The fuel such as food and oxygen (food-oxygen mixture) were in the bomb calorimeter. Thus, bomb calorimeter was covered by insulator to protect transferring into environmental. In addition, temperature increasing of water can know heat releasing of sample such carbohydrate, fat and protein, and there were difference energy content for heat combustion.

Heat combustion was heat energy content releasing by oxidizing reaction. Specific food was storage energy content in the food. Substrate such as glucose, palmitic acid and alanine released energy about 4.15, 9.40 and 5.65 Kcal per gram, respectively.

2.9 Amino acids

Protein are made of many monomers or amino acids link together, it composed of carbon atom in the central or known as the alpha (α) carbon interacted with carboxyl group (COOH), hydrogen atom and amino group (NH2). In every amino acid also contain another type of atom or known as the R group. R group or side chain will make each amino acid have specific characteristics such as pH, size and polarity. Chemical compositions of R group can identified the characteristics of amino acid. The hydrophobic or nonpolar amino acid like alanine, isoleucine, leucine, valine, methionine, phenylalanine, tryptophan, tyrosine and cysteine and the hydrophilic amino acidm included asparagine, glutamine, serine, threonine, aspartic acid, glutamic acid, arginine, histidine lysine, glycine and proline (source: sigmaaldrich.com).

2.9.1 Peptides

The number and sequence of amino acids determine protein's size, shape and function. Each amino acid is attached to another amino acid by covalent bond or known as a peptide bond. When amino acids attached together by peptide bond, the carboxyl group of one amino acid and the amino group of another amino acid combine and release water molecule. The reaction that combines amino acids and generate water molecule is known as dehydration reaction, therefore, the formation of peptide bond is an example of dehydration reaction.

2.10 Commercial probiotics for soyabean fermentation

Commercial probiotics for soyabean fermentation was used in this study is biological aquaculture additives (synbiotics: Engest$_{TM}$ UltraX). For preparation of Amino Peptide Concentrated (APC) solution used by White Cap (WC). In WC there two bacteria species found, they are *Bacillus licheniformis* and *Bacillus megaterium*. The total bacillus count is Min:8.0 x 10^9 cfu/gram and packing is 150 gram per bottle. advantages of white cap is:

- Creates synbiotics environment
- Hydrolyses organic substrates ex situ prior to introduce to shrimp ponds.
- Balances proper pH during day and night.
- Speeds up the natural biocolloids development.
- Environmental friendly, biodegradable component for broader activity spectrum.
- Works in fresh or salt water. (0-50 ppt)

Figure 2.7: Commercial probiotics White Cap & Yellow Cap.

In Yellow cap also be the same bacteria species like WC, *Bacillus licheniformis* and *Bacillus megaterium.* Here the total bacillus count is Min. 8.0 x 109 cfu/gram and packing is 150 gram per bottle. Advantages are:

Solubilizes organic substrates in shrimp ponds.

- Balances an ecosystem in pond environment.
- Speeds up the natural biocolloids development.
- Helps to break down left over feed.
- Balances proper pH during day and night.
- Works in fresh or salt water (0-50 ppt).
- Environmental friendly, biodegradable component for broader activity spectrum.

CHAPTER 3
METHODOLOGY

3.1 Materials

3.1.1 Raw materials:

Dehulled and fullfatted soya were bought from Thanakorn vegetable oil products Co,Ltd, Thiland. APC (Amino Peptide Concentrated solution), AFSY (Aquamimicry Fermented Soya) and FSY (Fermented Soya) were prepared in the lab of Aquaculture and Aquatic Resources Management, Asian Institute of Technology.

3.1.2 Equipment

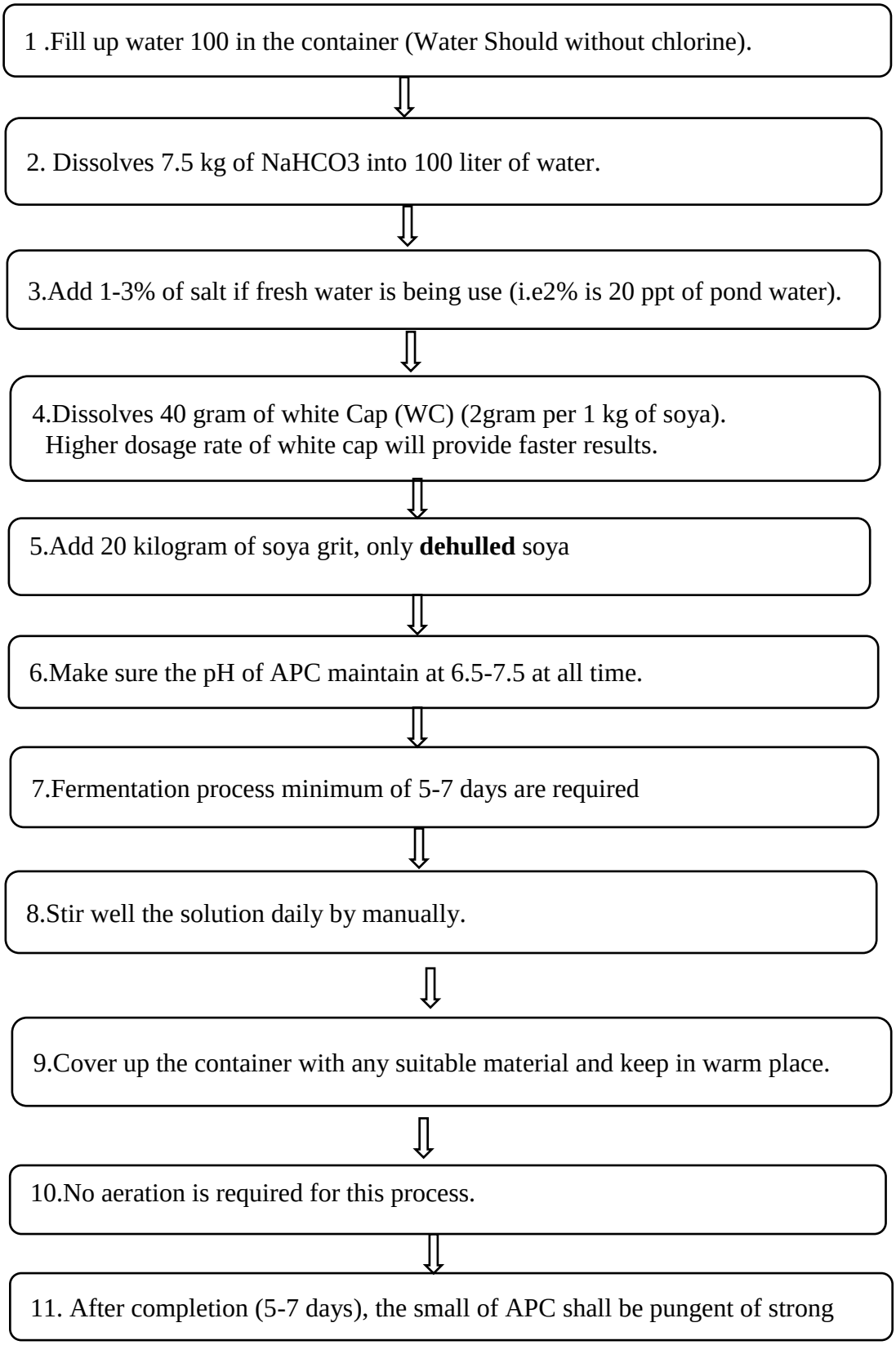
Moisture dishes, desiccator, incubator, autoclave, UV spectrophotometer, muffle furnace, petri dish, pipettes, tips, test tube, beakers, volumetric flask, conical flask, filter paper, digester, analytical balance, aluminum foil, digestion tubes, Kjeltec distilling unit, extraction cups, extraction thmbles, air oven, cotton wool, Color meter (Hunter Lab-Universal), refrigerator, Vortex mixer, Potter Thomas tissue grinder

3.1.3 Chemicals

Folin-ciocalteu's reagent, ethanol, methanol, $AgNO_3$, HNO_3, $CuSO_4$, NaOH solution Petroleum ether ,40-60^{0}C Ethanol ,Hydrochloric acid fuming, Concentrated H_2SO_4, sulphosalicylic acid, Tris HCl buffer, Boric acid, mixed indicator, n-Octanol,Acetone

3.2 Experiment design

3.2.1 Preparation of Amino Peptide Concentrated (APC) Solution (protocol)



⇩

12. The APC solution can be kept for two-four weeks prior to use.

3.2.2 Preparation of Aquamimicry Fermented Soya (AFSY) [protocol]

1.mixing the 1.4:1 ratio, 1.4l of water required for 1kg of soya.(80% of dehulled soya and 20% full fatted soya)

⇩

2. dissolves 3.5% of $NaHCO_3$ base on substrates to be fermented.

⇩

3.add 15% of solution A(APC), as above preparation into the mixture.

⇩

4.Dissolves 2 gm of White Cap (WC)along with 1 gram of Yellow Cap (YC), suitable for 10 kg of soya.

⇩

5.Add the amount of Soya requirement and mix thoroughly

⇩

6. Cover up the container with any suitable material and keep in warm place.

⇩

7. Make sure the pH of AFSY maintain at 6.8-7.2 at all time.

⇩

8. Fermentation process minimum of 12-24 hours required.

⇩

11.After completion (5-7 days), the small of APC shall be pungent of strong salted fish.

⇩

12.The APC solution can be kept for two-four weeks prior to use.

3.2.3 Preparation of Fermented Soya (FSY) [protocol]

Same protocol used for making AFSY , used to make FSY but the 3rd step which "add the 10% of APC ,as above preparation into the mixture" is omitted for making FSY.

3.3 Soyabean and fermented soyabean preparing

Dehulled and fullfatted soya were bought from Thanakorn vegetable oil products Co,Ltd. , and it stored in the store room. In addition, Dehulled and fullfatted soya were sieved through the 500 µm sieve to more all of a big particles out (broken parts and some small stone).

Figure3.1: Dehulled and fullfatted soya were sieved through the 500 µm sieve.

3.3.1 Preparing the Amino Peptide Concentrated (APC) Solution

Prepared APC according to the protocol in the lab of Aquaculture and Aquatic Resources Management, Asian Institute of Technology at room temperature ($25\dot{C}^{0}$) Thailand. and made 3kg of dehulled soya with 15l of water which was eliminated chlorine by aeration one night. Then dissolved WC (white cap: Engest® Synbiotics for Shrimp Farming from Baxel Company) with 6ml for 3kg of dehulled soya. Then add $NaHCO_3$ to maintain the pH in the solution. according to protocol pH of APC maintain at 6.5-7.5 at all time, 1.125kg of $NaHCO_3$ have to dissolved 15l of dechlorinated water and stir well the solution daily by manually for seven days, meanwhile every day recorded the pH and room temperature. For APC fermentation process goes seven days.

3.3.2 Preparing the Aquamimicry Fermented Soya (AFSY) Solution & Fermented Soya (FSY)

Here AFSY and FSY were prepared by the two time intervals 24hr and 48hr at same conditions. For AFSY every two time intervals done three replicates for each 24hr and 48hr. each replicate total product of AFSY was 500g. (1500g for three replicates which one time period 24hr or 48hr)

3.3.3 AFSY 24hr:

Three replicates were done (500g for each one replication) 2.1l of dechlorinated water used for 3 replicates. For preparing AFSY raw materials of dehulled soya 80% and the fullfatted

soya 20% used, according to that for one replication: 400g dehulled soya (for 3 replications 1200g used) and 100g of fullfatted soya (20%) was used (for 3 replications 300g used). Then dehulled and fullfatted soya was mixed with dechlorinated water. Next dissolved WC and YC. (white cap and yellow cap: Engest® Synbiotics for Shrimp Farming from Baxel Company) for one replicate 0.1 ml of WC and 0.05ml YC used. (for three replicates 0.3ml of WC and 0.15ml of YC used).

It is important to maintain pH at 6.8-7.2 in AFSY solution, for that $NaHCO_3$ is used. According to protocol $NaHCO_3$ used 3.5% of 100ml of dechlorinated water so 2100 ml of dechlorinated water (for 3 replicates) 73.5g of $NaHCO_3$ used for three replicates. (for one replicate 24.5g is used).important one is adding APC solution to AFSY because this is main different between AFSY and FSY. According to protocol 15% of APC mixed with the mixture by volume of dechlorinated water, so 2100ml of dechlorinated water APC was used 315g to all three replicates. (for one replicate 105g of APC was used) At last solution was mix thoroughly by manually. Fermentation was done for 24 hours.

3.3.4 AFSY 48hr:

Same procedure that used for AFSY 24hr was apply to this only different is the fermentation time
It is fermented up to 48hours by maintain the pH at 6.8-7.2 all the fermentation time in 25^0C.

3.3.5 FSY 24hr and FSY 48hr:

For making FSY only different is not adding the APC to the mixture, all other condition are same. Three replicates were done (500g for each one replication) 2.1l of dechlorinated water used for 3 replicates. For preparing FSY raw materials of dehulled soya 80% and the fullfatted soya 20% used, according to that for one replication: 400g dehulled soya (for 3 replications 1200g used) and 100g of fullfatted soya (20%) was used (for 3 replications 300g used). Then dehulled and fullfatted soya was mixed with dechlorinated water. Next dissolved WC and YC. (white cap and yellow cap: Engest® Synbiotics for Shrimp Farming from Baxel Company) for one replicate 0.1 ml of WC and 0.05ml YC used. (for three replicates 0.3ml of WC and 0.15ml of YC used). According to protocol $NaHCO_3$ used 3.5% of 100ml of dechlorinated water so 2100 ml of dechlorinated water (for 3 replicates) 73.5g of $NaHCO_3$ used for three replicates. (for one replicate 24.5g is used) for this we have to maintain the room temperature at 25^0C at all the time during 48 hours. FSY 48 also was done according to the procedure of FSY 24, only different is a fermentation time is increased up to the 48 hours. All other conditions applied in the same way.Done the FSY 24hr, FSY 48hr, AFSY 24hr and AFSY 48hr in same time and randomlydesigned the experiment.

Figure 3.1: Experiment design in the lab of Aquaculture and Aquatic Resources Management, Asian Institute of Technology.

3.3.6 Summary of fermentation:

- ✓ Treatments:

There are 12 treatments-

FSY
- 24hr-R_1. R_2 ,R_3
- 48hr-R_1, R_2, R_3

AFSY
- 24hr-R_1. R_2 ,R_3
- 48hr-R_1, R_2, R_3

- ✓ Replications: three replications per treatment 24hr and 48hr
- ✓ Experiment units: 12 units
- ✓ Time: 24 and 48 hours
- ✓ Place: lab of Aquaculture and Aquatic Resources Management, Asian Institute of Technology.

After fermentation time achieved (24hr and 48hr) kept the all fermented products in oven to make a powder form to do proximate analysis. In oven it kept 60^0C at two or three days until it dry. After all fermentation products get dried grind with the grinder until it made a powder form.

Figure 3.2: powder form of FSY 24hr, 48hr and AFSY 24hr and 48hr.

After that put all the samples in zip-block bag, labeled and put it at -4^0C, until the proximate analysis started.

3.4 Chemical composition determination

3.4.1 Moisture content

Oven dried cleaned empty moisture can and lid weight were obtained using an analytical measuring balance. Then 5g of the homogenized protein bar sample was weighed into the tared moisture dish. Homogenization of the sample was done using a mortar and pestle. Then the homogenized sample was then transferred into the pre weighed moisture determining dish. Then the moisture-determining can and the lid were transferred into the oven at 102^0C temperature for a period of 16 hours. Then after 16 hours' moisture, dishes were transferred into a desiccator.
26

After that, the weight of the cool down moisture dish, and the lid weight were obtained using analytical balance.
Weight of the dish = W0
Weight of the dish + wet sample = W1
Weight of the dish + dry sample = W2

Moisture content of sample % = $\frac{\{(W1- W0)-(W2 - W0)\}}{(W1- W0)} x100$

3.4.2 Crude protein (Micro - Kjeldahl method)

Approximately 0.2 g of samples was weighed in digestion tube (W). After that 1 spoon of Catalyst mixture and 10 ml of Sulfuric acid (H2SO4) (Conc.) were added into tube respectively. Then those samples were transferred into digester, and preheated at 420 ^{0}C for 1 hour (the color of sample was changed in to blue color solution). After cooling, 20 ml of distilled water was added into tube. Next, all samples were transferred to nitrogen distillation unit. 40 ml of 45% Sodium hydroxide (NaOH) was added in to samples within unit by the machine, and the sample has changed to black color solution. 25 ml of 4% Boric acid with 3 drops of Mix indicator was added in the flask for extraction of Ammonia (NH3), and then they were distilled for 5 minutes. After that all samples in the flask were titrated using 0.1 N Sulfuric acids (H2SO4) (V2) that was changed into to pink color solution. Blank (which mixed between 1 spoon of Catalyst mixture and 10 ml of Sulfuric acids) solution was analyzed every time when evaluating crude protein (V1).

25 ml of 0.1 N Sodium bicarbonate (Na2CO3) and 3 drops of Mixed indicator were mixed for checking standardization of acid solution every time during Crude protein determination. Next, those were titrated by 0.1 N Sulfuric acids (H2SO4) until it becomes pink, and follow the equation below to calculate Sulfuric acid concentration (N). Then, crude protein can be evaluated by formula below.

Standardization of acid solution:

Weigh approximately 0.4 g of the standard substance using am analytical balance (W1). Transfer sodium bicarbonate to a receive flask and add 40 mL of deionized H2O. Add 10 drops of mixed indicator and titrate to pink. Note the amount of titrant used (A1). Boil this solution for a few minutes. Cool rapidly to room temperature under running tap water. Continue the titration until the next pink colour change occurs. Note also the volume (A2).

Table 3.1: Standardization of acid

replication	A1	A2
R1	35.09	0.53
R2	35.71	0.44
R3	34.49	0.49

R1= $\frac{18.868*0.4010}{(35.09+0.53)}$ = 0.2124M

R1= $\frac{18.868*0.4004}{(35.71+0.44)}$ = 0.2089M

R1= $\frac{18.868*0.4000}{(34.49+0.49)}$ = 0.2157M

Average molarity of the acid solution (M) = 0.2123

N content (%) = $\frac{\text{(Titration- Blank)* Normality of the acid*14.007*100}}{\text{mg of sample}}$

Protein (%) = N content(%)* Conversion factor

Figure 3.3: Distillation of the digested sample (fermented sample solution)

Figure 3.4: Distillation of the digested sample (blank solution)

3.4.3 Crude Lipid Determination (Soxhlet method)

Materials: Dry, homogeneous sample (small and uniform particle size)
Equipments:
1. Analytical balance (4 d.p.)
2. Soxtec system HT 1043-001
3. Extraction thimbles 26 X 60 mm
4. Extraction cups
5. Air Oven (100°C)
6. Desiccator
7. Whatman GF/C filter paper (No.1)
8. Cotton wool

Reagents: 1. Petroleum ether (BP 40-60°C)

Procedure:

Place the required number of extraction cups (numbered) with boiling chips into an oven for 15 minutes, remove cups, cool in desiccator to room temperature, and weigh dry cups to 4 d.p. Weigh out 2-10 g (depending upon the lipid content) of the sample into a Whatman filter paper (4 d.p.). Wrap the sample and insert into a numbered extraction thimble and cover with a thin layer of cotton wool. Hook the thimble into a thimble holder. Repeat 2 and 4 for all samples. Switch on the heating unit of soxtec HT and pre-heat hot plates.Turn cool water supply to condenser unit and ensure the valve on the front arm of the condensers are turned on. Add 30-60 mL petroleum ether into extraction cups. Lower the control lever in the Soxtec HT into "RINSING" position, insert thimbles and raise the lever to 'BOILING' position. Lower the lever controlling position of hot plate and insert cups into the Soxtec HT. Make sure that all cups are properly fixed. Extract lipids for 30 minutes in "BOILING" position and 45 minutes in "RINSING" position. Turn off the valve on the side arm and collect excess petroleum ether. Release the cups and dry at 100°C for 30-60 minutes.Cool the cups in a desiccator and weigh.

Weight of the filter paper = W1
Weight of the filter paper + sample = W2
Weight of the cup + boiling chips = W3
Weight of the cup + chips + lipid = W4
Lipid content of sample (%) = $\frac{(W4-W3)}{(W2-W1)} * 100$

3. 5: crude lipid determination machine

3.4.4 Ash content

Materials: Dry or wet sample

Equipment:
1. Muffle furnace (thermostatically controlled at 550°C).
2. Desiccator with fresh silica gel desiccant
3. Silica crucibles

Procedure: Place the required number of crucibles into a muffle furnace for 15 minutes, remove dishes, cool in desiccator to room temperature, and weigh dry crucibles to the second decimal place. Transfer about 5 g of the prepared sample to the crucible (For dry

samples: weigh the crucible and contents, as rapidly as possible, to the nearest second decimal place). Place the crucibles inside the muffle furnace, as near to the center as possible and ash overnight at 550°C. Remove the crucibles from the muffle furnace, and place in a desiccator and allow to cool to room temperature (approx. 1 hour). Re-weigh each crucible + ash and calculate the percentage of ash as follows:
Weight of clean, dry crucible = W0
Weight of clean, dry crucible + dry sample = W1
Weight of clean, dry crucible + ash = W2
Ash Content of the Sample (%) = $\frac{W2-W0}{W1-W0} * 100$

3.4.5 Crude fiber determination

Materials:
Dry sample with small and uniform particle size.
Preferably a lipid free sample.

Apparatus:

1. Fibertec System consisting of: a Hot Extraction Unit 1010 b. Cold Extraction Unit 1011
2. Oven (100°C)
3. Muffle Furnace (500°C)
4. Analytical balance (4 d.p.)
5. Crucibles (porosity = 40-90 u)

Reagents:

1. Sulphuric acid - 0.128M (12.5 g of H_2SO_4 diluted to 1 litre of distilled water)
2. Sodium hydroxide - 0.313 M (12.5 g of NaOH dissolved in deionized water and diluted to 1 litre)
3. n-Octanol
4. Acetone

Procedure:

Glass crucibles were dried in hot air oven at 105ºC for 2 hours, and cooled in a desiccator for 30 minutes. Then, samples used for crude lipid determination were transferred carefully into glass crucibles (A), and inserted in to the Fibertec system. Next, 150 ml of reagent I (0.128 M Sulfuric acid: H_2SO_4 or 7.1 ml of H_2SO_4 in 1 L of distilled water) with 3 drops of Octanol were added into each sample through control panel, and boiled for 30 minutes. After that, samples were washed 3 times using hot distilled water (~30 ml). Then 150 ml of Reagent II (0.313 M Sodium hydroxide: NaOH or 12.5 g NaOH in 1 L of distilled water) with 3 drops of Octanol was added into control panel, and boiled for 30 minutes. Next samples were washed 3 times using hot distilled water (~30 ml), and 3 times of Acetone (~25 ml). After washing, glass crucibles were transferred into hot air oven at 105oC overnight or 16 hours, and then it was cooled in a desiccator for 30 minutes. Next, the weight (B) of samples were recorded, and burnt in a muffle furnace at 600oC for 4 hours. Then those were cooled in a desiccator for 30 minutes, and reweighed (C).

Sample weight = W1
Weight of the crucible + Dry residue = W2

Weight of the crucible + Ash = W3

3.4.6 Determination of protein content by Bradford protein assay

After collecting the supernatant, the BSA stock solution was diluted to 0.2, 0.4, 0.6, 0.8 and 1 mg per ml. 5 test tubes were contained 5 ml of dye solution, and were added 100 µl from each concentration to each tube. They were mixed and incubated at room temperature for 15 minutes. They were measured OD at 595 nm with blank of dye solution. The standard curve was plotted between concentration of BSA and OD value.
For the samples, 100 µl were taken out and mixed with 5 ml of the dye solution. It was measured OD at 595 nm also. The concentration of protein in samples will be finding from the standard curve with OD.

3.5 Physico -chemical properties

3.5.1 Determination of color

Color of the samples were measured using Hunter colorimeter described accordingly by Yudi pranoto *et al.* (2005) where the values were recorded as L^*, a^* and b^*. Sample was kept in sample holder and kept above the light source by covering lid. Then lightness (L^*), redness (a^*) and yellowish (b^*) values were evaluated. These values are L* black (-) to white (+), a* green (-) to red (+) and b* blue (-) to yellow (+). All the measurements were taken in triplicate and the average value was calculated.

3.5.2 pH determination

A portable pH meter which was standardized by using buffer of pH 7 was used for the pH determination. PH of the sample was determined directly by dipping glass electrode into the sample and wait for non-fluctuating value.

3.5.3 Bulk density

Bulk density for the fermented products & raw materials was determined by according to the method described by Adebowale, Adeyemi, & Oshodi, (2005).Initial weight of a measuring cylinder(M1) was taken. Then a known unique weight of the sample was transferred into the measuring cylinder by compressing the sample by tapping. The weight of the flour-containing cylinder was then taken (M2). The volume of the sample was determined (v).

$$\text{Bulk density} = \frac{M_2 - M_1}{v}$$

3.6 Anti-nutritional factors analysis

3.6.1 Tannin content

Accurately Weighed 0.5g of the powdered material was transferred to a 250mL conical flask. Add 75mL water. Heat the flask gently and boil for 30 min. Centrifuged at 2,000rpm for 20 min and collect the supernatant in 100mL volumetric flask and make up the volume. Transfer 1mL of the sample extract to a 100mL volumetric flask containing 75mL water.

Add 5mL of Folin-Denis reagent, 10mL of sodium carbonate solution and dilute to 100mL with water and Shaken well. Read the absorbance at 760nm after 30 min. Standard of tannic acid was used for the preparation of standard curve (Saxena, Mishra, Vishwakarma, & Saxena, 2013).

3.6.2 Phytate content

Phytate content on the sample was determined according to Eskin and latta 1980. Sample (2 g) was extracted using 2.4% HCL (10 ml) for 1 hour in mechanical shaker at ambient temperature. After 1 hour, centrifugation was done for 30 minutes at 3000 rpm. Then for phytate estimation supernatant was used. 3 mL of sample supernatant extract and 1 ml wade reagent (containing 0.03% solution of $FeCl_3.6H_2O$ and 0.3% of sulfosalicylic acid in water) was mixed well for 5 seconds in vortex. The absorbance was measured in spectrophotometer at 500 nm. Phytic acid was used as standard during analysis.

Standard curve preparation

A series of standard was prepared according to 10, 20, 30, 40, 50, 60 µg/ml by using phytic acid (analytical grade sodium phytate) with dissolving in 2.4% HCL. Then 3 ml of phytate standard were added into 15 ml centrifuged tube with 3 ml of water which was used as blank for setting zero. 1 ml of the wade reagent was added to each test tubes and mixing well for 5 seconds in vortex. Then after centrifugation for 10 minutes the absorbance was taken at 500 nm in the spectrophotometer. Phytate content of the sample was determined using given equation:

$$\text{Phytic acid (µg/gm)} = \frac{\text{Absorbance - Intercept}}{\text{Slope} \times \text{density} \times \text{weight of sample}}$$

3.7 Data Analysis

Data analysis of the response surface methodology was calculated by using analysis of variance (ANOVA). All the experiments were executed in triplicate and mean with standard deviation was also recorded. A commercial statistical package (IBM SPSS Statistics Data Editor, Version 23, USA) was used to perform for the statistical analysis and ANOVA was followed by Duncan test for comparing significance differences ($p<0.05$) within the samples.

CHAPTER 4
RESULTS AND DISCUSSION

Soyabean is an ethnic and traditionally fermented legumes product, during fermentation, nutritional value is increased with the reduction of anti-nutritional factors present in raw materials which ultimately effects to the feed. However, our concern of this research is to develop quality and better product from soyabean by adding APC with *Bacillus licheniformis* and *Bacillus megaterium.*

4.1 Proximate analysis of raw materials (Amino Peptide Concentrated (APC) Solution, Dehulled and fullfatted soya)

The proximate analysis of raw materials APC, dehulled and fullfat was performed by determining protein, moisture content, ash, fiber and energy content. The values analyzed during experiment were illustrated in the below Table 4.1. The protein content, ash, fiber, moisture, lipid and energy content of AFSY and FSY depends on the ratio of APC, dehulled and fullfat used during preparation

Table 4.1: Experimental results of proximate analysis of Amino Peptide Concentrated (APC) Solution, Dehulled and fullfatted soya. (Dry weight% basis)

Sample	Crude protein (%)	Crude lipid (%)	Crude fiber (%)	Ash (%)	Energy content
Dehull soybean meal	49.11±1.12	1.55±.01	7.02 ±1.39	9.66±0.02	17.16±0.16
Fullfat soubean meal	39.65±2.07	21.18±1.34	9.40±0.62	7.11 ±0.04	26.59±4.05
Amino Peptide Concentrated (APC)	38.13±0.09	0.89±0.11	8.43 ±0.08	37.49 ±0.31	14.76±0.04

The table indicates proximate composition (Mean±SE) of the raw materials in desired fermented products. But according to the protocol, For making FSY only different is not adding the APC to the mixture, all other condition are same, For preparing FSY raw materials of dehulled soya 80% and the fullfatted soya 20% used. For preparing AFSY raw materials of dehulled soya 80% and the fullfatted soya 20%. Next dissolved WC and YC. (white cap and yellow cap: Engest® Synbiotics for Shrimp Farming from Baxel Company) for one replicate 0.1 ml of WC and 0.05ml YC used.

It is important to maintain pH at 6.8-7.2 in AFSY solution, for that $NaHCO_3$ is used. According to protocol $NaHCO_3$ used 3.5% of 100ml of dechlorinated water. Important one is adding APC solution to AFSY because this is main different between AFSY and FSY. According to protocol 15% of APC mixed with the mixture by volume of dechlorinated water,

According to the proximate analysis of dehull soyabean, fullfat soyabean and Amino Peptide Concentrated (APC) solution ,according to the results dehull soyabean having high amount of crude protein% in dry basis, 49.11±1.12 but fullfat soyabean having high amount of crude lipid% comparing with dehulled soyabean and APC. Same while fullfat soyabean having high amount of crude fiber% (9.40±0.62) and energy content (26.59±4.05 kJ/g) comparing with other two raw materials.

Table 4.2: Experimental results of proximate analysis of Amino Peptide Concentrated (APC) Solution, Dehulled and fullfatted soyabean by the ratio and the total amount in the final FSY and AFSY Composition.

Sample before fermentation	%mixed before fermentation	Crude protein (%)	Crude lipid (%)	Crude fiber (%)	Ash (%)	Energy content (kJ/g)
Dehull soybean meal	0.8	39.2 ±1.12	1.24 ±.01	5.61 ±1.39	7.73 ±0.02	13.73±0.16
Fullfat souabean meal	0.2	7.93±2.07	4.24±1.34	1.42±0.62	1.42±0.04	5.35±4.05
Amino Peptide Concentrated (APC)	0.15	5.72 ±0.09	0.13 ±0.11	1.26±0.08	5.62 ±0.31	2.21±0.04
TOTAL(dehull + fullfat sybean meal)%		47.2 ±1.15	5.48±0.72	7.04±1.00	9.15±0.12	19.07±1.42
TOTAL(dehull + fullfat soybean meal +peptide)%		51.9 ±1.09	5.61±0.52	8.30±0.69	14.78±0.03	21.29±2.11

This table shows that before fermentation takes place and expected proximate values of the fermented soybean (FSY) and Aquamimicry Fermented Soya (AFSY).

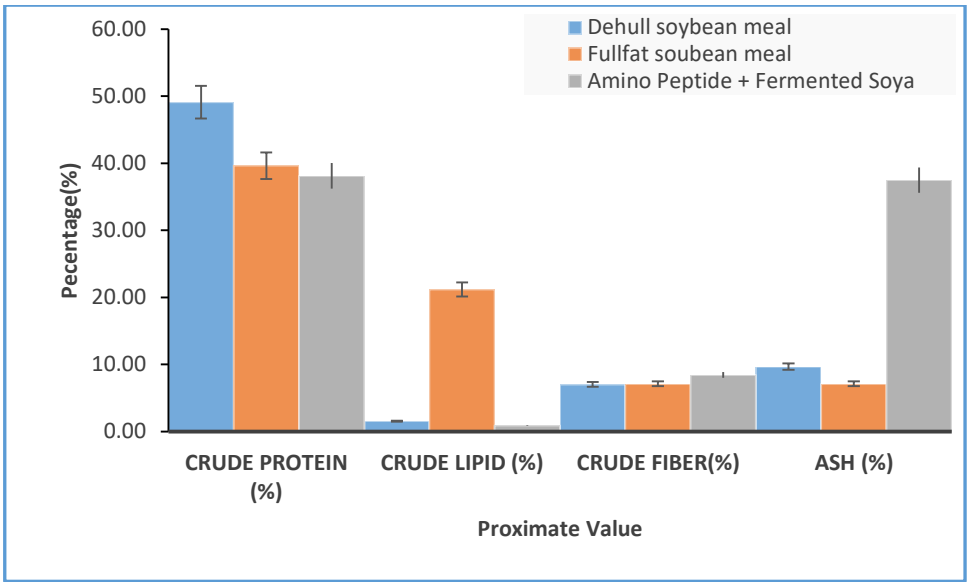


Figure 4.1: Graphically indication of proximate analysis of raw materials.

4.2 Effects of fermentation condition (24hr, 48hr) on the product after fermentation

Table 4.3:

Products after fermentation	Crude protein (%)	Crude lipid (%)	Crude fiber (%)	Ash (%)	Energy content
FSY 24	51.72±0.54	3.83±0.13	7.60±0.18	11.79±0.09	20.73±0.43
FSY 48	48.99±0.92	4.36 ±0.14	8.84±0.62	15.55 ± 2.58	20.47±0.36
AFSY 24	52.65±0.54	4.51±0.06	7.69±0.10	12.76±0.06	20.4 ± 0.04
AFSY 48	45.57±1.89	4.53±0.28	7.74±1.36	15.97±2.71	19.94 ± 0.02

Aquamimicry system was used aquamimicry fermented soya (Pathumthani 1 fragrant rice variety was bought from Thanakorn vegetable oil products Co,Ltd.). Therefore, the commercial probiotic (white cap: Engest® Synbiotics for Shrimp Farming from Baxel Company) for one replicate 0.1 ml of WC and 0.05ml YC used. And the APC added 15% by the volume of decolonized water.

The fermentation processing was allowed for 24 hours and 48 hours, but pH was adjusted around 6.0-7.0 during the fermentation period by sodium carbonate (Na_2CO_3).

The result showed that the crude protein of fermented soya was increased by probiotic bacteria with the time (FSY 24; 51.72±0.54%DW basis and AFSY 24:51.65±0.54 %DW basis of fermented soyabean. But the fermentation time is increased to 48h the crude

protein is decreased but not significantly,(FSY 48 is decreased to 48.99±0.92 and AFSY 48h is decreased up to 45.57±1.89) because The large protein molecules were became to small protein molecule, long peptide bond became short peptide bond of protein and amino acid.

Otherwise, crude lipid was deceased of fermented soyabean, (FSY 24h, 3.83±0.13%DW basis of FSY 24, but before fermented mixture(0h) 5.48±0.72 %DW. Therefore, energy content was also reduced of fermented soyabean (soya bean mixture before fermentation; 19.07±1.42 kJ/g DW and fermented soyabean; 18.73±0.43 kJ/g DW, respectively. In the case of ash content, the ash of fermented soyabean was increased around 11.79±0.09 %DW after fermentation. However, the crude fiber between rice bran and fermented rice bran were not increased too much.(crude fiber 7.04±1.00 in the mixture; and FSY 24 7.60±0.18**)**

If the time period is increased to 48hr the crude protein content is reduced, and the crude lipid is increased because of the bacterial activity, ash content increased by comparing with the 24h fermentation time period. Fermentation of soyabean, the nutrition was increased by many microbial during fermentation. A several researcher found the growth of microorganism on a substrate can change its chemical composition due to the production of exocellular enzymes to obtain nutrients, in addition, production of other metabolites. This metabolism can enrich the substrate, depending on the intrinsic components of the fermentation agent or by nutrients availability presented in the substrate. The microbial action turns the substrate components available to chemical or enzymatic extractive processes (Oliveira et al., 2010).

Lipid and fiber content also were not significantly different. The fiber content of fermented soyabean was higher than normal rice bran because of producing several polysaccharides, especially fungi (Oliveira et al., 2010). On the other hand, the ash and energy content were significantly different ($P<0.05$). In the case of energy content of soyabean had the value higher than fermented soyabean because of highly lipid content. The one of researcher found the lipid can provide energy content about 9 Kcal/g, but for the protein can provide 4 Kcal/g.

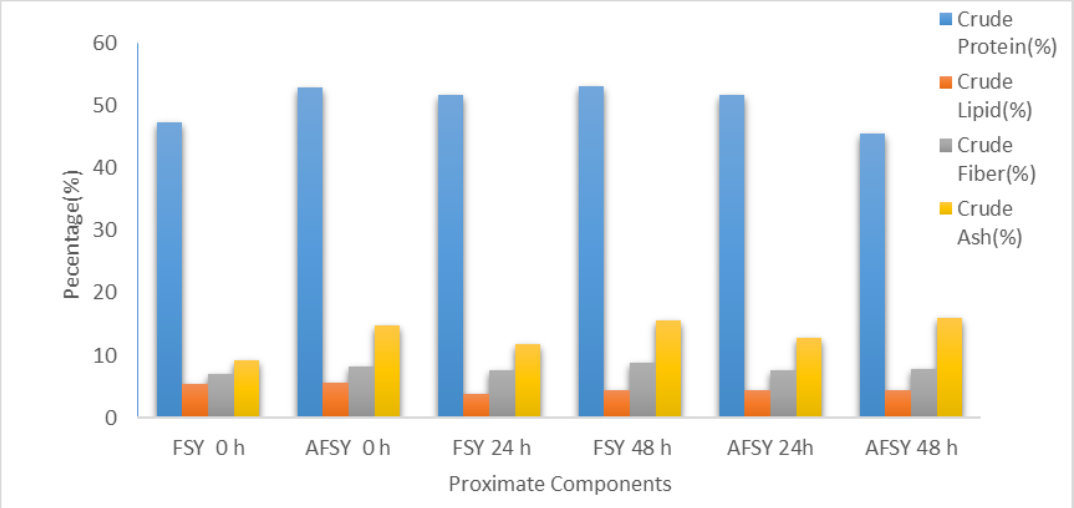


Figure 4.2: overall raw materials and product comparison.

4.3 Physico-chemical characteristic of raw materials

Table 4.4 Color and pH change of raw materials and fermented products

Sample	Color			pH
	L*	a*	b*	
Dehull Soya Bean	19.82±0.02[a]	6.82±0.03[c]	11.75±0.01[a]	6.42±0.02
Full Fat Soya Bean	42.13±0.79[b]	13.59±0.07[f]	27.01±0.690[c]	6.34±0.02
FSY 24	45.39±0.02[c]	11.65±0.02[d]	25.63±0.03[b]	7.07±0.04
FSY 48	51.32±0.03[d]	12.44±0.41[e]	27.56±0.04[d]	7.08±0.02
AFSY 24	60.53±0.02[e]	4.92±0.86[a]	27.63±0.04[e]	7.02±0.01
AFSY 48	69.00±0.32[f]	7.05±0.05[b]	25.72±0.02[f]	6.99±0.02

Values are the means ± standard deviation obtained from triplicate data. Different subscript letter in the same column (a-f) represent the significant difference ($p < 0.05$).

After fermentation, the pH of the raw material mixture prepared for FSY 24hr, FSY48hr, AFSY 24hr and AFSY 48hr the pH range in 6.8 to 7.2 because $NaHCO_3$ was added to the solution as a raw material and it maintain the pH during the fermentation period.

According to Markovic et al., (2013) consistent color dispensation of L*,a* and b* color space and the color attributes which are nearly related to the human perception, whereas the L*,a* and b* values are widely used in determination of the properties of the color attribute.

Similarly, when the color of the raw materials and the mixture of both raw material before and after fermentation was analyzed it was clearly shown that the L^* value (lightness) as well as yellowish (b^*) of the mixture of raw material decrease after fermentation whereas redness (a^*) increase with the fermentation time. From ANOVA table, it was clearly shown that fermentation have significant effect ($p < 0.05$) on the color values.

Table 4.5: bulk density values of the raw materials and fermented products

Samples	Bulk density
Dehull Soya Bean	0.88 ± 0.01^{e}
Full Fat Soya Bean	0.81 ± 0.01^{b}
FSY 24	0.83 ± 0.02^{d}
FSY 48	0.98 ± 0.01^{g}
AFSY 24	0.89 ± 0.01^{f}
AFSY 48	0.82 ± 0.02^{c}
APC	0.79 ± 0.01^{a}

Density for the protein bar was determined by according to the method described by Adebowale, Adeyemi, & Oshodi, (2005). After fermentation process the bulk densities are increase.

4.4 Anti-nutritional constituents (tannin and phytate)

Table 4.6: Change in anti-nutritional components of raw materials and product

Sample	Tannin	Phytate
Raw materials (dehull 0.8 full fats 0.2)	1.48 ± 0.07^{abc}	$2.98^{e}\pm0.33$
FSY 24 hours	0.27 ± 0.10^{ab}	$2.74^{d}\pm0.02$
FSY 48 hours	0.07 ± 0.00^{a}	$1.87^{b}\pm0.01$
AFSY 24 hours	0.14 ± 0.00^{a}	$2.45^{c}\pm0.01$
AFSY 48 hours	0.02 ± 0.21^{a}	$1.58^{a}\pm0.01$

Values are the means ± standard deviation obtained from triplicate data. Different subscript letter in the same column (a-e) represent the significant difference ($p< 0.05$).

Anti-nutritional constituents like tannin, phytate was also found to be reduced in significant amount after fermentation with comparing the raw materials.

CHAPTER 5
CONCLUSION AND RECOMMENDATIONS

This research was continued to develop soyabean feed by value added by adding APC to soyabean fermentation, because farmer point of view, adding APC will enhance the nutritional value and the amino acid profile.by optimizing the time 24hr and 48hr . according to proximate results, comparing crude protein content 48 hr reduce the amount because The result showed that the crude protein of fermented soya was increased by probiotic bacteria with the time (FSY 24; 51.72±0.54%DW basis and AFSY 24:51.65±0.54 %DW basis of fermented soyabean. But the fermentation time is increased to 48h the crude protein is decreased but not significantly,(FSY 48 is decreased to 48.99$\pm$0.92 and AFSY 48h is decreased up to 45.57$\pm$1.89) because The large protein molecules were became too small protein molecule, long peptide bond became short peptide bond of protein and amino acid. After fermentation the products pH was the range with 6.5-7.5 at all-time because of we adding $NaHCO_3$ to the fermented solution.

The physical properties (bulk density, color, and pH) and anti-nutritional constituents of dehulled soya, fullfat soya, AFSY 24 and 48, FSY 24 and 48 were analyzed before and after fermentation. After fermentation, the anti-nutritional constituents like tannin, phytate present in the raw material used was reduced significantly because of fermentation from 1.48 to 0.02 mg/g, 2.98 to 1.58 mg/g respectively. It was observed that the product prepared with fermentation of *Bacillus licheniformis* and *Bacillus megaterium* was found to have high functional property and lower anti-nutritional constituents in compare to traditionally prepared product. (dehulled and fullfat soya), and the color values and bulk density will increase by the fermentation increase.

Recommendations

- ✓ Analyze the amino acid profile and fatty acid profile of AFSY, FSY, and APC to know that the adding APC will infect the amino acid profile of AFSY.

- ✓ This results is very interesting for Aqua industry and yet more scope on finding on trypsin, oligosaccharide and nutritional profiling will help more in exploring this area of research for substantiality.

- ✓ Doing the in-vitro digestibility of AFSY, FSY and APC for tilapia by gut content analysis.

REFERENCES

Battock, M., & Azam-Ali, S. (1998). Fermented fruits and vegetables. *FAO Agricultural services bulletin*(134).

Bourdichon, F., Casaregola, S., Farrokh, C., Frisvad, J. C., Gerds, M. L., Hammes, W. P. Hansen, E. B. (2012). Food fermentations: microorganisms with technological beneficial use. *Int J Food Microbiol, 154*(3), 87-97. doi: 10.1016/j.ijfoodmicro.2011.12.030

Breidt, F., & Fleming, H. P. (1997). Using lactic acid bacteria to improve the safety of minimally processed fruits and vegetables. *Food technology (USA)*.

Busch, J., Vanhanen, L., & Savage, G. (2003). Chemical analysis and consumer acceptance of taro. *Proceedings of the Nutrition Society, 28*, 109.

Chettri, R., & Tamang, J. P. (2008a). Microbiological evaluation of maseura, an ethnic fermented legume-based condiment of Sikkim.

AOAC. 2000. Official Methods of Analysis of AOAC International, 17th Ed. AOAC International, Gaithersburg, MD.

APHA, (American Public Health Association) 2005 Standard Methods for the Examination of Water and Wastewater, 21st edn. American Wastewater Association and Water Environment Federation, Washington, DC.

Jantrakajorn, S., M. Lukkana and J. Wongtauatchai. 2014. Serological and molecular characterization Streptococcus iniae in cultured Nile tilapia (Oreochromis niloticus) in Thailand. Thia Vet Med. 44(1):49-58.

Kramsri, B. and O. Chavalparit. 2012. Production of bioextract using bio-sludge as co-digestion material. Department of Environmental Engineering, Faculty of Engineering, Chulalongkorn University, Bangkok.460-467.

Krummenauer, D., S. Peixoto, R.O. Cavalli, L.H. Poersch and W. Wasielesky Jr. 2011. Superintensive culture of white shrimp, *Litopenaeus vannamei*, in a biofloc technology system in Southern Brazil at different stocking densities. Journal of the world aquaculture society. 42(5): 726-733.

Lopez-Elias, J.A., A. Moreno-Arias, A. Miranda-Baeza, L.R. Martinez-Cordova, M.E. Rivas-Vega and E. Marquez-Rios. 2015. Proximate composition of bioflocs in culture systems containing hybrid red tilapia fed diets with varying levels of vegetable meal inclusion. North American Journal of Aquaculture. 77: 102-109.

Liping, L and K Fitzsimmons. 2011. Better science, better fish, better life proceedings of the ninth international symposium on tilapia in aquaculture. Aquafish Collaborative Research Support Program. 9-18.

Maillard, V.M., G.D. Boardman, J.E. Nyland and D.D. Kuhn. 2005. Water quality and sludge characterization at raceway-system trout farms. Aquaculture Engineering.

33: 271-284.

Melville, J. 2014. Bomb calorimetry and heat of combustion. UC Berkeley College of Chemistry.

Moongngarm, A., N. Daomukda and S. Khumpika. 2012. Chemical composition, phytochemicals, and antioxidant capacity of rice bran, rice bran layer, and rice germ.
APCBEE Procedia. 2:73-79.

Namkorn, R., S. Silapanuntakul, P. Vatanasomboon and T. Prechthai. 2015. Co-compost production from sewage sludge mixed with vegetable wastes and scum. Department of Environmental Health Science, Faculty of Public Health, Mahidol University, Ratchathewi Campus, Bangkok, 10400, Thailand. 95-103.

Noonpan, P., R. Chiachana. 2014. Effects of nitrogen and phosphorus on eutrophication in water bodies and nitrogen and phosphorus removal. Department of environmental engineering of Kasetsart University 88: 57-67.

Olsen, S.R. and L.E. Sommers. 1982. Phosphorus, pp. 403-427, in D. Buxton, ed., Methods of soil analysis: Chemical and microbiological properties, 2nd ed., Soil Science Society of Amarica, Madison, Wisconsin.

Oliveira, M. S., Feddern, V., Kupski, L., Cipolatti, E. P., Badiale-Furlong, E. and Souza-Soares, L. A. 2011. Changes in lipid, fatty acids and phospholipids composition of whole rice bran after solid-state fungal fermentation. Bioresource Technology. 102: 8335–8338.

Pantachai, W. (2016). Effect of ammonium chloride and rice bran fermentation on the biofloc system:small scale experiment. A special study submitted in partial fulfillment of the requirements for the degree of Master of Science in Aquaculture and Aquatic Resources Management, Asian Institute of Technology.

Roselien C., Tom D., Peter B. and Willy V., 2012. Biofloc technology in aquaculture : Beneficial effect and future challenges. Aquaculture (356-357), 351-356.

Rokocy, J.E., J.J. Danaher, D.S. Bailey and R.C. Shultz. 2008. Development of a biofloc system for the production of tilapia. Aquaculture.

Rail, T. and S. Mishra. 2006. Morphology and functional properties of corn, potato and tapioca starches. Food Hydrocolloids. 20:557-566.

Rajkumar, M., P.K. Pandey, R. Aravind, A. Vennila, V. Bharti and C.S. Purushothaman. 2015. Effect of different biofloc system on water quality, biofloc composition and growth performance in *Litopenaeus vannamei* (Boone, 1931). Aquaculture Research.

Silva, K.R.D., W. Wasielesky Jr and P.C. Abreu. 2013. Nitrogen and phosphorus dynamics
in the biofloc production of the pacific white shrimp, *Litopenaeus vannamei*. Journal
of the world aquaculture society. 44(1): 30-41.

Sakkaravarthi, K. and G. Sankar. 2015. Identification of effective organic carbon of biofloc shrimp culture system. Journal of Biological Sciences. 15(3): 144-149.

Sriyasak, P., C. Chitmanat, N. Whangchai, J. Promya and L. Lebel. 2015. Effect of water de-stratification on dissolved oxygen and ammonia in tilapia ponds in Northern Thailand. Int Aquat Res.

Srikerdkruen, R. (2016). Preliminary experiment on rice bran fermentation enhance by baker's yeast and commercial probiotics. A special study submitted in partial fulfillment of the requirements for the degree of Master of Science in Aquaculture and Aquatic Resources Management, Asian Institute of Technology.

Schveitzer, R., R. Arantes, P.F.S. Costodio, C.M. do Espirito Santo, L.V. Arana, W.Q. Seiffert and E.R. Andreatta. 2013. Effect of different biofloc levels of microbial activity, water quality and performance of Litopenaeus vannamei in a tank system operated with no water exchange. Aquaculture Engineering. 56:59-70.

Sumantha, A., Peepa, P., Sandhya, C., Szakacs, G., Soccol, C. R. and Pandey, A. 2006. Rice bran as a substrate for proteolytic enzyme production. Brazilian archives of biology and technology. 49(5): 843-851.

Supriyati, Haryati, T., Susanti, T. and Susana, I. W. R. 2015. Nutritional value of rice bran fermented by Bacillus amyloliquefaciens and humic substances and its utilization as a feed ingredient for broiler chickens. Asian Australas. J. Anim. Sci. 28(2): 231-238.

APPENDICES
APPENDICES: A

Statistic analysis (Using SPSS version 16)
Independent-Samples T-test and correlation.
Randomized Complete Block Design ; RCBD (two-way ANOVA)

Proximate composition of Fermented products (using independent-sample T-test).

A 1:Descriptive statistics of Ash content

Dependent Variable: Ash Content (% d.b)

Formulation	Fermentation Time (h)	Mean	Std. Deviation	N
AFSY	24 hrs.	12.7533	.05859	3
	48 hrs.	15.9633	2.70178	3
	Total	14.3583	2.45203	6
FSY	24 hrs.	11.7900	.09165	3
	48 hrs.	15.5467	2.57877	3
	Total	13.6683	2.62624	6
Total	24 hrs.	12.2717	.53211	6
	48 hrs.	15.7550	2.37317	6
	Total	14.0133	2.44905	12

A 2: Tests of Between-Subjects Effects

Dependent Variable: Ash Content (% d.b)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	38.053[a]	3	12.684	3.634	.064
Intercept	2356.482	1	2356.482	675.137	.000
Form	1.428	1	1.428	.409	.540
Time	36.401	1	36.401	10.429	.012
Form * Time	.224	1	.224	.064	.806
Error	27.923	8	3.490		
Total	2422.458	12			
Corrected Total	65.976	11			

R Squared = 0.577 (Adjusted R Squared = 0.418)

A 3:Tests of Between-Subjects Effects

Dependent Variable: Ash Content (% d.b)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	38.053[a]	3	12.684	3.634	.064
Intercept	2356.482	1	2356.482	675.137	.000
Form	1.428	1	1.428	.409	.540
Time	36.401	1	36.401	10.429	.012
Form * Time	.224	1	.224	.064	.806
Error	27.923	8	3.490		
Total	2422.458	12			
Corrected Total	65.976	11			

R Squared = 0.577 (Adjusted R Squared = 0.418)

A 4: Descriptive Statistics of fiber content

Dependent Variable: Fiber Content (% d.b)

Formulation	Fermentation Time (h)	Mean	Std. Deviation	N
AFSY	24 hrs.	7.6933	.10408	3
	48 hrs.	7.7433	1.36137	3
	Total	7.7183	.86395	6
FSY	24 hrs.	7.6033	.18583	3
	48 hrs.	8.8400	.62193	3
	Total	8.2217	.79205	6
Total	24 hrs.	7.6483	.14345	6
	48 hrs.	8.2917	1.12110	6
	Total	7.9700	.83278	12

A 5: **Tests of Between-Subjects Effects**

Dependent Variable: Protein Content (% d.b)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	99.871[a]	3	33.290	26.786	.000
Intercept	30582.803	1	30582.803	24607.490	.000
Form	42.038	1	42.038	33.824	.000
Time	17.280	1	17.280	13.904	.006
Form * Time	40.554	1	40.554	32.630	.000
Error	9.943	8	1.243		
Total	30692.617	12			
Corrected Total	109.814	11			

R Squared = .909 (Adjusted R Squared = .876)

A 6: Descriptive Statistics of energy content

Dependent Variable: Energy Content (% d.b)

Formulation	Fermentation Time (h)	Mean	Std. Deviation	N
AFSY	24 hrs.	20.4750	.03536	2
	48 hrs.	19.9400	.01414	2
	Total	20.2075	.30966	4
FSY	24 hrs.	20.7350	.43134	2
	48 hrs.	20.4650	.36062	2
	Total	20.6000	.36009	4
Total	24 hrs.	20.6050	.29149	4
	48 hrs.	20.2025	.36782	4
	Total	20.4038	.37508	8

A 7: Descriptive Statistics of lipid content

Dependent Variable: Lipid Content (% d.b)

Formulation	Fermentation Time (h)	Mean	Std. Deviation	N
AFSY	24 hrs.	4.5100	.05568	3
	48 hrs.	4.5267	.27610	3
	Total	4.5183	.17837	6
FSY	24 hrs.	3.8300	.12767	3
	48 hrs.	4.3567	.14295	3
	Total	4.0933	.31290	6
Total	24 hrs.	4.1700	.38273	6
	48 hrs.	4.4417	.21757	6
	Total	4.3058	.32898	12

Dependent Variable: Ash Content (% d.b)

Formulation	Fermentation Time (h)	Mean	Std. Deviation	N
AFSY	24 hrs.	12.7533	.05859	3
	48 hrs.	15.9633	2.70178	3
	Total	14.3583	2.45203	6
FSY	24 hrs.	11.7900	.09165	3
	48 hrs.	15.5467	2.57877	3
	Total	13.6683	2.62624	6
Feed	24 hrs.	14.0633	.03786	3
	48 hrs.	14.0633	.03786	3
	Total	14.0633	.03386	6
Total	24 hrs.	12.8689	.98987	9
	48 hrs.	15.1911	2.05810	9
	Total	14.0300	1.97025	18

A 8: Multiple Comparisons

Dependent Variable: Ash Content (% d.b)

	(I) Formulation	(J) Formulation	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Tukey HSD	AFSY	FSY	.6900	.88079	.720	-1.6598	3.0398
		Feed	.2950	.88079	.940	-2.0548	2.6448
	FSY	AFSY	-.6900	.88079	.720	-3.0398	1.6598
		Feed	-.3950	.88079	.896	-2.7448	1.9548
	Feed	AFSY	-.2950	.88079	.940	-2.6448	2.0548
		FSY	.3950	.88079	.896	-1.9548	2.7448

Based on observed means.

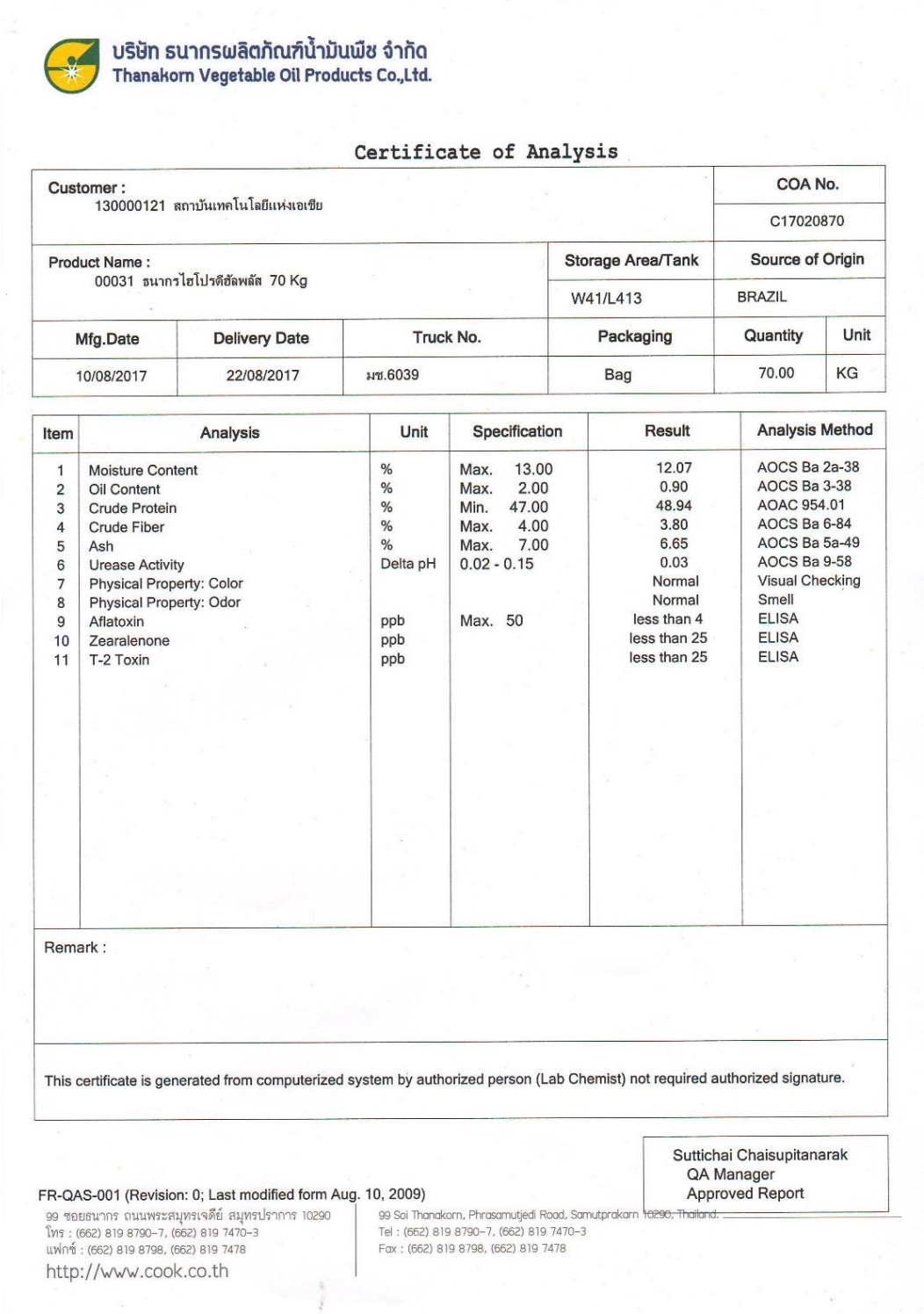
The error term is Mean Square(Error) = 2.327.

A 9: Proximate analysis of overall products

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
lipid	FSY 24 h	3	3.8300	.12767	.07371	3.5128	4.1472	3.69	3.94
	AFSY 24h	3	4.5100	.05568	.03215	4.3717	4.6483	4.45	4.56
	commercial fedd	3	4.9967	.21221	.12252	4.4695	5.5238	4.85	5.24
	Total	9	4.4456	.52312	.17437	4.0435	4.8477	3.69	5.24
ash	FSY 24 h	3	11.7900	.09165	.05292	11.5623	12.0177	11.69	11.87
	AFSY 24h	3	12.7533	.05859	.03383	12.6078	12.8989	12.71	12.82
	commercial fedd	3	14.0633	.03786	.02186	13.9693	14.1574	14.02	14.09
	Total	9	12.8689	.98987	.32996	12.1080	13.6298	11.69	14.09
protein	FSY 24 h	3	51.7167	.53314	.30781	50.3923	53.0410	51.22	52.28
	AFSY 24h	3	51.6500	.53842	.31086	50.3125	52.9875	51.15	52.22
	commercial fedd	3	47.9033	.73009	.42152	46.0897	49.7170	47.18	48.64
	Total	9	50.4233	1.96207	.65402	48.9152	51.9315	47.18	52.28
fiber	FSY 24 h	3	7.6033	.18583	.10729	7.1417	8.0650	7.39	7.73
	AFSY 24h	3	7.6933	.10408	.06009	7.4348	7.9519	7.61	7.81
	commercial fedd	3	4.4000	.36661	.21166	3.4893	5.3107	4.08	4.80
	Total	9	6.5656	1.63841	.54614	5.3062	7.8249	4.08	7.81
energy	FSY 24 h	2	20.7350	.43134	.30500	16.8596	24.6104	20.43	21.04
	AFSY 24h	2	20.4750	.03536	.02500	20.1573	20.7927	20.45	20.50
	commercial fedd	2	18.7600	.04243	.03000	18.3788	19.1412	18.73	18.79
	Total	6	19.9900	.97933	.39981	18.9623	21.0177	18.73	21.04

APPENDICES:B

B 1: Dehull soya bean analysis report



บริษัท ธนากรผลิตภัณฑ์น้ำมันพืช จำกัด
Thanakorn Vegetable Oil Products Co.,Ltd.

Certificate of Analysis

Customer : 130000121 สถาบันเทคโนโลยีแห่งเอเชีย			COA No. C17020870	
Product Name : 00031 ธนากรไฮโปรตีอัลพลัส 70 Kg			Storage Area/Tank W41/L413	Source of Origin BRAZIL
Mfg.Date	Delivery Date	Truck No.	Packaging	Quantity / Unit
10/08/2017	22/08/2017	มช.6039	Bag	70.00 KG

Item	Analysis	Unit	Specification	Result	Analysis Method
1	Moisture Content	%	Max. 13.00	12.07	AOCS Ba 2a-38
2	Oil Content	%	Max. 2.00	0.90	AOCS Ba 3-38
3	Crude Protein	%	Min. 47.00	48.94	AOAC 954.01
4	Crude Fiber	%	Max. 4.00	3.80	AOCS Ba 6-84
5	Ash	%	Max. 7.00	6.65	AOCS Ba 5a-49
6	Urease Activity	Delta pH	0.02 - 0.15	0.03	AOCS Ba 9-58
7	Physical Property: Color			Normal	Visual Checking
8	Physical Property: Odor			Normal	Smell
9	Aflatoxin	ppb	Max. 50	less than 4	ELISA
10	Zearalenone	ppb		less than 25	ELISA
11	T-2 Toxin	ppb		less than 25	ELISA

Remark :

This certificate is generated from computerized system by authorized person (Lab Chemist) not required authorized signature.

Suttichai Chaisupitanarak
QA Manager
Approved Report

FR-QAS-001 (Revision: 0; Last modified form Aug. 10, 2009)

99 ซอยธนากร ถนนพระสมุทรเจดีย์ สมุทรปราการ 10290
โทร : (662) 819 8790-7, (662) 819 7470-3
แฟกซ์ : (662) 819 8798, (662) 819 7478
http://www.cook.co.th

99 Soi Thanakorn, Phrasamutjedi Road, Samutprakarn 10290, Thailand.
Tel : (662) 819 8790-7, (662) 819 7470-3
Fax : (662) 819 8798, (662) 819 7478

B 2: Crude protein analysis

B 3: Crude lipid analysis

B 5: Energy content analysis