EVALUATION OF SPENT BREWER'S YEAST AS AN ALTERNATIVE FISH FEED

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ABSTRACT

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The increase in the world population results in a rising protein demand which become the most important factor in accelerating the development of the aquaculture industry. Fishes require main nutrients such as protein, fat, carbohydrate, vitamins and minerals for growth and development. Protein source within the fish feed contributes to the major cost in the fish industry. Thus, an evaluation of single cell protein, the spent brewer's yeast (SY) as a feed material was carried out to determine its potential application in fish farming. The nutritional composition of SY was determined and crude enzyme extracts from digestive tract of two types of local farmed fish, the tilapia and catfish were characterized and used in protein digestibility study on SY. From the proximate examination of the SY, the contents of crude protein, moisture, crude lipid, ash, fiber and nitrogen-free extract (NFE) were 30.51±0.27%, 17±0.42%, 1.03±0.18%, 8.45±1.01%, 4.48±0.60% and 38.54±1.31% respectively. The protease activity of tilapia and catfish was higher at the pH range from 9 to 12. The amylase activity of crude enzymes from the digestive tract of tilapia and catfish was higher at pH 6, 7, 8 and 12; pH 7, 8, 11 and 12 accordingly. pH drop method was used to carry out *in*

vitro protein digestibility of spent brewer's yeast by crude digestive enzymes of fishes. The relative protein digestibility (RPD) of spent brewer's yeast by tilapia was 41.07% whereas RPD of SY by catfish was 35.14%. However, these values are not representative enough to conclude that spent brewer's yeast can substitute fishmeal completely in tropical fish diet. Yet, the determined RPD of spent brewer's yeast and the fish digestive enzymes characterization can be used as the base information for the feed preparation of tilapia and catfish.

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DECLARATION

I hereby declare that the project report is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree in UTAR or other institutions.

……..

LOONG JIN MUN

APPROVAL SHEET

This project report entitled **"EVALUATION OF SPENT BREWER'S YEAST AS AN ALTERNATIVE FISH FEED"** was prepared by LOONG JIN MUN and submitted as partial fulfilment of the requirements for the degree of Bachelor of Science (Hons) Biochemistry at Universiti Tunku Abdul Rahman.

Approved by:

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PERMISSION SHEET

It is hereby certified that **LOONG JIN MUN** (ID: **09 ADB 04443**) has completed this final year project entitled **"EVALUATION OF SPENT BREWER'S YEAST AS AN ALTERNATIVE FISH FEED"** supervised by **Dr. CHANG YING PING** from the Department of Chemical Science, Faculty of Science.

I hereby to give permission to my supervisor to write and prepare manuscripts of these research findings for publishing in any form, if I do not prepare it within six (6) months from this date, provided that my name is included as one of the authors for this article. The arrangement of the name depends on my supervisor.

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LIST OF ABBREVIATION

g	Gravitational force
Δ	Change in value
Abs	Absorbance
BSA	Bovine Serum Albumin
CA	Crude Ash
CF	Crude Fiber
CP	Crude Protein
DNS	3,5-dinitrosalicyclic acid
EE	Crude Fat or Crude Lipid
et al.	et alia (and others)
FAO	Food and Agriculture Organization
H_2SO_4	Sulphuric acid
HCl	Hydrochloric acid
kcal	kilocalorie
KCl	Potassium chloride
N	Normality
Na_2CO_3	Sodium carbonate
Na_2HPO_4	Disodium hydrogen phosphate
NaOH	Sodium hydroxide
NFE	Nitrogen-free Extract
Nm	Nanometer

RPD	Relative Protein Digestibility
SC	*Saccharomyces cerevisiae*
SCP	Single Cell Protein
SY	Spent Brewer's Yeast
Syn	Synonymy
TCA	Trichloroacetic acid
VHCl	Volume of standard HCl (mL)
wt	Weight

CHAPTER 1

INTRODUCTION

Global population has grown substantially in the past decade, reaching 7 billion in 2012, compared with 6 billion in 2000. This increasing world population indirectly rising the demand of protein for human consumption and animal production. Moreover, it is predicted that for the coming 20 years, there will be more than 8 billion people standing on the earth (Heyden, 2010). There are more than one billion people suffered from hunger or were undernourished in 2009. Therefore, there is the need to find new food source with promising high protein and nutrient to solve food demand problem.

Aquaculture has an important role in addressing food insecurity by enhancing the supply and consumption of fish and other marine and freshwater products, which are commonly rich sources of protein, essential fatty acids, vitamins and minerals. Therefore, the highly desirable nutrient profile and excellent source of high-quality animal protein of fish could provide significant nutrients source in promoting nutritional wellbeing among most population groups. Nevertheless, aquaculture is one of the fastest-growing animal food producing sector and currently accounts for more than 60% global fish production between year 2000 (32.4 million tons) and 2008 (52.5 million tons) (FAO Fisheries and Aquaculture Department, 2011). In addition, world fish food supply has outpaced global population growth in the last

five decades. Hence, aquaculture has been suggested to have the greatest potential in fulfilling the protein demand supply gap. Though, the growth of aquaculture is limited by the low availability and higher price of all quality aqua feed ingredients. In other word, the financial viability of aquaculture investments is highly dependent on the cost paid for aqua feeds, which generally account for 50–70 % of production cost (FAO Fisheries and Aquaculture Department, 2011).

Aqua feeds are used for feeding omnivorous fishes such as tilapia and catfish, carnivorous fishes such as salmon and tuna, and crustacean species such as craps and lobsters. There are three types of feed ingredients used for the production of aqua feeds that can be categorized based on their origin (FAO Fisheries And Aquaculture Department, 2012):

(1) animal nutrient sources which include both aquatic and terrestrial animals such as fish meal and poultry meal;

(2) plant nutrient sources such as sunflower seed and soy bean; and

(3) microbial nutrient sources such as bacteria and fungi.

Among these feed ingredients stated above, fishmeal and fish oil are highly favored ingredients in aqua feeds. This is because these ingredients are high in protein, mineral and essential fatty acids, high palatability and digestibility and can improve immunity and survival rate of fishes (Rana, Siriwardena and Hasan,

2009). It has been estimated that, by 2012, 60 percent of world fishmeal production and 88 percent of world fish oil production will be used by aquaculture (Huntington and Hasan, 2009).

Conversely, the increased competition between the expanding aquaculture and livestock sectors for a limited supply of fishmeal and fish oil continues to drive the price upwards and that the price could reach a level where the use of fishmeal and fish oil may no longer be financially viable (FAO of the United Nations, 2006). Hence, due to the limited availability and the rising price of fishmeal, an impressive amount of studies have been carried out in recent decades to reduce dependency on fishmeal. Some of such studies that have been conducted are evaluation of the suitability of single cell protein (SCP) to substitute fishmeal in fish diets. Since 1970's, researchers (Attack and Matty, 1979; Avnimelech and Mokady, 1988; Beck et al., 1979; Bhosale, 1997; Davies and Wareham, 1988; Kiesling and Askbrandt, 1993; Lara-Flores, Olvera-Novoa and Lopez-Madrid, 2003; Mahnken et al., 1980; Matty and Smith, 1978, cited in Bob-Manuel and Alfred-Ockiya, 2011) suggest that the SCPs have significant potentials in their utilization in aqua feeds.

Single cell protein (SCP) is including unicellular and filamentous algae, fungi and bacteria which can be produced by controlled fermentation processes. SCP production can be based on raw carbon substrates which are available in large

quantities and inexpensive such as agricultural or cellulosic waste products and industrial waste which would otherwise cause an environmental hazard. Some of agro-based wastes such as crop peel, cereal husks, sugar cane (bagasses) and waste from coconut and mango are lignocelluloses and accumulate in considerable amount thereby posing environmental and public nuisance (Bob-Manuel and Alfred-Ockiya, 2011). Thus, utilization of wastes as substrate for SCP production could reduce pollutant and provide a solution for waste disposal problem.

The SCP that has chosen for this project is spent brewer's yeast. "Each stage of the brewing process produces waste," says Juan Jurado, Competence Center Manager Filtration & Separation at Alfa Laval. He stated that for every 1,000 tonnes of beer produced, 137 to 173 tonnes of solid waste is created (Reducing waste in beer production, 2011). Brewer's yeast biomass is the second major by-product from brewery industry (after brewer spent grain); however, it is still underutilized, being basically used as animal feed (Ferreira, Pinhos and Tavarela, 2010). Therefore, exploring the potential of spent brewer's yeast utilization may solve the waste disposal and also pollution problem. In addition, applications for this agro-industrial by-product as a source of nutrients for human and fish nutrition is having great potential in achieving zero-waste operational target in brewery industries by utilizing brewery waste as zero cost substrate for SCP production. Hence, the conversion of brewery wastes to SCP in providing fishes a good protein source need to be evaluated by extending the study on *in vitro* digestibility by tropical fishes such as catfish and tilapia.

In order to be a viable alternative feedstuff to fishmeal for aqua feeds, the candidate ingredient, spent brewer's yeast must possess certain characteristics that are compatible with fishmeal such as wide availability, competitive price, as well as ease of handling, shipping, storage and use in feed production (Gatlin III et al., 2007). Yet, the foremost quality is it must contain certain nutritional constituents, for instance, low levels of fiber, starch (especially non-soluble carbohydrates) and anti-nutrients, and have relatively high protein content, high nutrient digestibility, and reasonable palatability (Gatlin III et al., 2007). Therefore, its chemical composition which includes the contents of moisture, ash, protein, fiber, lipid and non-nitrogen substances governs its utilization.

The main objectives of this project are to quantify important constituents in SY and to evaluate the protein digestibility of spent brewer's yeast using crude enzyme extracts from the digestive tract of tilapia and catfish. Besides, it is anticipated that data collected from this study can aid in the following:

1) to achieve zero-waste operational target in brewery industries by utilizing brewery waste as zero cost substrate for SCP production and

2) to minimize the environmental impact by reducing the amount of the agriculture and industrial waste disposed into the river or soil.

CHAPTER 2

LITERATURE REVIEW

2.1 Single Cell Protein

The protein obtained from microbial source is known as "Single Cell Protein" (SCP). Bacteria, moulds, yeasts, green and blue-green algae are widely used as source of single cell protein. Among those, blue-green algae are the most frequently used organism because its cell wall lack of cellulose and are easily digestible. It has high protein content with wide amino acid spectrum, higher protein-carbohydrate ratio than forages and low fat content. Moreover, it is environmental friendly because it can be grown on waste and thus helps in recycling waste. Apart from nutritional value, a protein should have desirable functional properties for its incorporation in food. SCP has fulfilled all the above requirements for its inclusion as diet supplement for both human and livestock especially in the developing countries of Africa and the world at large (Haider, AL-Barhawi and Hassan, 1989, cited in Adedayo, Ajiboye and Odaibo, 2011). Since long time ago, microorganisms have been employed in the production of high protein food such as cheese and fermented soybean products. The main nutritional component in both types of food is protein. Thus, the ability of microorganisms in upgrading low protein organic material to high protein food has been exploited by industries. For example in Germany during the First World War, the growth of *Saccharomyces cerevisiae* (SC) was exploited for human

consumption. Another example is during the Second World War, *Candida arborea* and *C. utilis* were used and about 60% of the country prewar food input was replaced (Kahlon, 1991; Litchfield, 1983). The table below shows the nutrients composition of the main group of microorganisms:

Table 2.1: Average composition of the main group of microorganisms.

Nutrients	**(% dry weight)**			
	Fungi	Algae	Yeast	Bacteria
Protein	30-45	40-60	45-55	50-65
Fat	2-8	7-20	2-6	1.5-3.0
Ash	9-14	8-10	5-9.5	3-7
Nucleic Acid	7-10	3-8	6-12	8-12

(Miller and Litsky, 1976)

2.1.1 Spent brewer's yeast

The brewing industry generates quite large amounts of by-products and wastes but the spent grain, spent hops and yeast are being the most common. However, all these wastes can be readily recycled and reused, as well as spent brewer's yeast. Yeast has been the first microorganism which was recognized for its importance as animal feed supplement almost a century ago. Yeast contains about 50 – 55 % protein, high protein–carbohydrate ratio than forages, good balance of amino acids and rich in β–complex vitamins, thus, suitable as poultry feed as well. A study by

Santin, et al. (2001; 2003) showed that the cell wall of SC can improve the intestinal mucosa aspects and correlated with the improvement in growth performance of broilers supplemented with cell wall of SC. Researchers like Churchil, Mohan and Viswanathan (2000) and Yadav, Srivastava and Shukla (1994) claim that broilers fed with 0.2 to 1 % brewer's yeast had better weight gain and feed conversion. Result from Nilson, Peralta and Miazzo (2004) is agreed with previous studies which also stated that the broilers receiving yeast to replace part of the premix feed had better average weight gain and feed conversion ratio. In addition, Sentihilkumar, Kadirvel and Vijaykumar (1997) reported an improvement in broiler productive values when incorporating 5 to 20 % yeast in the diets.

Constituents from spent brewer's yeast may be applied as functional ingredients for food production as well as health supplements for fishes. Zechner-Krpan, et al. (2010) reported that β-glucans isolated from brewer's yeast are mainly for food production and immunostimulation. It also stated that β-glucans from different origin have the potential to be used as food thickeners or fat replacers, dietary fibers, viscosity imparting agents, emulsifiers, and films.

Apart from that, spent brewer's yeast is a natural diet additives that shown to have immunostimulant properties which affects non-specific immunity and protection against disease (Siwicki, Anderson and Rumsey, 1994), improve growth of some

fish species (Oliva-Teles and Goncalves, 2001; Lara-Flores, Olvera-Novoa and Lopez-Madrid, 2003; Li and Gatlin III, 2003, 2004), provide desirable flesh colouration or pigmentation in salmonid fish (Johnson, Conklin and Lewis, 1977; Whyte and Sherry, 2001), and may possibly serve as an alternative protein source to fishmeal (Cheng, Hardy and Huige, 2004; Oliva-Teles and Goncalves, 2001; Rumsey, Kinsella and Hughes, 1990, 1991; Sanderson and Jolly, 1994) or added to aquaculture diets as partial replacement for fishmeal (Li and Gatlin III, 2003). However, according to Lim, Lam and Ding (2005) and Rumsey, Kinsella and Hughes (1991), application of yeast in the diet of cultured fish may not be absolutely beneficial. This is because yeast supplements are deficient in sulfated amino acids, particularly methionine (Oliva-Teles and Goncalves, 2001), which restricts their extensive use as the sole protein source.

2.2 Nutritional Composition of Spent Brewer's Yeast

The proximate or Weende analysis of feed is a quantitative method to determine different macronutrients in feed so that can be used in formulating a diet as a protein or energy source for the finished feedstuffs and as a requirement to be met during formulation. Basically it is the partition of feed compounds into six categories by means of common chemical properties. The categories are moisture, crude ash (CA), crude protein (CP), ether extracts (crude fats or lipids; EE), crude fiber (CF) and nitrogen-free extractives (NFE) (Olvera-Novoa, Martinez-Palacios and Real de Leon, 1994).

2.2.1 Moisture content

The feed sample is initially dried at 105 °C for 12 hours. The weight loss of the sample is determined and the crude water fraction is calculated. It is necessary to know the water content of each component especially in prepared feed to ensure the moisture content below 8% and 14% as a control measure to prevent contamination by insects, fungi and bacteria.

2.2.2 Crude ash content

Ashing the sample at 550 °C for 12 hours removes the carbon from the sample, thus, all organic compounds are removed. By calculating the weight loss of the feed sample from the dry matter to crude ash (CA) content mathematically determines the organic matter fraction. Ash remaining in the crucible is considered as the total inorganic content in the sample.

2.2.3 Crude fat content

Fats and lipids are extracted continuously with petroleum ether, after evaporation of the solvent the residue remaining is the ether extract (EE) fraction or the crude fat.

2.2.4 Crude protein content

The nitrogen content of the food is the basis for calculating the crude protein (CP) content of the feed and it is the most important dietary nutrient in a commercial operation. The method established by Kjeldahl converts the nitrogen present in the sample after digested in sulphuric acid to ammonia which is determined by titration. By multiplying the nitrogen content of sample in % obtained via Kjeldahl analysis with 6.25 will give an approximate protein content of the sample.

2.2.5 Crude fiber content

One of the fractions of insoluble carbohydrates in a feed sample is crude fiber. This fraction is not soluble in a defined concentration of alkalis and acids. There are cellulose, hemicellulose and lignin in this fraction. After the sample is digested in sulphuric acid and sodium hydroxide and the residue being calcined, the difference in weight after calcination represents the fiber content.

2.2.6 Nitrogen-free extract

Soluble carbohydrates such as sugars, starch and hemicelulose, and other non-nitrogen soluble organic compounds are defined as nitrogen-free extractives (NFE). This soluble carbohydrate is the cheapest and most abundant energy source for animal. Besides, it acts as a building block for other nutrients and stored as fat if dietary excess. The metabolizable energy (ME) values of carbohydrates for fish

range from near zero for cellulose to about 3.8 kcal/g for easily digested sugars (Smith, n.d.). This fraction again is not determined chemically it is rather calculated by subtracting CP, EE and CF from organic matter.

The table below shows the proximate compositions of brewer yeast:

Table 2.2: Reported average proximate composition of Brewer yeast (*S. cerevisiae*) meal.

Single Cell Protein		**Average composition (% by weight)**							
		H_2O[1]	CP[2]	EE[3]	CF[4]	NFE[5]	Ash[6]	Ca[7]	P[8]
Brewer yeast	Min	7.0	43.8	0.8	2.4	24.3	6.6	0.12	1.26
(*S.cerevisiae*)	Max	8.6	49.4	1.7	3.9	39.4	12.1	0.25	1.45
	Mean	7.6	46.1	1.3	2.9	34.0	8.1	0.18	1.37

Source: Tacon, Metian and Hasan (2009)

[1] water; [2] crude protein; [3] lipid or ether extract; [4] crude fiber; [5] nitrogen-free extractives; [6] ash; [7] calcium; [8] phosphorus.

2.3 Fish Gut Enzyme Characterization

The quality of a given feed diet is directly proportional to its ability to support growth whereas its nutritional value is determined by the digestibility and absorption ability of the animal (Akintunde, 1985). According to Tengjaroenkul, Smith and Smith (2000), the ability of fish to utilize ingested nutrients depends on the presence of appropriate enzymes in appropriate locations in the wall and along the lumen of the intestinal tract. Tengjaroenkul, Smith and Smith (2000) proposed

that there are various intestinal enzymes involved in digestive and absorptive processes in tilapia fish, such as amylase, pepsin, trypsin, esterases and alkaline phosphatase.

Thus, the characteristics of amylase and protease enzyme from both the stomach and the intestine of the herbivorous and carnivorous fishes are important for its digestion. Assays of fish gut enzymes may provide information about its nutritional physiology and the potential nutritional problem and to know the nutritional limiting factor. Moreover, a comparative study of the activity of fish digestive proteolytic enzymes and amylase with different nutritional habits can reveal the capacity of different species to utilize protein and carbohydrates (Hidalgo, Urea and Sanz, 1999).

2.3.1 Characterization of protease: Optimum pH

From the study by Klahan, Areechon and Engkagul (2009), it demonstrates that variations in the digestive enzyme activity (protease, amylase and lipase) were depended on sizes of Tilapia and the organ. The protease activity was high in small-sized fish; and more active in the intestine (Klahan, Areechon and Engkagul, 2009). The results from Klahan, Areechon and Engkagul (2009) were in line with the work of Kuz'mina and Ushakova (2007), which showed the protease activity of 620 g turbot decreased considerably at pH 5.0 and increased at pH 8.5. The studies indicated that size of the fish influences the levels of enzymatic activities.

Generally, pepsin is utilized as a low-pH proteolytic enzyme and after that its role are taken over by alkaline proteases, which are most active in an alkaline environment (Moyle and Cech, 2000, cited in De Silva and Anderson, 1995). Although alkaline protease is initially low activity in early juvenile stages, the general protein digestion is heavily dependent on the alkaline tryptic rather than the acidic peptic enzymes.

Lundstedt, Melo and Moraes (2002) reported that the feeding habits govern the digestive pattern of Brazilian catfish (*Pseudoplatystoma coruscans*) via the distribution and activity of digestive enzymes along the gut lumen. In the study, the higher proteolytic activity was found in acidic pH of stomach rather than in intestine. Moreover, the presence of trypsin and chymotrypsin has been detected in the stoamch. Another study by Sudaporn, Kringsak and Yuwadee (2010) also detected the presence of acidic protease and alkaline protease with high protease activity in the stomach of Mekong Giant Catfish after feeding with a combination of fishmeal and dried *Spirulina* powder. However, only alkaline protease was found in the intestine with a high proteinase activity.

2.3.2 Characterization of amylase: Optimum pH

Carbohydrase (α-amylase) is produced in the pancreas and has been identified in pancreatic juice, stomach and intestines (Klahan, Areechon and Engkagul, 2009). Carbohydrase hydrolysis activity apparently responds to the level of dietary

carbohydrate and is differs from species to species and inter-related to their feeding habits (Klahan, Areechon and Engkagul, 2009). The products from carbohydrate hydrolysis catalyzed by carbohydrase are polysaccharides, oligosaccharides and monosaccharides, which are easier to be absorbed. Al-Tameemi, Aldubaikul and Salman (2010) has reported that the activity of amylase differs from species to species and appears to be related to their feeding habits based on his study on bunny *Barbussharpeyi* (herbivorous), common carp *Cyprinuscarpio* (omnivorous) and shilik *Aspiusvorax* (carnivorous). Furthermore, fishes are poikilothermic and vary considerably in their feeding habits and temperature preferences, so diversity of their digestive enzymes could be expected (Godfrey and Reichelt, 1983, cited in El-Beltagy, El-Adawy and El-Bedawey 2005).

2.4 *In vitro* Protein Digestibility

Fishes require some main nutrients such as protein, fat, carbohydrate, vitamins and minerals for growth (anabolism) and for energy (catabolism), but the requirements vary by species. Among those nutrients, proteins are the most required nutrients for the animal. Fishes use proteins as their energy source, yet, due to the high cost of proteins, fats and carbohydrates are preferred as energy source in feeds (Fenerci and Sener, 2005). In spite of this, other researchers (Demir, 1996; Nose, 1989; Sener and Yıldız, 1998, cited in Ali, Haque and Shariful, 2009) also claimed that

proteins must be used only for growth in fish. The fate of dietary protein after ingestion is dependent on its digestibility.

The *in vitro* techniques that can be used to estimate the digestibility of total protein is a multienzyme technique. This technique evaluates the use of the multienzyme to react on a wide variety of ingredients as well as food laboratory to estimate the protein digestibility. An immediate and rapid decline in pH of the solution continuously within 10 min was noted by authors that it was caused by the freeing of carboxyl groups from the protein chain by the proteolytic enzymes (Boucher, 2008). This pH of the solution after 10 min was correlated to *in vivo* protein digestibility measured in rats and the correlation was 0.90 (Hsu, Vavak and Miller, 1977). However, this procedure has not been widely utilized to estimate protein digestibility. Its limitations are: (1) the digestibility of structurally stable proteins will be underestimated using this technique due to short incubation time (Porter, Swaisgood and Catignani, 1984); and (2) the buffering capacity of the food tested can influence the pH of the solution which will alter the 10 min pH drop (Hsu, Vavak and Miller, 1977).

Various approaches have been tried in order to develop reliable and cost-efficient methods for the evaluation of protein digestibility. Chong, Hashim and Ali (2002) had compared dry matter and protein digestibility in discus fish (*Symphysodon aequifasciata*) assessed by three different methods: (1) the *in vitro* protocols (Hsu,

Vavak and Miller, 1977; Satterlee, Marshall and Tennyson, 1979; Lazo, Romaire and Reigh, 1998); (2) *in vitro* digestion using gut extract from the discus fish; and (3) *in vivo* digestibility assessed in feeding trials with fish itself. It has been found that relative digestibility measured in simple steps which involving only a few proteases in a single reaction step correlated well with digestibility measured *in vivo*. Hence, *in vitro* digestibility experiments can be a very useful tool for screening feed ingredients and reducing the number of dietary treatments to be tested in growth-trial studies and thus much more cost efficient. In this project, we are using protocol from Lazo (1994), cited in Sultana, Ahmed and Chisty (2010), the pH drop method to evaluate the *in vitro* methods for the protein digestibility of different feed ingredients. The protein digestibility (PD) was calculated as the percentage of magnitude of pH drop (-Δ pH) ratio of the ingredient and casein (Lazo, 1994, cited in Sultana, Ahmed and Chisty, 2010).

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

Spent brewer's yeast slurry, a by-product from brewery was kindly provided by Chemical Industries (Malaya) SdnBhd, Ipoh, Perak. Both Tilapia and Catfish were bought from Kim Seng Fishery, Temoh, Perak. Both fishes were acclimated for one week before they were subject for enzyme extraction.

3.1.1 Spent brewer's yeast preparation and pretreatment

Sample preparation was carried out by using the method of Sombutyanuchit, Suphantharika and Verduyn (2001). Pretreatment began by centrifuging yeasts at 10,000 ×*g* for 10 min at 4 ^{0}C to remove beer liquor. Then, the yeast pellet obtained was adjusted to 15% solids content with distilled water. The mixture was adjusted to around pH 9 with 1M sodium carbonate (Na_2CO_3) at 20 ^{0}C. The mixture was stirred by magnetic stirrer for 30 min and then centrifuged immediately at 10,000 ×*g* for 10 min at 4 ^{0}C. Later, the yeast cell paste was washed three to four times with 1M hydrochloric acid (HCl) and lastly with distilled water until the pH was around pH 7. After that, the yeast cell paste was allowed to dry at 35 ^{0}C for at least 4 hrs until no more solid clump. The SY sample was homogenized by grinding and

sieving and was kept in bottle sealed with parafilm and stored in desiccators before further analysis.

3.1.2 Crude fish gut enzyme preparation

Crude fish gut enzymes were extracted based on the method of Ali, Haque and Shariful (2009). The gastrointestinal tract and the stomach were collected from acclimatized tilapia and catfish as stated in 3.3, and weighed. The live specimen was grinded and centrifuged at 12,000 rpm for 15 min at 4 ^{0}C. The upper lipid layer of supernatant was discarded and the supernatant was stored at -20 ^{0}C.

3.2 Chemical Reagents

3.2.1 Chemicals for sample preparation and pretreatment

Sodium carbonate was purchased from QRëC™ (Asia) Sdn Bhd (Selangor, Malaysia). Hydrochloric acid was purchased from Thermo Fisher Scientific (M) Sdn Bhd (Selangor, Malaysia). All other chemicals were of analytical grade.

3.2.2 Chemicals for proximate analysis

Petroleum ether with boiling point range of 60-80 ^{0}C was purchased from Sigma-Aldrich (M) Sdn Bhd (Selangor, Malaysia). Whatman filter paper was purchased from Chemopharm Sdn Bhd (Selangor, Malaysia). Boric acid, potassium sulphate

and copper (II) sulphate pentahydrate were purchased from SYSTERM® (Selangor, Malaysia). Sodium hydroxide was purchased from QRëC™ (Asia) Sdn Bhd (Selangor, Malaysia). Sulphuric acid was purchased from Merck Sdn Bhd (Selangor, Malaysia). Methyl red and bromocresol green were purchased from UNI-Chem (New Territories, Hong Kong) Kjeldahl digestion and distillation unit was purchased from C. Gerhardt (Königswinter, Germany). Fritted filter funnel was purchased from Sigma-Aldrich (M) Sdn Bhd (Kuala Lumpur, Malaysia). All other chemicals were of analytical grade.

3.2.3 Chemicals for pH characterization and enzyme assays of fish gut enzyme

Glycine, sodium citrate, azocasein and citric acid were purchased from HmbG® Reagent Chemicals (Selangor, Malaysia). Sodium dihydrogen phosphate, casein, disodium hydrogen phosphate (Na_2HPO_4), 3,5-dinitrosalicyclic acid (DNS) and sodium bicarbonate are purchased from Merck Sdn Bhd (Selangor, Malaysia). Maltose, starch, potassium sodium tartrate tetrahydrate and trichloroacetic acid (TCA) was purchased from QRëC™ (Asia) Sdn Bhd (Selangor, Malaysia). Sodium sulphate was purchased from UNI-Chem (New Territories, Hong Kong). Potassium chloride (KCl) was purchased from SYSTERM® (Selangor, Malaysia). Phenol, Bradford reagent and Bovine Serum Albumin (BSA) were purchased from Sigma-Aldrich (M) Sdn Bhd (Kuala Lumpur, Malaysia). All other chemicals were of analytical grade.

3.3 Proximate Analysis

The nutritional composition of SY sample from 3.1.1 was analyzed in triplicate according to Weende proximate analyses (Fisheries and Aquaculture Department, 1994).

3.3.1 Determination of moisture content

Approximately 10 g of processed SY sample was placed in drying oven at 105 ^{0}C for at 6 hrs and was allowed to cool down before weighed. Later, the sample was dried and weighed every one hour consecutively for a few hours until a constant weight was obtained. The moisture content of the sample can be calculated by applying the following formula:

$$\text{Moisture content (\%)} = \frac{\text{wt of processed sample} - \text{wt of dried sample}}{\text{wt of processed sample}} \times 100$$

3.3.2 Determination of ash content

Approximately 3 g of defatted, dry sample was weighed and placed in a crucible prior to ashing in a furnace. The sample was heated at 550 ^{0}C for 12 hrs and was allowed to cool. The weight of ash was obtained by weighing the crucible on the analytical balance. The crude ash content can be obtained by using the formula below:

$$\text{Ash content (\%)} = \frac{\text{wt of ash}}{\text{wt of processed sample}} \times 100$$

3.3.3 Determination of crude lipid content

Approximately 5 g of weighed sample was put inside a bag made of muslin cloth and placed in a soxhlet extraction unit. The unit was then connected to a round bottom flask containing 2/3 full of petroleum ether (boiling point is 60-80 ^{0}C). The petroleum ether was brought to boil for 6 hrs. Then, the ether was evaporated in a fume hood and the flask was allowed to cool down at room temperature. The fat content was calculated by using the formula below:

$$\text{Crude lipid content (\%)} = \frac{\text{wt of round bottom flask with fat – wt of clean round bottom flask}}{\text{wt of processed sample}} \times 100$$

3.3.4 Determination of crude protein content

Approximately 1 g of weighed defatted sample (wrapped by Whatman filter paper) was transferred to Kjeldahl flask which containing 7.0 g potassium sulphate (K_2SO_4), 0.8 g copper (II) sulphate pentahydrate ($CuSO_4.5H_2O$) and 15 mL 98% sulphuric acid (H_2SO_4). Then the flask was put into a preheated Kjeldahl digestion unit (C. Gerhardt, Germany) and digested for 30 min. Later, the temperature was raised to 380 ^{0}C and extra 5 mL of H_2SO_4 was added to wash down the organic particles that adhered to the flask wall. The solution was further boiled for 1 or 2 hrs until it turned clear and colourless, and then it was left aside to cool down. Before crystallization occurred, 50 mL distilled water was added. After that, the flask was transferred to Kjeldahl distillation unit or Vapodest 10 (C. Gerhardt, Gemany) and a titration flask containing 25 mL 4% boric acid with pH indicators

(0.1 mL of 0.1% methyl red and 0.5 mL of 0.1% bromocresol green) was placed on the receiving platform. An aliquot of 60 mL 40% sodium hydroxide (NaOH) was dispensed into flask and steam distilled until approximately 100 mL distillate was collected. The titration flask from receiving platform was titrated against 0.1M HCl and the end point was recorded when the colour changed from blue to red. The formula involved are:

For standard HCl titrant:

$$\%\text{Nitrogen} = \frac{\text{VHCl required for sample} - \text{VHCl required for blank}}{\text{sample wt (g)}} \times \text{N (acid standard)} \times 1.4007$$

Crude protein content (%) = %Nitrogen × 6.25

3.3.5 Determination of crude fiber content

Approximately 3 g of weighed defatted sample was placed in a round bottom flask and 200 mL of 0.255N H_2SO_4 was added into it. The flask was attached to a condenser and was boiled for exactly 30 min. Fritted funnel was preheated with boiling distilled water. At the same time, the flask was left aside to rest for 1 min at the end of the boiling period before filtration. Then, extra 50 mL boiling distilled water was added to wash the residue before it was transferred into a flask containing 200 mL 0.313M NaOH and boiled for 30 min as before. Again, the boiling solution was rested for 1 min before filter through a preheated fritted funnel. Later, the residue was washed with 50 mL boiling distilled water, 25 mL 1.25% H_2SO_4, two washes with 50 mL boiling distilled water and finished with 25

mL petroleum ether. After that, the fritted funnel was placed at 105 ^{0}C for 12 hrs and then was cooled in a dryer. Then, the funnel with the dry residue inside was weighed before placing into a furnace at 550 ^{0}C for 3hrs. Lastly, the weight of funnel with ash inside was obtained by weighing them on an analytical balance. The calculation involved in determining crude fiber content is shown below:

$$\text{Crude fiber content (\%)} = \frac{\text{wt of funnel with dry residue} - \text{wt of funnel with ash}}{\text{wt of processed sample}} \times 100$$

3.3.6 Determination of nitrogen-free extract (NFE) content

The result was obtained by subtracting the percentages calculated for each nutrient from 100. The calculation involved is shown below:

NFE (%) = 100 - moisture - crude protein - crude lipid - crude fiber - ash

3.4 Tests on Fish Gut Enzymes

The crude fish gut enzymes prepared from 3.1.2 was analyzed on its protein concentration and assayed for amylase activity and protease activity. Tilapia and catfish were used hereafter to represent the crude fish gut enzymes from the respective fish.

3.4.1 Determination of protein concentration

An aliquot of 10 µL Bradford reagent was added into 2 tubes that containing 10 µL of 5 times dilution of crude enzyme from tilapia and catfish and a blank tube containing 10 µL distilled water. All of the tubes were incubated at room temperature for 10 min before taking the absorbance reading at 595 nm. The mg of enzyme from both species was determined from the BSA standard curve constructed from 3.4.2.

3.4.2 Preparation of standard curve for bovine serum albumin (BSA)

Based on the method of Sigma-Aldrich, Inc. (n.d.), Bradford reagent was added into 5 tubes that containing different concentration of BSA (mg/mL): 0.02, 0.04, 0.06, 0.08 and 0.1, and a blank tube with distilled water. All of the tubes were incubated at room temperature for 10 min before taking the absorbance reading at 595 nm. A standard curve of absorbance reading against concentration of BSA was plotted.

3.4.3 Amylase assay

Based on the method of Worthington Biochemical Corporation (1993), 2 tubes with 0.5 mL of respective fish crude enzymes (tilapia and catfish) and a blank tube with 0.5 mL distilled water were incubated at 25 ^{0}C for 3-4 min. At time intervals, 0.5 mL of 1% starch solution was added into three tubes and incubated exactly 3

min. After that, 1 mL of 1% DNS was added to each tubes and all the tubes were incubated in a boiling water bath for 5 min. Later, they were cooled at room temperature and 10 mL of distilled water was added. Lastly, the absorbance readings of all three tubes were taken at 540 nm. The micromole of maltose released by the enzyme in the tubes was determined from the maltose standard curve as determined in 3.4.3. The unit of enzyme/mg can be determined from the formula bellow:

$$\text{Units/mg} = \frac{\text{micromoles maltose released}}{\text{mg enzyme in reaction mixture x 3min}}$$

3.4.4 Preparation of standard curve for maltose

Maltose, the product of hydrolysis by amylase was determined based on a standard curve. Based on the method of Worthington Biochemical Corporation (1993), 0.5 mL of starch solution was added into 6 tubes that containing 1 mL of different maltose concentrations (µmol/mL): 0.5, 1.0, 1.5, 2.0, 2.5 and 5.0, and a blank tube with 1 mL distilled water. Then, 1 mL of 1% DNS was added into those seven tubes and was incubated in boiling water bath for 5 min. After that, all the tubes were cooled to room temperature and 10 mL distilled water was added. Lastly, the absorbance readings of all seven tubes were taken at 540 nm. A standard curve of absorbance reading against micromoles of maltose was plotted.

3.5 pH Characterization of Fish Gut Enzymes

3.5.1 Characterization of protease activity

Protease activity was determined by measuring the increase in cleavage of short chain polypeptides based on the method of Bezerra et al. (2005) using azocasein as substrate and determine enzyme activity from pH 2 to 13. The pH buffers used were: 0.1M glycine-HCl pH 2; 0.1M citrate buffer pH 3-5; 0.1M phosphate buffer pH 6-8; 0.05M carbonate buffer pH 9-10; 0.05M Na_2HPO_4 buffer pH 11; and 0.1M KCl-NaOH buffer pH12-13. 500 µL of 1% azocasein was incubated with 20 µL crude enzymes from tilapia and catfish and 200 µL buffer solution in two different eppendorf tubes for 60 min at 30 ^{0}C. Another blank tube with the same preparation except the 20 µL of crude enzymes was replaced with distilled water was prepared. Five hundred microliter of 20% TCA was then added into three tubes to stop reaction. 15 min later, three tubes were centrifuged at 10,000 $\times g$ for 10 min. One militer of supernatant was added into 1.5 mL of 1M NaOH in a glass cuvette and the absorbance reading of three tubes was measured at 440nm. The protease activity was defined as the change in absorbance per min per mg protein of enzyme extract (ΔAbs min^{-1} mg protein^{-1}).

3.5.2 Characterization of amylase activity

The amylase activity was monitored in triplicate by the DNS method (Bernfeld, 1951) with slight modification. The amylase activity was determined by using starch as a substrate with a buffer solution at pH 5, 6, 7, 8, 9, 11 and 12 as in 3.5.1.

Tubes with label B, 5, 10, 15, 20 and 30 were prepared. Tube B was the blank with incubation time for 5 min and the incubation period (in min) for the other tubes was as stated in the label. Five hundred microliter of 1% starch was incubated with 50 µL crude enzymes and 400 µL buffer solution at 30 ^{0}C for different period of time. At the end of the incubation time of each tubes, 1.5 mL of 1% DNS was added into it and was boiled for 5 min. After that, 1.5 mL of distilled water was added into it and was left aside to cool down. Lastly, the absorbance reading of every tube was read at 550nm against blank (Tube B). The amylase specific activity was defined by the µmol of maltose produced per min per mg protein at the specified condition.

3.6 *In vitro* Protein Digestibility

In vitro protein digestibility assay of SY was conducted in triplicate using pH drop method. A weighed SY sample prepared from 3.1.1, which was an equivalent amount of ingredient that provided 160 mg of crude protein was soaked with 20 mL distilled water for overnight at 4 ^{0}C. On the next day, the pH of the mixture was adjusted to pH 8 using 0.1M NaOH and then 2 mL of crude enzyme from either tilapia or catfish was added. The pH of the mixture was recorded at every minute interval for 10 min by pH meter. Casein was chosen as the reference protein. The protein digestibility was calculated as the percentage of magnitude of pH drop (-ΔpH) ratio of the SY and casein (Lazo, 1994, cited in Sultana, Ahmed

and Chisty, 2010). The equation that used to calculate the relative protein digestibility (RPD) of SY is as follows:

$$\text{RPD (\%)} = \frac{\text{-}\Delta\text{pH of processed spent brewer's yeast}}{\text{-}\Delta\text{pH of casein}} \times 100$$

3.7 Statistical Analysis

Results were expressed as mean± standard deviation. The amylase specific activity of crude enzyme extracts from tilapia and catfish was subjected to statistical evaluation performed by t-test. A value of $p<0.05$ was considered significant. The statistical program used was SAS® software.

CHAPTER 4

RESULTS

4.1 Nutritional Constituents of Spent Brewer's Yeast

Nutritional constituents of SY were expressed as percentage. With refer to Figure 4.1, the moisture content, crude protein, crude lipid, ash, crude fiber and nitrogen-free extract (NFE) of SY are 17.00±0.42%, 30.51±0.27%, 1.03±0.18%, 8.45±1.01%, 4.48±0.60% and 38.54±1.31% respectively.

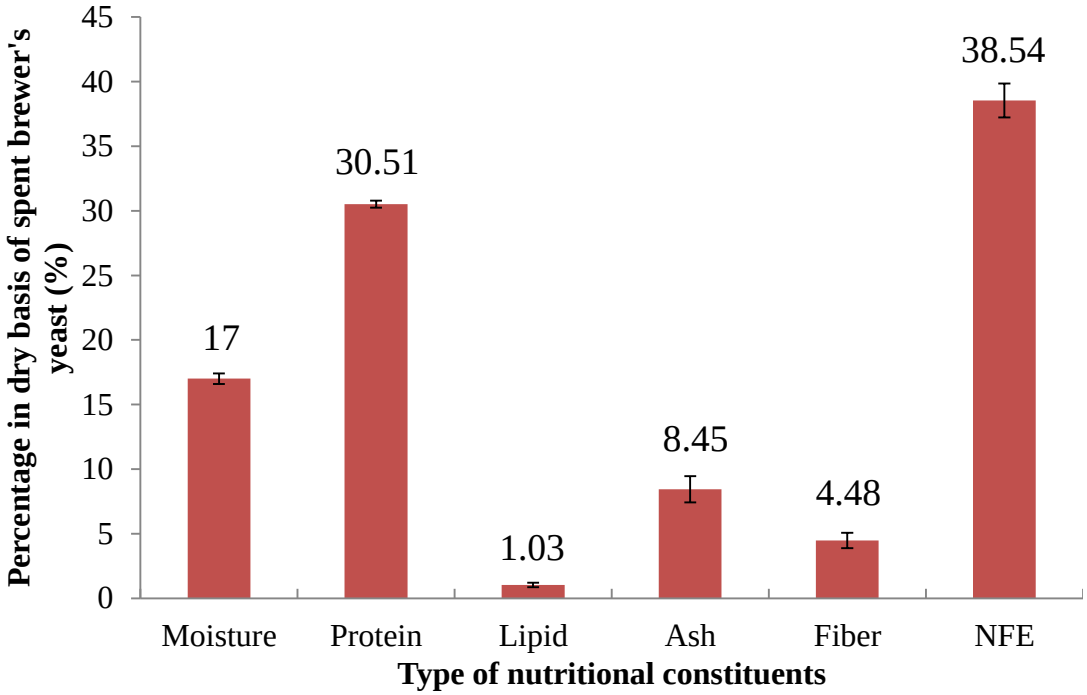


Figure 4.1: Type of nutritional constituents of spent brewer's yeast (SY). Values represent mean ± standard error (n=3).

4.2 Amylase Specific Activities of Fish Gut Enzyme as a Function of pH

The highest amylase specific activity of both fish gut enzymes was at pH 7.

Table 4.2: Amylase specific activities of fish gut enzyme as a function of pH.

pH	Amylase specific activity (μmol $min^{-1}mg^{-1}$)	
	Catfish	Tilapia
5	0.18 ± 0.02^{e}	6.22 ± 0.13^{d}
6	0.35 ± 0.00^{e}	17.38 ± 1.67^{b}
7	0.80 ± 0.03^{e}	26.73 ± 0.13^{a}
8	0.64 ± 0.03^{e}	11.85 ± 1.29^{c}
9	0.27 ± 0.01^{e}	1.65 ± 0.21^{e}
11	0.50 ± 0.00^{e}	1.66 ± 0.05^{e}
12	0.49 ± 0.05^{e}	10.94 ± 0.71^{c}

* ^{abcde}Mean values in the same column with different letters are significantly different ($p<0.05$).
* Mean values that share a common superscript letter between columns or in the same column are not significantly different ($p>0.05$).

4.2.1 Amylase specific activity of crude catfish gut enzyme as a function of pH

The lowest amylase specific activity of crude catfish gut enzyme was at pH 5 which is 0.18±0.02µmol $min^{-1}mg^{-1}$ while the highest was at pH 7 which was 0.80±0.03µmol $min^{-1}mg^{-1}$. Among the alkali pHs, amylase specific activity at pH 8 was the highest, 0.64±0.03 µmol $min^{-1}mg^{-1}$, followed by 0.50±0.00 µmol $min^{-1}mg^{-1}$ at pH 11 and 0.49±0.05 µmol $min^{-1}mg^{-1}$ at pH 12. The amylase specific activity at pH 6 and 9 were 0.35±0.00 and 0.27±0.01 µmol $min^{-1}mg^{-1}$ respectively.

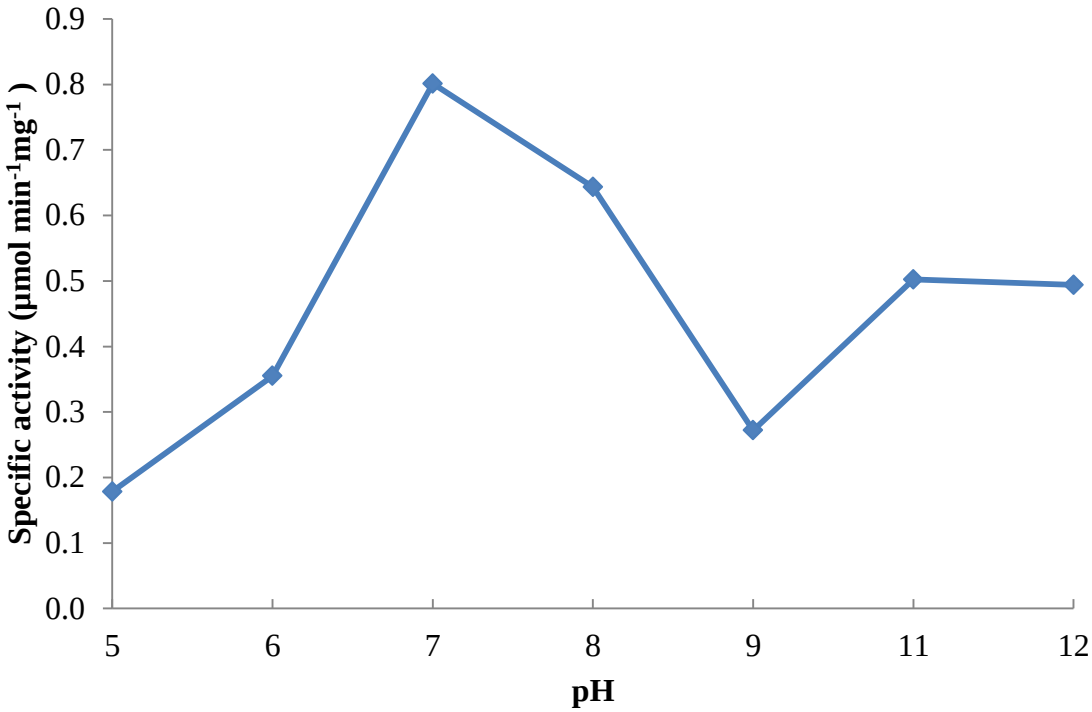


Figure 4.2a: Amylase specific activity of crude catfish gut enzyme as a function of pH. Values represent mean ± standard error (n=3).

4.2.2 Amylase specific activity of crude tilapia gut enzyme as a function of pH

The amylase specific activity of crude tilapia gut enzyme was highest at pH 7, 26.73±0.13 µmol $min^{-1}mg^{-1}$. However, the amylase specific activity was higher at acidic pH than in alkali pH. Amylase specific activity at pH 6 (17.38±1.67µmol $min^{-1}mg^{-1}$) was higher than at pH 8 (11.85±1.29µmol $min^{-1}mg^{-1}$), 9 (1.65±0.21µmol $min^{-1}mg^{-1}$), 11 (1.66±0.05µmol $min^{-1}mg^{-1}$), and 12 (10.94±0.71 µmol $min^{-1}mg^{-1}$). Although the amylase specific activity at pH 5 (6.22±0.13 µmol $min^{-1}mg^{-1}$) was lower than at pH 8, it still higher than at pH 9, 11 and 12.

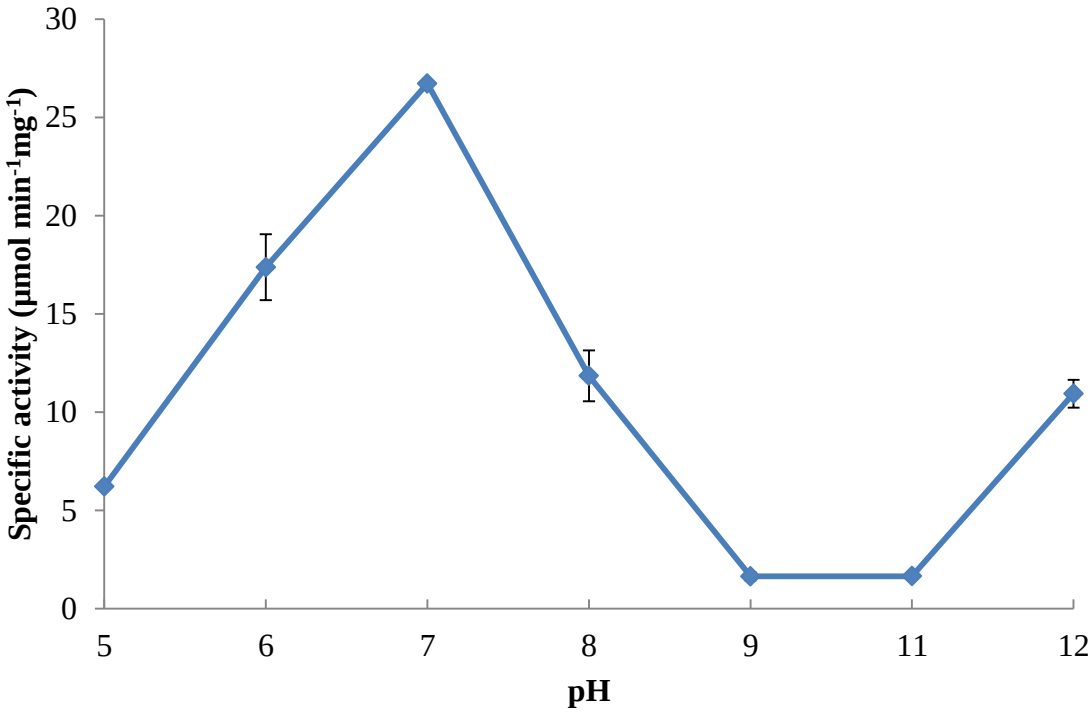


Figure 4.2b: Amylase specific activity of crude tilapia gut enzyme as a function of pH. Values represent mean ± standard error (n=3).

4.3 Protease Specific Activities of Fish Gut Enzyme as a Function of pH

Based on the Table 4.3, the protease specific activity of both fishes gut enzyme in mU/mg were plotted against a pH range of 2 to 13. Crude tilapia gut enzyme had a higher protease specific activity than the crude catfish gut enzyme. The highest protease specific activity of crude tilapia gut enzyme was 2.938 mU/mg at pH 10. Contrary, the highest protease activity of crude catfish gut enzyme was 0.649 mU/mg at pH 12.

Table 4.3: Protease specific activities of fish gut enzyme as a function of pH.

pH	Protease specific activity (mU/mg)	
	Catfish	Tilapia
2	0.285	0.081
3	0.125	0.115
4	0.171	0.209
5	0.062	0.324
6	0.073	0.912
7	0.036	1.162
8	0.125	1.398
9	0.296	1.472
10	0.327	2.938
11	0.306	1.479
12	0.649	1.553
13	0.042	0.142

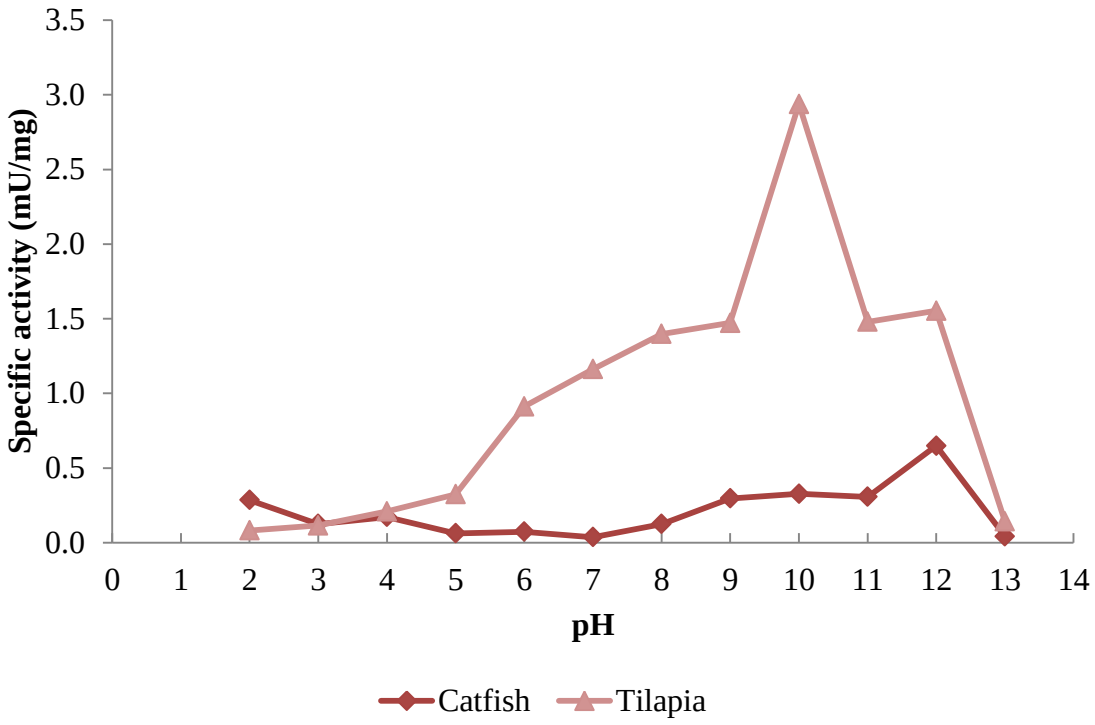


Figure 4.3: Protease specific activities of fish gut enzyme as a function of pH.

4.4 pH Change of Casein and Spent Brewer's Yeast

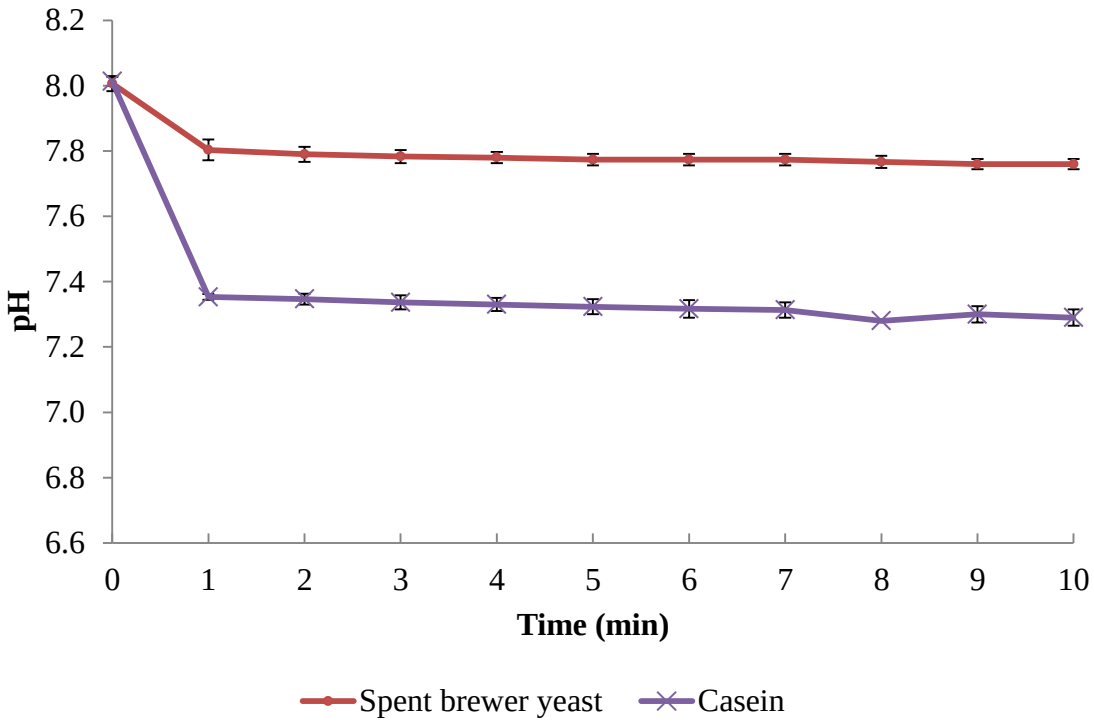


Figure 4.4a: pH change of casein and spent brewer's yeast. Values represent mean ± standard error (n=3).

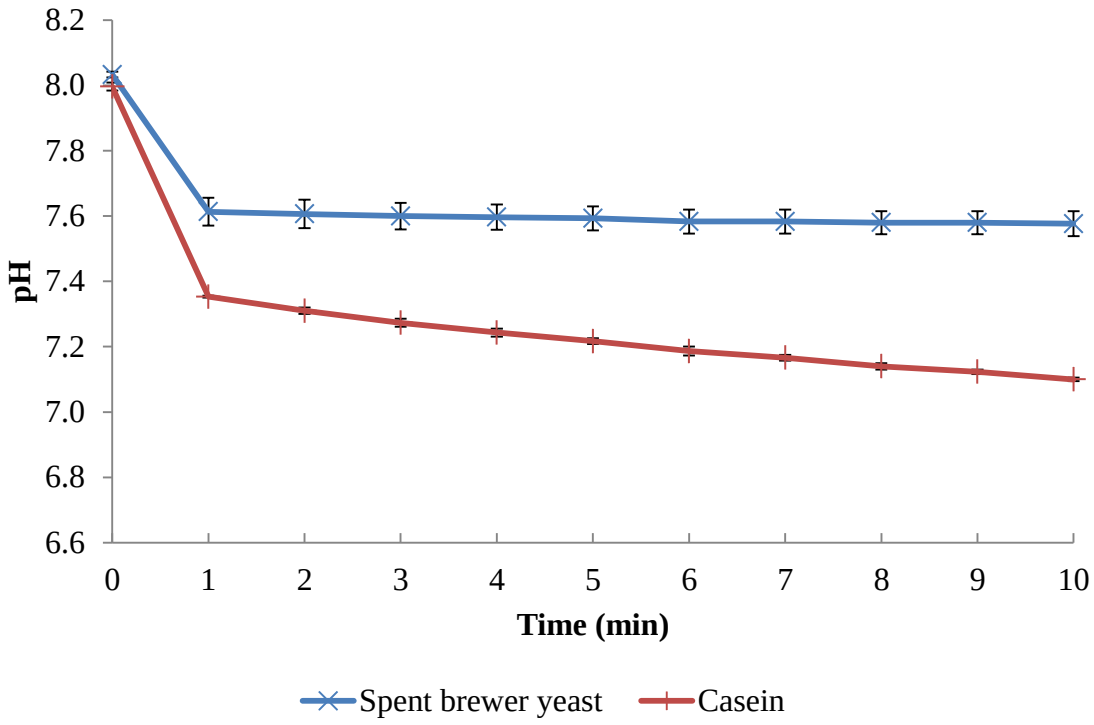
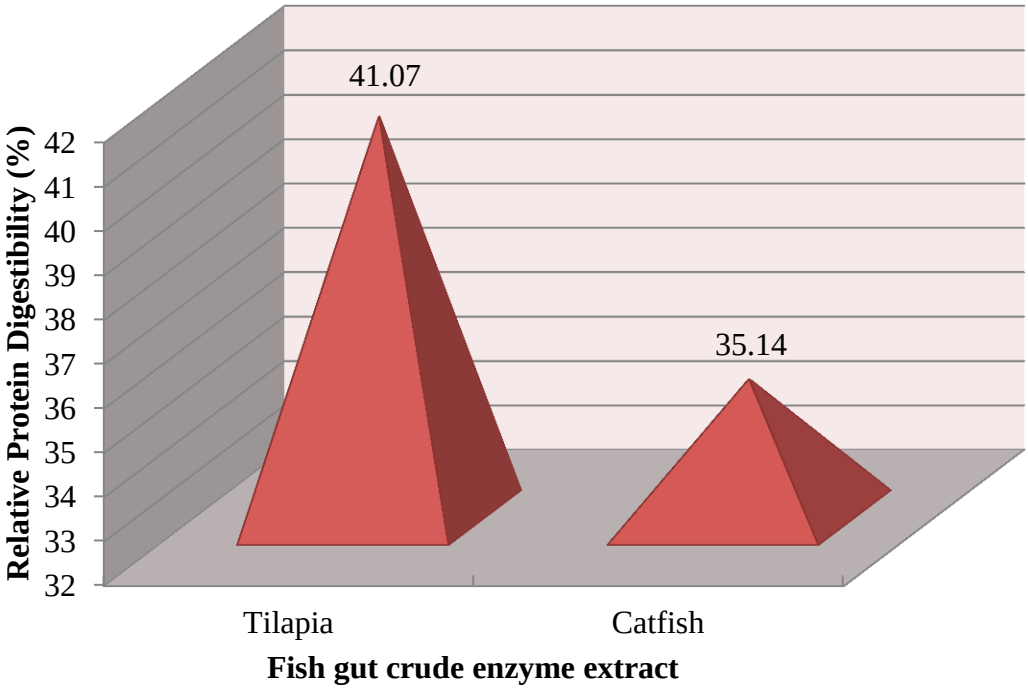


Figure 4.4b: pH change of casein and spent brewer's yeast. Values represent mean ± standard error (n=3).

4.5 The Relative Protein Digestibility of Spent Brewer's Yeast



* Relative protein digestibility was calculated from the gradient of curve of spent brewer's yeast against gradient of curve of casein within the same plot and times with 100%.

Figure 4.5: The relative protein digestibility of spent brewer's yeast.

CHAPTER 5

DISCUSSION

5.1 Analysis on Nutritional Composition

From the result of proximate analysis showed in Figure 4.1, the moisture content, crude protein content, crude lipid content, ash content, fiber content and nitrogen-free extract (NFE) content of spent brewer's yeast (SY) are 17±0.42%, 30.51±0.27%, 1.03±0.18%, 8.45±1.01%, 4.48±0.60% and 38.54±1.31% respectively. The result is slightly different from the Tacon, Metian and Hasan (2009) in which the crude moisture and crude fiber in our result is higher, lower in crude protein and the rest remain almost the same.

Table 5.1: Comparison of nutritional compositions of spent brewer's yeast.

Brewer's yeast	**Average composition (% by weight)**					
	Moisture	Crude protein	Crude lipid	Crude fiber	NFE	Ash
Result range from Tacon, Metian and Hasan (2009)	7.0-8.6	43.8-49.4	0.8-1.7	2.4-3.9	24.3-39.4	6.6-12.1
Result from this project	17±0.42	30.51±0.27	1.03±0.18	4.48±0.60	38.54±1.31	8.45±1.01

The major constituents of spent brewer's yeast are its protein and NFE content which both of them contribute to almost 70% of the total nutritional content. NFE is acts as an energy source in the diet. It is because NFE is a type of carbohydrate which included soluble sugar and starch. Therefore, instead of functioning as energy source, it is also a building block for other nutrients. Although fiber also is a type of carbohydrate which is the major nutrition constituent in plant based feed ingredient, excess fiber content could reduce the digestibility of nutrient (Ayuba and Iorkohol, 2012). Yet, there are high standard error shown in NFE (more than 1), ash (more than 1) and fiber (more than 0.5). The lower the standard error which means nearer to zero, more accurate and reliable the result is. This may due to the imprecision of crude ash, crude fiber and NFE as well as crude protein determined by Weende proximate anaylse (Evonik Industries, n.d.). From the same website, it stated that modern methods has been established such as to determined crude ash via atomic absorption spectroscopy, crude protein via near infrared spectroscopy and method developed by Van Soest to detect different components of the cell wall to specify the NFE and crude fiber fraction.

5.2 pH Characterization of Fish's Amylase Enzyme

Based on Figure 4.2.2, fish gut enzymes from tilapia for hydrolysis of the starch substrate displayed high amylase specific activity at 6, 7, 8 and 12. This result were comparable with Moreau, Desseaux and Santimone (2001), Rathore, Kumar and Chakrabarti (2005), Klahan, Areechon and Engkagul (2009) and Li, Li and

Wu (2006) who also found that fish gut enzymes exhibits relative higher activity of amylase at a pH of 6, 7 and 12. Meanwhile, fish gut enzymes from catfish for hydrolysis of the starch substrate displayed high amylase specific activity at 7, 8, 11 and 12. These results were comparable to Sudaporn, Kringsak and Yuwadee (2010) who also found that catfish gut enzymes exhibits relatively higher activity of amylase at a pH of 6, 7, 8, 11 and 12. Effect of pH on both of the amylase activities from different fish species is significantly different ($p<0.05$). Yet, there is no significant different in the amylase activity at pH 9 and 11 between both fishes. Despite, both tilapia and catfish gut enzymes have the highest amylase specific activity at pH 7, a neutral pH in this study. The amylase activity obtained were 0.80±0.03 μmol $min^{-1}mg^{-1}$ and 26.73±0.13 μmol $min^{-1}mg^{-1}$ for catfish and tilapia respectively. Wong (1995) has reported that the optimum pH for amylase activity varies depending on the source of the enzyme, with a range of pH values reported for amylase in mammals of 6.0-7.0 and 4.8-5.8 for *Aspergillus oryzae*, 5.85-6.0 for *Bacillus subtilis* (Wong, 1995 cited in Klahan, Areechon and Engkagul, 2009). Both of the fishes showed highest specific activity at the same pH range. We believe that this is due to the fish gut crude enzymes used in this study, were collected from the empty digestive tracts which include stomach and intestine of fasted fishes. Thus, only enzymes located in the intestinal mucus and stomach lumen had been extracted and assayed. Li, Li and Wu (2006) has reported that the amylase from the intestine part of the digestive system of tilapia also has maximum activity at pH range of 6-7. We have found that the magnitude of amylase specific activity at specific pH was influenced by the types of fish as well. The amylase activity from tilapia gut enzymes apparently was higher as compared

to catfish gut enzymes throughout the pH range studied. This could be due to tilapia is an indigenous herbivorous fish; tilapia demonstrates greater activity of carbohydrase (α-amylase) compared to carnivorous and omnivorous fish (Fish, 1960; Agrawal et al., 1975; Das and Tripathi, 1991; Opuszynski and Shireman, 1995, cited in Tengjaroenkul, Smith and Smith, 2000). In contrast, catfish is classified as a type of omnivorous fish (Fisheries and Aquaculture Department, 2001).

5.3 pH Characterization of Fish's Protease Enzyme

The protease activity of tilapia and catfish gut enzymes for hydrolysis of the azocasein substrate displayed high specific activity at pH 9, 10, 11 and 12. The highest specific activity of tilapia's protease was at pH 10 while the catfish was at pH 12. Both fishes apparently were using alkaline protease for protein digestion. Protease activity of tilapia gut enzyme was higher than of the catfish. There was an increase of protease specific activity in both of the tilapia and catfish at the pH range of 3-4. This indicates that the possibility of the presence of another protease which may be an acidic protease (acidic pepsin) from stomach. Lacking of functional acid secreting stomach may negatively affect protein digestion because under denaturing acid conditions (pH 2 to 5) of a functional gastric stomach, proteins are exposed to proteolytic active pepsin (Jany, 1974, Ronnestad et al., 2003, Tonheim et al., 2005, cited in Tonheim, Nordgreen and Ronnestad, 2007). In turn, the proteolysis ingested dietary proteins is accelerated. However, this

increase could not be seen obviously in Figure 4.3 as compared with the alkaline protease that may originate from intestines which having a sharp and nice peak. On the other hand, the protease activity of tilapia started to increase from pH 5 to pH12. This probably indicates the increase of activity of the alkaline proteolytic enzyme to digest dietary protein from spent brewer's yeast. However, the protease activity of catfish started to increase from pH 8 to pH 12. This indicates that the protease activity of catfish was mainly contributed by alkaline protease (chymotrypsin and trypsin). Both of the protease activity from fishes dropped at pH 13. This may due to the pH 13 is too alkaline and not favorable for protease to react with dietary protein. The variations of optimum pH in digestive enzyme activity (amylase and protease) depend on the fish species and source of the enzyme. But, the protease and amylase activity may also relate to the feeding habits of fish. This is supported by the study of De Silva and Anderson (1995), cited in Klahan, Areechon and Engkagul (2009) which noted that *Oreochromismossambicus* developed a higher level of amylase activity when their diet were changed to a starch-rich diet. Amylase responds to the level of dietary carbohydrate. From the observations, different digestive enzyme activity in different fish species can be used as a basis for suitable feed formulation for effective utilization by fish.

5.4 *In vitro* Protein Digestibility of Spent Brewer's Yeast

Figure 4.4a and 4.4b showed the *in vitro* protein digestibility by tilapia and catfish's gut enzyme extracts using the pH drop method of Lazo single enzyme assay (Lazo, 1994, cited in Sultana, Ahmed and Chisty, 2010). Casein is normally used as reference standard for comparing its digestion to that of other proteins in feed ingredient and the evaluation of protein nutritional quality in *in vivo* and *in vitro* experiments. This is because casein exhibits a rate of *in vitro* digestibility between 83 and 92%, thus this supports the use of casein as a reference standard (FDA 1991, cited in Clark, 2003).

The *in vitro* protein digestibility of spent brewer's yeast was different dependent on the types of fish gut enzyme extract. Relative protein digestibility of spent brewer's yeast by tilapia showed a higher rate (41.07%) as compared to catfish (35.14%). The feed ingredient used is constant and the origin of enzyme is varied. From the result of proximate analysis (Figure 4.1), protein percentage of spent brewer's yeast is the second major nutrient other than nitrogen-free extract (30.51±0.27%). Although the same protein percentage of spent brewer's yeast was given to both of the fish enzymes, different digestibility was showed. This may be explained by the significant higher protease activity in tilapia gut enzymes as compared to catfish gut enzymes. The digestibility of any protein depends on the ability of fish to utilize the nutrient after digest. The responsibility for digestion of the feed ingredient that the fish consumed relies on the enzyme which is the

proteases. It is because the protease acts as a catalyst that transforms feed ingredient into absorbable form (Nelson and Cox, 1982, cited in Sultana, Ahmed, and Chisty, 2010). The higher relative protein digestibility in tilapia than in catfish probably may due to herbivorous and omnivorous like tilapia is less choosy about the feed ingredient (Klahan, Areechon and Engkagul, 2008). Even though tilapia have been categorized as herbivorous that possess morphological and physiological adaptations for the utilization of high fiber diets, many are well-known for their ability to utilize a wide variety of foods. The variety of foods includes aquatic larvae and insects as well as algae, weeds and macrophytes (Lowe-McConnell, 1975, Bowen, 1982, Trewavas, 1983, cited in Tengjaroenkul, Smith and Smith, 2000). Moreover, formulated feeds for tilapia normally resembles to omnivorous fish which contain mainly animal proteins (Maina et al., 2002). Therefore, tilapia has higher protein digestibility than catfish. In fact, the relative protein digestibility of spent brewer's yeast by both of the fish enzymes is low. Thus, it could explain that why there is limited research or study on the 100% replacement of fishmeal to spent brewer's yeast, but normally can be seen in present research as a combination diet with fishmeal or other feed ingredients. For examples, the report by Matty and Smith (1978) cited in Bob-Manuel and Alfred-Ockiya (2011) which showed that 20% inclusion of yeast (*Candida lypolytica*) was accepted by rainbow trout, and 50% yeast substituted diet was better utilized by the fish than the 100% fishmeal diet observed by Bob-Manuel and Alfred-Ockiya (2011). It is because feeding fish with more than one protein source will promote growth performance due to the synergistic effect of combining two biological compounds may have superior effect than individually applied for fish

diets (Hossain and Jauncey, 1989, Sogbesan et al., 2004, cited in Bob-Manuel and Alfred-Ockiya, 2011). Nevertheless, it could be recommended to fish farmers and fish feed technologists to make use of this under-utilized protein source in feed formulation for tilapia and catfish as well.

CHAPTER 6

CONCLUSIONS

In short, the objectives of this project have been achieved in which the important composition and the protein digestibility of spent brewer's yeast through *in vitro* digestibility study have been determined. The crude protein content, moisture content, crude lipid content, ash content, fiber content and nitrogen-free extract (NFE) content are 30.51±0.27%, 17±0.42%, 1.03±0.18%, 8.45±1.01%, 4.48±0.60% and 38.54±1.31% respectively.

Both of the protease activity of tilapia and catfish was high at pH range of 9 to 12. The digestive protease enzyme from both tilapia and catfish prefers alkaline pH. In contrast, the amylase activity of tilapia was high at pH of 6, 7, 8 and 12 whereas the amylase activity of catfish was high at pH 7, 8, 11 and 12. The digestive amylase from tilapia prefers slightly acidic to alkali pH for optimum enzyme activity. Yet, the digestive amylase from catfish prefers neutral to alkali pH for optimum enzyme activity.

Apart from that, the relative protein digestibility (RPD) of spent brewer's yeast by Tilapia is 41.07% whereas by catfish is 35.14%. The digestibility of spent brewer's yeast is high in Tilapia than in Catfish, thus, spent brewer's yeast is more

suitable for feed formulation for the Tilapia. However, it could not be an alternative protein source for Tilapia in replacing fishmeal completely in diet preparation because the relative protein digestibility is just nearly to 50% (partially digestible). Despite of this, spent brewer's yeast still can be included in feed formulation for any species since it has been effectively utilized in a combination feed diet with coupling to other feed ingredient to reduce the cost of complete utilization of fishmeal.

REFERENCES

Adedayo, M. R., Ajiboye, E. A., Akintunde, J. K. and Odaibo, A., 2011. Single Cell Proteins: As Nutritional Enhancer. *Advances in Applied Science Research*, **2** (5), pp. 396-409.

Akintunde, E. A., 1985. Digestive enzymes in the Digestive enzymes in the gut of *Sarotherodon galilaeus* (Syn. Tilapia galilaea, family Cichlidae) of the Lake Kainji, Nigeria. *Nigerian Journal of Science*, **18**, pp. 22-25.

Ali, H., Haque, M. M., Chowdhury, M. M. R. and Shariful, M. I., 2009. In vitro protein digestibility of different feed ingredients in Thai koi (*Anabas testudineus*). *J. Bangladesh Agril. Univ.*, **7** (1), pp. 205-210.

Al-Tameemi, R., Aldubaikul, A. and Salman, N. A., 2010. Comparative study of α-amylase activity in three Cyprinid species of different feeding habits from Southern Iraq. *Turkish Journal of Fisheries and Aquatic Sciences*, **10** (3), pp. 411-414.

Ayuba, V. O. and Iorkohol, E. K., 2012. Proximate Compostion of Some Commercial Fish Feeds Sold in Nigeria. *Journal of Fisheries and Aquatic Science*, **8**, pp. 248-252.

Bernfeld, P., 1951. Enzymes of Starch Degradation and Synthesis. In: NORD, F. F., ed. *Advances in Enzymology and Related Areas of Molecular Biology*. New York: Interscience Publishers, Inc., pp. 379-424.

Bezerra, et al., 2005. Alkaline proteinase from intestine of Nile tilapia (*Oreochromis niloticus*). *Process Biochemistry*, **40** (5), pp. 1829-1834.

Bob-Manuel, F. G. and Alfred-Ockiya, J. F., 2011. Evaluation of yeast single cell protein (SCP) diets on growth performance, feed conversion and carcass composition of Tilapia*Oreochromis niloticus(L.)* fingerlings. *African Journal of Biotechnology*, **10** (46), pp. 9473-9478.

Boucher, S., 2008. *Evaluation of in vitro methods to estimate digestibility of amino acids in the rumen undegraded protein fraction of feedstuffs*. Ph. D. University of New Hampshire. Available at http://books.google.com.my/books?hl=en&lr=&id=dUiSSwh1HoC&oi=fnd&pg=PR3&dq=Evaluation+of+in+Vitro+Methods+to+Estimate+Digestibility+of+Amino+Acids+in+the+Rumen+Undegraded+Protein+Fraction+of+Feedstuffs&ots=HBjkVfA7KQ&sig=s3SLUbFARZvHkAZPDIbVAMgxUd8 [Accessed 3 March 2012].

Cheng, Z. J., Hardy, R. W. and Huige, N. J., 2004. Apparent digestibility coefficients of nutrients in brewer's and rendered animal by-products for

rainbow trout (*Oncorhynchus mykiss* (Walbaum)). *Aquaculture Research*, **35** (1), pp. 1-9.

Chong, A. S. C., Hashim, R. and Ali, A. B., 2002. Assessment of dry matter and protein digestibilities of selected raw ingredients by discus fish (*Symphysodon aequifasciata*) using *in vivo* and *in vitro* methods. *Aquaculture Nutrition*, **8** (3), pp. 229-238.

Churchil, R.R., Mohan, B. and Viswanathan, K., 2000. Effect of supplementation of broiler rations with live yeast culture. *Cheiron*, **29** (1-2), pp. 23-27.

Clark, D. R., 2003. *Improving in vitro protein digestibility and determining protein Nutritional quality by modeling*. MSc. The University of Georgia. Available through Universiti Tunku Abdul Rahman Library website http://library.utar.edu.my [Accessed 20 March 2013].

De Silva, S. S. and Anderson, T. A., 1995. *Fish Nutrition in Aquaculture*. 1st ed. Melbourne: Chapman & Hall London.

El-Beltagy, A. E., El-Adawy, T. A., Rahma, E. H. and El-Bedawey, A. A., 2005. Purification and Characterization of An Alkaline Protease From The Viscera of Bolti Fish (Tilapia Nilotica). *Journal of Food Biochemistry*, **29**, pp. 445-458.

Evonik Industries, n.d. *Feeding strategies.* [Online]. Available at: http://feed-additives.evonik.com/product/feed-additives/en/about/healthy-nutrition/feeding-strategies/pages/feed-analysis.aspx [Accessed 24 March 2013].

FAO of the United Nations. 2006.*Report of the expert consultation on Global Forest Resources Assessment: Towards FRA 2010* [pdf]. Available at: http://www.fao.org/forestry/11187-1-0.pdf [Accessed at 30 March 2013].

FAO Fisheries and Aquaculture Department, 2012. *The State of World Fisheries and Aquaculture 2012.* [Online]. Available at: http://www.fao.org/docrep/016/i2727e/i2727e.pdf [Accessed 20 December 2012].

FAO Fisheries and Aquaculture Department, 2011. *World Aquaculture 2010.* [Online]. Available at: http://www.fao.org/docrep/014/ba0132e/ba0132e.pdf [Accessed 18 December 2012].

Fenerci, S. and Sener, E., 2005. *In Vivo* and *in Vitro* Protein Digestibility of Rainbow Trout (*Oncorhynchus mykiss* Walbaum, 1972) Fed Steam Pressured or Extruded Feeds. *Turkish Journal of Fisheries and Aquatic Sciences*, **5**, pp. 17-22.

Ferreira, I. M. P. L. V. O., Pinhos, O. and Vieira, E. and Tavarela, J. G., 2010. Brewer's Saccharomyces yeast biomass: Characteristics and potential applications. *Trends in Food Science and Technology*, **21** (2), pp. 77-84.

Fisheries and Aquaculture Department, 1994. *Nutrition of fish and crustaceans: a laboratory manual.* [Online]. Available at: http://www.fao.org/docrep/field/003/AB479E/AB479E00.htm [Accessed 30 March 2013].

Fisheries and Aquaculture Department, 2001. *Rice-Fish System.* [Online]. Available at: http://www.fao.org/docrep/005/Y1187E/y1187e18.htm#TopOfPage [Accessed 24 March 2013].

Gatlin III, D. M., et al., 2007. Expanding the utilization of sustainable plant products in aquafeeds – a review. *Aquaculture Research*, **38**, pp. 551-579.

Heyden, Johan van der, 2010. *Population of the entire world, yearly, 1950-2100.* [Online]. Available at: http://www.geohive.com/earth/his_history3.aspx [Accessed 19 December 2012].

Hidalgo, M. C., Urea, E. and Sanz, A., 1999. Comparative study of digestive enzymes in fish with different nutritional habits. Proteolytic and amylase activities. *Aquaculture*, **170** (3-4), pp. 267-283.

Hsu, H. W., Vavak, D. L., Satterlee, L. D. and Miller, G. A., 1977. A multienzyme technique for estimating protein digestibility. *Journal of Food Science*, **42** (5), pp. 1269-1273.

Huntington, T.C. and Hasan, M.R., 2009. Fish as feed inputs for aquaculture–practices, sustainability and implications: a global synthesis. *In*: Hasan, M.R. and Halwart, M., eds. *Fish as feed inputs for aquaculture: practices, sustainability and implications*. Rome: FAO, pp. 1-61.

Johnson, E. A., Conklin, D. E. and Lewis, M. J., 1977. The yeast *Phaffia rhodozyma* as a dietary pigment source for salmonids and crustaceans. *Journal of the Fisheries Research Board of Canada*, **34** (12), pp. 2417-2421.

Kahlon, S. S., 1991. Single-Cell protein from moulds and higher fungi. *In*: Arora, D., Mukerji, K.G. and Marth, E.H., eds. *Handbook of Applied Mycology*. New York: Marcel Dekker, Inc., pp. 499-539.

Klahan, R., Areechon, N., Yoonpundh, R. and Engkagul, A., 2009. Characterization and Activity of Digestive Enzymes in Different Sizes of Nile Tilapia (*Oreochromis niloticus L.*). *Kasetsart Journal*, **43** (1), pp. 143-153.

Klahan, R., Areechon, N., Yoonpundh, R. and Engkagul, A., 2008. *In vitro* protein, lipid and starch digestibility in various sizes of Nile Tilapia (*Oreochiromis Niloticus Linnaeus*). *International Symposium on Tilapia in Aquaculture*, **8**, pp. 875-886.

Kuz'mina, V. V., and Ushakova, N. V., 2007. The effect of temperature, pH, and heavy metals (copper, zinc) upon proteinase activities in the potential preys for typical and facultative piscivores. *Journal of Ichthyology*, **47** (9), pp. 791-801.

Lara-flores, M., Olvera-Novoa, M. A., Guzman-Mendez, B. E. and Lopez-Madrid, W., 2003. Use of the bacteria *Streptococcus faecium* and *Lactobacillus acidophilus*, and the yeast *Saccharomyces cerevisiae* as growth promoters in Nile tilapia (*Oreochromis niloticus*). *Aquaculture*, **216** (1-4), pp. 193-201.

Lazo, J. P., Romaire, R. P. and Reigh, R. C., 1998. Evaluation of Three *In Vitro* Enzyme Assays for Estimating Protein Digestibility in the Pacific White Shrimp *Penaeus vannamei*. *Journal of The World Aquaculture Society*, **29** (4), pp. 441-450.

Li, P. and Gatlin III, D. M., 2004. Dietary brewers yeast and the prebiotic GroBiotick™ AE influence growth performance, immune responses and resistance of hybrid striped bass (*Morone chrysops* × *M. saxatilis*) to *Streptococcus iniae* infection. *Aquaculture*, **231** (1-4), pp. 445-456.

Li, P. and Gatlin III, D. M., 2003. Evaluation of brewers yeast (*Saccharomyces cerevisiae*) as a feed supplement for hybrid striped bass (*Morone chrysops* × *M. saxatilis*). *Aquaculture*, **219**, 681-692.

Li, J. S., Li, J. L. and Wu, T. T., 2006. Ontogeny of protease, amylase and lipase in the alimentary tract of hybrid Juvenile tilapia (*Oreochromis niloticus* × *Oreochromis aureus*). *Fish Physiology and Biochemistry*, **32** (4), pp. 295-303.

Lim, E. H., Lam, T.J. and Ding, J. L., 2005. Single-cell protein diet of a novel recombinant vitellogenin yeast enhances growth and survival of first-feeding tilapia (*Oreochromis mossambicus*) larvae. *Journal of Nutrition*, **135** (3), pp. 513-518.

Litchfield, John H., 1983. Single-Cell Proteins. *Science*, **219**, pp. 740-746.

Lundstedt, L. M., Melo, J. F. B. and Moraes, G., 2002. Induction of Digestive Enzymes in The Brazilian Catfish (Pseudoplatystoma coruscans), [online] Available at: <http://www-heb.pac.dfo mpo.gc.ca/congress/2002/Biochem/lundstedt1.pdf>. [Accessed 1 April 2013].

Maina, et al., 2002. Digestibility and feeding value of some feed ingredients fed to tilapia*Oreochromis niloticus (L). Aquaculture Research*, **33** (11), pp. 853-862.

Miller, Brinton M. and Litsky, W., 1976. *Industrial Microbiology*. Minneapolis: McGraw-Hill Companies.

Moreau, Y., Desseaux, V., Koukiekolo, R., Marchis-Mouren, G. and Santimone, M., 2001. Starch digestion in tropical fishes: isolation, structural studies and inhibition kinetics of α-amylases from two tilapias *Oreochromis niloticus* and *Sarotherodon melanotheron*. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, **128** (3), pp. 543-552.

Nilson, A., Peralta, J. M. F. and Miazzo, R. D., 2004. Use of brewers yeast (*S. cerevisiae*) to replace part of the vitamin mineral premix in finisher broiler diets. *XXII World's Poultry Congress, Istanbul, Turkey*, 495.

Oliva-Teles, A. and Goncalves, P., 2001. Partial replacement of fishmeal by brewers yeast (*Saccharomyces cerevisiae*) in diets for sea bass (*Dicentrarchus labrax*) juveniles. *Aquaculture 202*, pp. 269-278.

Olvera-Novoa, M. A., Martínez-Palacios, C. A., Real de León, E., 1994. *Proximate Analyses*. [Online]. Available at: http://www.fao.org/docrep/field/003/AB479E/AB479E00.htm [Accessed 1 March 2013].

Porter, D. H., Swaisgood, H. E. and Catignani, G. L., 1984. Characterization of an immobilized digestive enzyme system for determination of protein digestibility. *Journal of Agricultural and Food Chemistry*, **32** (2), pp. 334-339.

Rana, K. J., Siriwardena, S. and Hasan, M. R., 2009. *Impact of Rising Feed Ingredient Prices on Aquafeeds and Aquaculture Production*. [Online]. Available at: http://www.fao.org/docrep/012/i1143e/i1143e.pdf [Accessed 27 December 2012].

Rathore, R. M., Kumar, S. and Chakrabarti, R., 2005. Digestive enzyme profile of *Cyprinus carpio* during ontogenic. *Journal of the World Aquaculture Society*, **36**,pp. 37-39.

Reducing waste in beer production. 2011. [Online]. Available at: http://www.alfalaval.com/about-us/press/product-press/Pages/reduced-waste.aspx [Accessed 18 July 2012].

Rumsey, G. L., Kinsella, J. E., Shetty, K. J. and Hughes, S. G., 1991. Effect of high dietary concentrations of brewer's dried yeast on growth performance and liver uricase in rainbow trout (*Oncorhynchus mykiss*). *Animal Feed Science and Technology*, **33** (3-4), pp. 177-183.

Rumsey, G. L., Hughes, S. G. and Kinsella, J. L., 1990. Use of dietary yeast Saccharomyces cerevisiae nitrogen by lake trout. *Journal of the World Aquaculture Society*, **21** (3), pp. 205-209.

Sanderson, G. W., and Jolly, S. O., 1994. The value of Phaffia yeast as a feed ingredient for salmonid fish. *Aquaculture*, **124** (1-4), pp. 193-200.

Santin, et al., 2003. Evaluation of the efficacy of *Saccharomyces cerevisiae* cell wall to ameliorate the toxic effects of aflatoxin in broilers. *International Journal of Poultry Science*, **2** (5), pp. 341- 344.

Santin, et al., 2001. Performance and intestinal mucosa development of broiler chickens fed diets containing *Saccharomyces cerevisae* cell wall. *The Journal of Applied Poultry Research*, **10** (3), pp. 236- 244.

Satterlee, L. D., Marshall, H. F. and Tennyson, J. M., 1979. Measuring protein quality. *Journal of the American Oil Chemists' Society*, **56** (3), pp. 103-109.

Sentihilkumar, M., Kadirvel, R., and Vijaykumar, R. (1997). The use of dried yeast sludge in broiler ration. *Indiana Journal of Poultry Science*, **32** (2), 137-141.

Sigma-Aldrich, Inc., n.d.. *Product information- Bradford Reagent.* [Online]. Availbale at: http://www.sigmaaldrich.com/etc/medialib/docs/Sigma/Bulletin/b6916bul.Par.0001.File.tmp/b6916bul.pdf [Accessed 7 April 2013].

Siwicki, A. K., Anderson, D. P., and Rumsey, G. L., 1994. Dietary intake of immunostimulants by rainbow trout affects non-specific immunity and protection against furunculosis. *Veterinary Immunology and Immunopathology*, **41**, pp. 125-139.

Smith, R. R., n.d. *Chapter 2. Nutritional Bioenergetics in Fish.* [Online]. Available at: http://www.fao.org/docrep/X5738E/x5738e03.htm#3. energy requirements of fish [Accessed 25 March 2013].

Sombutyanuchit, P., Suphantharika, M. and Verduyn, C., 2001. Preparation of 5'-GMP-rich yeast extracts from spent brewer's yeast. *World Journal of Microbiology & Biotechnology*, **17** (2), pp. 163-168.

Sudaporn, T., Kringsak, M. A. and Yuwadee, P., 2010. Characterization of Amylase, Cellulase and Proteinase Enzyme in Stomach and Intestine of the Mekong Giant Catfish Fed with Various Diets Consisting of Spirulina. *Current Research Journal of Biological Sciences*, **2** (4), pp. 268-274.

Sultana, Z., Ahmed, Md. S., Iqball, Md. S. and Chisty, Md. A. H., 2010. Determination of In Vitro Protein Digestibility of Different Feed Ingredients for Nilotica (*Oreochromis nilotica*). *Bangladesh Research Publications Journal*, **4** (1), pp. 87-94.

Tacon, A. G. J., Metian, M. and Hasan, M. R., 2009. *Feed ingredients and fertilizers for farmed aquatic animals: sources and composition.* [Online]. Available at: http://www.fao.org/docrep/012/i1142e/i1142e.pdf [Accessed 2 January 2013].

Tengjaroenkul, B., Smith, B. J., Caceci, T. and Smith, S. A., 2000. Distribution of intestinal enzyme activities along the intestinal tract of cultured Nile tilapia, *Oreochromis niloticus L*. *Aquaculture*, **182**, pp. 317-327.

Tonheim, S. K., Nordgreen, A., Hogoy, I., Hamre, K. and Ronnestad, I., 2007. In vitro digestibility of water-soluble and water-insoluble protein fractions of some common fish larval feeds and feed ingredients. *Aquaculture*, **262**, pp. 426-435.

Whyte, J. N. C., and Sherry, K. L., 2001. Pigmentation and composition of flesh of Atlantic salmon fed diets supplemented with the yeast *Phaffia rhodozyma*. *North American Journal of Aquaculture*, **63** (1), pp. 52-57.

Worthington Biochemical Corporation, 1993. *Amylase, Alpha Assay*. [Online]. Available at: http://www.worthington-biochem.com/AA/assay.html [Accessed 5 April 2013].

Yadav, B. S., Srivastava, K. K. and Shukla, P. K., 1994. Effect of supplementation of the broiler ration with live yeast culture on nutrient utilization and meat production. *Indian Journal of Animal Nutrition*, **11** (4), pp. 225-227.

Zechner-Krpan, et al., 2010.Characterization of β -Glucans Isolated from Brewer's Yeast and Dried by Different Methods. *Food Technol. Biotechnol.*, **48** (2), pp. 189- 197.

APPENDIX A

Table 1: The nutritional composition of spent brewer's yeast (SY) based on dry basis.

Nutritional composition	Triplicate (% dry basis)			Average (%)
	T1	T2	T3	
Moisture	17.20	16.20	17.60	17.00±0.42
Crude Protein	29.98	30.71	30.85	30.51±0.27
Crude lipid	0.83	1.38	0.87	1.03±0.18
Ash	9.37	6.42	9.55	8.45±1.01
Crude Fiber	3.28	5.00	5.16	4.48±0.60
Nitrogen-free extract	39.35	40.29	35.98	38.54±1.31

APPENDIX B

Table 2a: Absorbance reading of amylase from tilapia at pH 5.

Time (min)	Absorbance reading (A)		
	T1	T2	T3
5	0.080	0.091	0.062
10	0.109	0.177	0.099
15	0.153	0.194	0.145
20	0.203	0.271	0.211
30	0.391	0.436	0.378

Table 2b: Absorbance reading of amylase from tilapia at pH 6.

Time (min)	Absorbance reading (A)		
	T1	T2	T3
5	0.126	0.151	0.122
10	0.204	0.253	0.199
15	0.305	0.413	0.340
20	0.590	0.710	0.646
30	0.873	1.182	0.903

Table 2c: Absorbance reading of amylase from tilapia at pH 7.

Time (min)	Absorbance reading (A)		
	T1	T2	T3
5	0.353	0.370	0.290
10	0.757	0.720	0.502
15	1.017	0.967	0.842
20	1.397	1.375	1.268
30	1.747	1.724	1.593

Table 2d: Absorbance reading of amylase from tilapia at pH 8.

Time (min)	Absorbance reading (A)		
	T1	T2	T3
5	0.056	0.052	0.092
10	0.236	0.281	0.216
15	0.332	0.328	0.309
20	0.478	0.476	0.430
30	0.816	0.653	0.615

Table 2e: Absorbance reading of amylase from tilapia at pH 9.

Time (min)	Absorbance reading (A)		
	T1	T2	T3
5	0.037	0.037	0.025
10	0.052	0.063	0.051
15	0.064	0.066	0.065
20	0.073	0.077	0.095
30	0.120	0.112	0.131

Table 2f: Absorbance reading of amylase from tilapia at pH 11.

Time (min)	Absorbance reading (A)		
	T1	T2	T3
5	0.009	0.014	0.01
10	0.014	0.023	0.024
15	0.024	0.032	0.038
20	0.048	0.053	0.050
30	0.089	0.096	0.102

Table 2g: Absorbance reading of amylase from tilapia at pH 12.

Time (min)	Absorbance reading (A)		
	T1	T2	T3
5	0.130	0.139	0.204
10	0.199	0.236	0.217
15	0.236	0.315	0.289
20	0.421	0.565	0.460
30	0.673	0.739	0.677

APPENDIX C

Table 3a: Maltose released by tilapia's amylase at pH 5.

Time (min)	Maltose released (µmol/mL)			Maltose released (µmol)		
	T1	T2	T3	T1	T2	T3
5	0.26	0.31	0.17	3.23	3.89	2.15
10	0.40	0.72	0.35	4.96	9.03	4.37
15	0.61	0.80	0.57	7.60	10.05	7.12
20	0.85	1.17	0.89	10.59	14.65	11.06
30	1.75	1.96	1.68	21.83	24.52	21.05

Calculation

Maltose released by tilapia's amylase in µmol/mL was determined by substituting the absorbance readings from the Table 2a into the "y" of the equation, y = 0.209x + 0.026 from Figure 1. Then, the maltose released by tilapia's amylase in µmol was calculated by multiplying maltose released to amylase assay volume which is 12.5 mL.

Table 3b: Maltose released by tilapia's amylase at pH 6.

Time (min)	Maltose released (µmol/mL)			Maltose released (µmol)		
	T1	T2	T3	T1	T2	T3
5	0.48	0.60	0.46	5.98	7.48	5.74
10	0.85	1.09	0.83	10.65	13.58	10.35
15	1.33	1.85	1.50	16.69	23.15	18.78
20	2.70	3.27	2.97	33.73	40.91	37.08
30	4.05	5.53	4.20	50.66	69.14	52.45

Calculation

Maltose released by tilapia's amylase in µmol/mL was determined by substituting the absorbance readings from the Table 2b into the "y" of the equation, y = 0.209x + 0.026 from Figure 1. Then, the maltose released by tilapia's amylase in µmol was calculated by multiplying maltose released to amylase assay volume which is 12.5 mL.

Table 3c: Maltose released by tilapia's amylase at pH 7.

Time (min)	Maltose released (µmol/mL)			Maltose released (µmol)		
	T1	T2	T3	T1	T2	T3
5	1.56	1.65	1.26	19.56	20.57	15.79
10	3.50	3.32	2.28	43.72	41.51	28.47
15	4.74	4.50	3.90	59.27	56.28	48.80
20	6.56	6.45	5.94	82.00	80.68	74.28
30	8.23	8.12	7.50	102.93	101.56	93.72

Calculation

Maltose released by tilapia's amylase in µmol/mL was determined by substituting the absorbance readings from the Table 2c into the "y" of the equation, y = 0.209x

+ 0.026 from Figure 1. Then, the maltose released by tilapia's amylase in µmol was calculated by multiplying maltose released to amylase assay volume which is 12.5 mL.

Table 3d: Maltose released by tilapia's amylase at pH 8.

Time (min)	Maltose released (µmol/mL)			Maltose released (µmol)		
	T1	T2	T3	T1	T2	T3
5	0.14	0.12	0.32	1.79	1.56	3.95
10	1.00	1.22	0.91	12.56	15.25	11.36
15	1.46	1.44	1.35	18.30	18.06	16.93
20	2.16	2.15	1.93	27.03	26.91	24.16
30	3.78	3.00	2.82	47.25	37.50	35.23

Calculation

Maltose released by tilapia's amylase in µmol/mL was determined by substituting the absorbance readings from the Table 2d into the "y" of the equation, y = 0.209x + 0.026 from Figure 1. Then, the maltose released by tilapia's amylase in µmol was calculated by multiplying maltose released to amylase assay volume which is 12.5 mL.

Table 3e: Maltose released by tilapia's amylase at pH 9.

Time (min)	Maltose released (µmol/mL)			Maltose released (µmol)		
	T1	T2	T3	T1	T2	T3
5	0.05	0.05	0.00	0.66	0.66	-0.06
10	0.12	0.18	0.12	1.56	2.21	1.50
15	0.18	0.19	0.19	2.27	2.39	2.33
20	0.22	0.24	0.33	2.81	3.05	4.13
30	0.45	0.41	0.50	5.62	5.14	6.28

Calculation

Maltose released by tilapia's amylase in µmol/mL was determined by substituting the absorbance readings from the Table 2e into the "y" of the equation, y = 0.209x + 0.026 from Figure 1. Then, the maltose released by tilapia's amylase in µmol was calculated by multiplying maltose released to amylase assay volume which is 12.5 mL.

Table 3f: Maltose released by tilapia's amylase at pH 11.

Time (min)	**Maltose released (µmol/mL)**			**Maltose released (µmol)**		
	T1	T2	T3	T1	T2	T3
5	-0.08	-0.06	-0.08	-1.02	-0.72	-0.96
10	-0.06	-0.01	-0.01	-0.72	-0.18	-0.12
15	-0.01	0.03	0.06	-0.12	0.36	0.72
20	0.11	0.13	0.11	1.32	1.61	1.44
30	0.30	0.33	0.36	3.77	4.19	4.55

Calculation

Maltose released by tilapia's amylase in µmol/mL was determined by substituting the absorbance readings from the Table 2f into the "y" of the equation, y = 0.209x + 0.026 from Figure 1. Then, the maltose released by tilapia's amylase in µmol was calculated by multiplying maltose released to amylase assay volume which is 12.5 mL.

Table 3g: Maltose released by tilapia's amylase at pH 12.

Time (min)	Maltose released (µmol/mL)			Maltose released (µmol)		
	T1	T2	T3	T1	T2	T3
5	0.50	0.54	0.85	6.22	6.76	10.65
10	0.83	1.00	0.91	10.35	12.56	11.42
15	1.00	1.38	1.26	12.56	17.28	15.73
20	1.89	2.58	2.08	23.62	32.24	25.96
30	3.10	3.41	3.11	38.70	42.64	38.94

Calculation

Maltose released by tilapia's amylase in µmol/mL was determined by substituting the absorbance readings from the Table 2g into the "y" of the equation, y = 0.209x + 0.026 from Figure 1. Then, the maltose released by tilapia's amylase in µmol was calculated by multiplying maltose released to amylase assay volume which is 12.5 mL.

APPENDIX D

Table 4: Amylase specific activity of tilapia at pH 5, 6, 7, 8, 9, 11 and 12.

pH	Specific activity (µmol $min^{-1}mg^{-1}$)			Average(µmol $min^{-1}mg^{-1}$)
	T1	T2	T3	
5	6.02	6.46	6.18	6.22 ± 0.13^{d}
6	15.28	20.69	16.18	17.38 ± 1.67^{b}
7	26.99	26.57	26.63	26.73 ± 0.13^{a}
8	14.37	11.08	10.10	11.85 ± 1.29^{c}
9	1.56	1.34	2.05	1.65 ± 0.21^{e}
11	1.61	1.61	1.75	1.66 ± 0.05^{e}
12	10.79	12.24	9.80	10.94 ± 0.71^{c}

* Means with the same letter are not significantly different.

Calculation

Specific activity of a particular pH in µmol/min was the gradient from the curve of maltose released (µmol) against time from every triplicate. Then, the gradient was divided by the amount of enzyme in the reaction mixture (mg) determined by Bradford assay (Table 16 of Appendix L) to obtain the specific activity in µmol $min^{-1}mg^{-1}$.

APPENDIX E

Table 5a: Absorbance reading of amylase from catfish at pH 5.

Time (min)	Absorbance reading (A)		
	T1	T2	T3
5	0.035	0.021	0.034
10	0.057	0.046	0.048
15	0.066	0.070	0.062
20	0.072	0.084	0.074
30	0.092	0.091	0.087

Table 5b: Absorbance reading of amylase from catfish at pH 6.

Time (min)	Absorbance reading (A)		
	T1	T2	T3
5	0.038	0.031	0.039
10	0.054	0.064	0.051
15	0.085	0.070	0.081
20	0.105	0.108	0.102
30	0.150	0.145	0.152

Table 5c: Absorbance reading of amylase from catfish at pH 7.

Time (min)	Absorbance reading (A)		
	T1	T2	T3
5	0.072	0.081	0.069
10	0.142	0.141	0.154
15	0.163	0.172	0.195
20	0.252	0.265	0.243
30	0.338	0.347	0.318

Table 5d: Absorbance reading of amylase from catfish at pH 8.

Time (min)	Absorbance reading (A)		
	T1	T2	T3
5	0.036	0.035	0.034
10	0.041	0.047	0.063
15	0.072	0.068	0.083
20	0.120	0.139	0.109
30	0.247	0.241	0.230

Table 5e: Absorbance reading of amylase from catfish at pH 9.

Time (min)	Absorbance reading (A)		
	T1	T2	T3
5	0.011	0.006	0.008
10	0.016	0.013	0.015
15	0.030	0.034	0.036
20	0.061	0.067	0.064
30	0.085	0.094	0.092

Table 5f: Absorbance reading of amylase from catfish at pH 11.

Time (min)	Absorbance reading (A)		
	T1	T2	T3
5	0.036	0.027	0.030
10	0.057	0.050	0.055
15	0.063	0.060	0.078
20	0.130	0.105	0.110
30	0.190	0.187	0.195

Table 5g: Absorbance reading of amylase from catfish at pH 12.

Time (min)	Absorbance reading (A)		
	T1	T2	T3
5	0.042	0.049	0.050
10	0.052	0.060	0.080
15	0.093	0.090	0.095
20	0.120	0.146	0.141
30	0.172	0.226	0.204

APPENDIX F

Table 6a: Maltose released by catfish's amylase at pH 5.

Time	Maltose released (µmol/mL)			Maltose released (µmol)		
(min)	T1	T2	T3	T1	T2	T3
5	-0.09	-0.10	-0.09	-1.12	-1.29	-1.13
10	-0.07	-0.08	-0.08	-0.84	-0.98	-0.96
15	-0.06	-0.05	-0.06	-0.73	-0.68	-0.78
20	-0.05	-0.04	-0.05	-0.66	-0.51	-0.63
30	-0.03	-0.03	-0.04	-0.41	-0.42	-0.47

* Calculations are the same as shown in Appendix C.

Table 6b: Maltose released by catfish's amylase at pH 6.

Time	Maltose released (µmol/mL)			Maltose released (µmol)		
(min)	T1	T2	T3	T1	T2	T3
5	-0.09	-0.09	-0.09	-1.08	-1.17	-1.07
10	-0.07	-0.06	-0.07	-0.88	-0.76	-0.92
15	-0.04	-0.05	-0.04	-0.49	-0.68	-0.54
20	-0.02	-0.02	-0.02	-0.24	-0.21	-0.28
30	0.03	0.02	0.03	0.32	0.26	0.34

Table 6c: Maltose released by catfish's amylase at pH 7.

Time	Maltose released (µmol/mL)			Maltose released (µmol)		
(min)	T1	T2	T3	T1	T2	T3
5	-0.05	-0.04	-0.06	-0.66	-0.54	-0.69
10	0.02	0.02	0.03	0.22	0.21	0.37
15	0.04	0.05	0.07	0.48	0.59	0.88
20	0.13	0.14	0.12	1.59	1.76	1.48
30	0.21	0.22	0.19	2.67	2.78	2.42

Table 6d: Maltose released by catfish's amylase at pH 8.

Time (min)	Maltose released (µmol/mL)			Maltose released (µmol)		
	T1	T2	T3	T1	T2	T3
5	-0.09	-0.09	-0.09	-1.11	-1.12	-1.13
10	-0.08	-0.08	-0.06	-1.04	-0.97	-0.77
15	-0.05	-0.06	-0.04	-0.66	-0.71	-0.52
20	0.00	0.01	-0.02	-0.06	0.18	-0.19
30	0.12	0.12	0.11	1.53	1.46	1.32

Table 6e: Maltose released by catfish's amylase at pH 9.

Time (min)	Maltose released (µmol/mL)			Maltose released (µmol)		
	T1	T2	T3	T1	T2	T3
5	-0.11	-0.12	-0.12	-1.42	-1.48	-1.46
10	-0.11	-0.11	-0.11	-1.36	-1.39	-1.37
15	-0.09	-0.09	-0.09	-1.18	-1.13	-1.11
20	-0.06	-0.06	-0.06	-0.79	-0.72	-0.76
30	-0.04	-0.03	-0.03	-0.49	-0.38	-0.41

Table 6f: Maltose released by catfish's amylase at pH 11.

Time (min)	Maltose released (µmol/mL)			Maltose released (µmol)		
	T1	T2	T3	T1	T2	T3
5	-0.09	-0.10	-0.09	-1.11	-1.22	-1.18
10	-0.07	-0.07	-0.07	-0.84	-0.93	-0.87
15	-0.06	-0.06	-0.05	-0.77	-0.81	-0.58
20	0.01	-0.02	-0.01	0.07	-0.24	-0.18
30	0.07	0.06	0.07	0.82	0.78	0.88

Table 6g: Maltose released by catfish's amylase at pH 12.

Time (min)	Maltose released (µmol/mL)			Maltose released (µmol)		
	T1	T2	T3	T1	T2	T3
5	-0.08	-0.08	-0.07	-1.03	-0.94	-0.93
10	-0.07	-0.06	-0.04	-0.91	-0.81	-0.56
15	-0.03	-0.03	-0.03	-0.39	-0.43	-0.37
20	0.00	0.02	0.02	-0.06	0.27	0.21
30	0.05	0.10	0.08	0.59	1.27	0.99

APPENDIX G

Table 7: Amylase specific activity of catfish at pH 5, 6, 7, 8, 9, 11 and 12.

pH	Specific activity (µmol $min^{-1}mg^{-1}$)			Average(µmol $min^{-1}mg^{-1}$)
	T1	T2	T3	
5	0.16	0.21	0.16	0.18 ± 0.02^{f}
6	0.35	0.35	0.36	0.35 ± 0.00^{d}
7	0.82	0.84	0.74	0.80 ± 0.03^{a}
8	0.67	0.67	0.59	0.64 ± 0.03^{b}
9	0.25	0.29	0.27	0.27 ± 0.01^{e}
11	0.50	0.50	0.51	0.50 ± 0.00^{c}
12	0.42	0.58	0.48	0.49 ± 0.05^{c}

* Means with the same letter are not significantly different.

*Calculations are the same as shown in Appendix D.

APPENDIX H

Table 8: Absorbance reading and specific activity of tilapia's protease at pH range of 2-13.

pH	Absorbance (A)	Specific activity (mU/mg)
2	0.012	0.081
3	0.017	0.115
4	0.031	0.209
5	0.048	0.324
6	0.135	0.912
7	0.172	1.162
8	0.207	1.398
9	0.218	1.472
10	0.435	2.938
11	0.219	1.479
12	0.23	1.553
13	0.021	0.142

Calculation

Specific activity was calculated by dividing absorbance reading to assay time, 60 min and then divided by the enzyme concentration. The enzyme concentration is total enzyme (mg/mL) showed in Appendix L multiplied with the assay volume, 0.02mL.

APPENDIX I

Table 9: Absorbance reading and specific activity of catfish's protease at pH range of 2-13.

pH	Absorbance (A)	Specific activity (mU/mg)
2	0.055	0.285
3	0.024	0.125
4	0.033	0.171
5	0.012	0.062
6	0.014	0.073
7	0.007	0.036
8	0.024	0.125
9	0.057	0.296
10	0.063	0.327
11	0.059	0.306
12	0.125	0.649
13	0.008	0.042

Calculation

Specific activity was calculated by dividing absorbance reading to assay time, 60 min and then divided by the enzyme concentration. The enzyme concentration is total enzyme (mg/mL) showed in Appendix L multiplied with the assay volume, 0.02mL.

APPENDIX J

Table 10: pH change of casein and spent brewer's yeast by using crude gut enzyme of tilapia.

Time(min)	pH change in casein			pH change in spent brewer's yeast		
	T1	T2	T3	T1	T2	T3
0	7.98	7.99	8.02	8.02	8.05	8.03
1	7.35	7.35	7.36	7.64	7.67	7.53
2	7.29	7.32	7.32	7.64	7.66	7.52
3	7.25	7.29	7.28	7.63	7.65	7.52
4	7.22	7.26	7.25	7.63	7.64	7.52
5	7.2	7.23	7.22	7.63	7.63	7.52
6	7.16	7.2	7.2	7.62	7.62	7.51
7	7.15	7.17	7.18	7.62	7.62	7.51
8	7.12	7.15	7.15	7.62	7.61	7.51
9	7.11	7.13	7.13	7.62	7.61	7.51
10	7.09	7.1	7.11	7.62	7.61	7.5

Table 11: pH change of casein and spent brewer's yeast by using crude gut enzyme of catfish.

Time(min)	pH change in casein			pH change in spent brewer's yeast		
	T1	T2	T3	T1	T2	T3
0	8.04	8.01	7.99	8.05	7.97	8
1	7.35	7.37	7.34	7.86	7.75	7.8
2	7.33	7.38	7.33	7.83	7.75	7.79
3	7.32	7.38	7.31	7.82	7.75	7.78
4	7.31	7.37	7.31	7.81	7.75	7.78
5	7.3	7.37	7.3	7.8	7.74	7.78
6	7.29	7.37	7.29	7.8	7.74	7.78
7	7.29	7.36	7.29	7.8	7.74	7.78
8	7.28	7.28	7.28	7.79	7.73	7.78
9	7.27	7.35	7.28	7.78	7.73	7.77
10	7.26	7.34	7.27	7.78	7.73	7.77

APPENDIX K

Table 12: Relative protein digestibility (%) of spent brewer's yeast by crude gut enzyme of Tilapia and Catfish.

Type of fish enzyme	Relative protein digestibility (%) on spent brewer's yeast
Tilapia	41.07
Catfish	35.14

APPENDIX L

Table 13: Absorbance reading of maltose standard curve.

Maltose concentration (µmol/mL)	Absorbance (A)
0.5	0.107
1	0.224
1.5	0.327
2	0.473
2.5	0.594
5	1.047

Table 14: Absorbance reading of BSA standard curve.

BSA concentration (mg/mL)	Absorbance (A)
0.02	0.277
0.04	0.359
0.06	0.472
0.08	0.528
0.10	0.605

Table 15: Absorbance reading and total enzyme in both fishes based on BSA standard curve.

Fish type	Absorbance (A)			Amount of enzyme (mg/mL)	Amount of enzyme (mg)
	T1	T2	Average		
Tilapia	0.626	0.792	0.709	0.123	0.123
Catfish	0.857	0.868	0.863	0.160	0.160

* Total enzyme assay volume is 1mL.

APPENDIX M

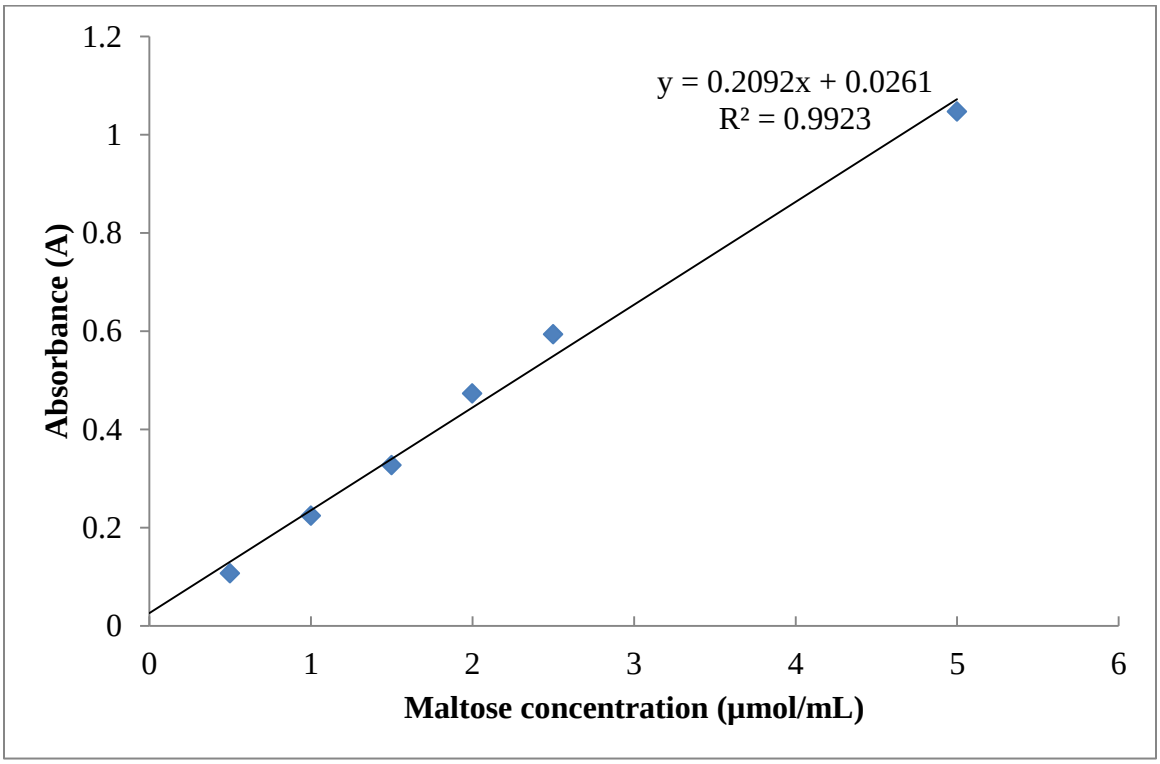


Figure 1: Maltose standard curve.

APPENDIX N

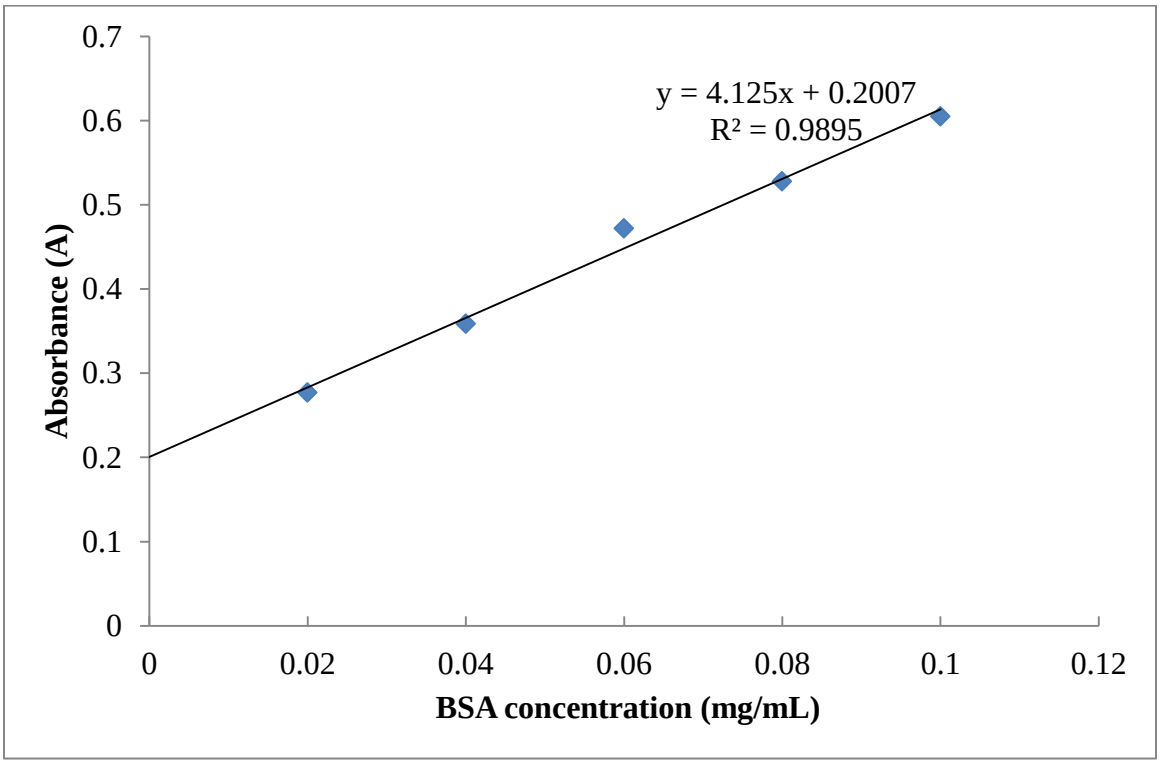


Figure 2: Bovine Serum Albumin (BSA) standard curve.