UNIVERSITY OF NAIROBI

FISH PROTEIN HYDROLYSATE FROM DAGAA *(Rastrineobola argentea)* AS NUTRITIONAL COMPONENT IN MICROBIAL CULTURE MEDIA

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DECLARATION

This is my original work and it has not been presented for award of a degree in any other University.

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DEDICATION

I dedicate this work to my parents, science and all those interested in the development of Kenya when it comes to Biotechnological products.

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LIST OF ABBREVIATIONS

AAS	Atomic absorption spectrophotometry
ANOVA	Analysis of variance
AOAC	Association of analytical chemists
BSA	Bovine serum albumin
DFH	Dagaa fish hydrolysate
DNS	Dinitrosalicylic
DPH	Dagaa protein hydrolysate
DPH-FM	Dagaa protein hydrolysate formulated media
FH	Fish hydrolysate
FPH	Fish protein hydrolysate
LB	Luria Bertani
NaCl	Sodium chloride
NaOH	Sodium hydroxide
SD	Sabouraud’s dextrose
SDA	Sabouraud’s dextrose agar
SDB	Sabouraud’s dextrose broth
TSB	Tryptic soy broth
UK	United Kingdom
USA	United States of America

ABSTRACT

Dagaa is a small pelagic fish found in Lake Victoria. It is mainly used as a nutrient source in the animal feed industry as well as for human consumption among the poor and the middle class. Fish protein hydrolysate as a source of nutrient in culture media has been explored in several fish species such as Silver Carp, Yellow Tuna, Yellow Stripe Trevally, Herring and Mackerel. Dagaa could also be an excellent candidate for culture media since it is rich in proteins, lipids and minerals. This would also add value to the underutilized fish species and minimize post-harvest losses of up to 50% during rainy seasons. In the current study, Dagaa as a source of nitrogen in culture media was explored by first preparing the Dagaa Protein Hydrolysate (DPH) using Alcalase enzyme. Proximate analysis for protein, lipid, moisture /dry weight and ash contents of DPH was performed and compared with that of Dagaa, Dagaa Fish Hydrolysate (DFH), Standard media Luria Bertani (LB) and Sabouraud's Dextrose Broth (SDB). Mineral analysis was also performed on all these samples. The potential of DPH as a nitrogen source in microbial growth media was investigated using; *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis* and *Rhodobacter capsulatus* for bacterial growth studies while *Metarhizium anisopliae* and *Beauveria bassiana* were used for fungal growth. Potential of DPH to sustain production of commercial valued enzyme amylase was also investigated with *R. capsulatus* and *B. subtilis* in DPH-Starch media. This was done using Dinitrosalicylic (DNS) acid assay. *M. anisopliae* was analyzed for sporulation (conidia production) rate in DPH-Dextrose media. Proximate and mineral analysis indicated that there was significant difference ($p<0.05$) in the nutritional and mineral profiles of DPH and the two standard media (LB and SD). The protein content of DPH was higher than that of LB and SDB while the mineral content of DPH was lower than that of LB but slightly higher than that of SDB. LB had more Sodium content than DPH. Investigation of the potential of DPH as a nitrogen source in media indicated that in all microorganisms used in this study, there was no significant difference ($p>0.05$) in DPH-FM and DPH-Dextrose as compared to standard media in the growth of microorganisms. The potential for DPH-Starch to sustain production of commercial valued amylase indicated that there was significant difference ($p<0.05$) in the sustenance of amylase production in formulated DPH-Starch and standard media by *R. capsulatus* and *B. subtilis*. DPH-Starch media gave better production of amylase enzyme in both microbes than the standard media. There was no significant difference ($p>0.05$) in the sporulation rates of *M. anisopliae* in DPH-Dextrose and the standard media. Hence DPH formulated media can be substituted as a potential culture media for microbial growth. The cost of formulation is low and provides an alternative means of value addition to Dagaa.

CHAPTER ONE
1.0 INTRODUCTION

1.1 Background

Culture of microorganisms is a very important part of research especially when used in molecular biology and biotechnology and is also important in foundational and basic diagnostic methods (Nair, 2008). Nutritional demands of microbes grown on culture media are usually unique to the microorganism being cultured, but all culture media usually have macronutrients such as carbohydrates, lipids and proteins as well as micronutrients (minerals and vitamins) (Oberhardt et al., 2015; Prentice, 2005). Macronutrients are usually required in large amounts to maintain cell structure and metabolism of microorganisms while micronutrients are required in small amounts for enzyme function and maintenance of protein structure (Prentice, 2005).

The growth of microbes on culture media is highly dependent on whether the media meets the nutritional demands of the microbe. Current commercial culture media in the market contain at least a nitrogen source, a carbohydrate source and other nutritional components (Nair, 2008). The nitrogen source is usually the most expensive in preparation of culture medium (Aspmo et al., 2005; Martone et al., 2005). Currently in the market for the preparation of LB media (a nutrient media) made up of Sodium chloride, Yeast extract and Tryptone, Sodium Chloride (NaCl) is priced at €39.20 for a 1kg formulation, Yeast Extract is priced at €222 for a 1kg formulation while Tryptone the nitrogen source is priced at € 406 for a 1kg formulation all by Sigma Aldrich, USA (Sigma Aldrich, 2016). The nitrogen source in media can be derived primarily from bovine or porcine origins such as meat, internal organs, gelatine and milk but also from plants and yeast. However, due to outbreaks of bovine and porcine diseases, use of peptones of non-meat origin is becoming increasingly important (Ovissipour et al., 2009).

Several fish and fish by-products have been used to prepare fish peptone. The formulated media from fish protein hydrolysates has been shown to produce similar yields of microbes in comparison with standard media (Beaulieu et al., 2009). Peptone prepared from Silver Carp head using Alcalase enzyme was found to have a protein concentration of 20.7% (Safari et al., 2011). The growth of *Vibrio*

anguillarum in Silver Carp head peptone was not different from the growth of this bacterium in standard peptone media (Safari et al., 2011). Alcalase hydrolysate from Silver Carp filleting was also reported to perform better than Tryptic Soy Broth (TSB) media in the growth of *Staphylococcus aureus* (Fallah et al., 2015). Therefore, fish peptone can be substituted for traditional peptone sources with the advantage of fish peptone being more economical and less susceptible to disease (Ovissipour et al., 2009).

In Kenya, Lake Sardine or Silver Cyprinid, locally known as Dagaa (*Rastrineobola argentea*), accounts for the second largest fish catch at Lake Victoria (Nyeko, 2008; Salehe, 2012). It is relatively cheap and available as compared to Nile Perch (Owaga et al., 2010). Despite the large harvest of Dagaa, most of the catches are lost due to endogenous enzyme spoilage as well as lack of adequate preservation methods during the rainy season. The post-harvest losses can be managed by exploitation of the high nutritive content of Dagaa through preparation of Dagaa Fish Hydrolysate (DFH) and Dagaa Protein Hydrolysate (DPH) (Ogonda et al., 2014). Proximate studies carried out on Dagaa have shown that it is rich in protein (19.1-21.7%) and lipids (1.8-3.4%) (Ogonda et al., 2014).

This study therefore aimed at carrying out proximate analysis of macronutrients and mineral analysis of Dagaa and its derivatives i.e. Dagaa fish hydrolysate (DFH) and Dagaa protein hydrolysate (DPH) and also to determine the efficacy of DPH as a nutritional component in microbial culture media. DPH formulated media would provide a more economically rewarding avenue for Dagaa harvests as well as an alternative to the usually expensive commercial culture media in the market.

1.2 Research Problem Statement

Fish hydrolysates contain the macronutrients; proteins, fats and some important minerals (Abdulazeez et al., 2013; Fallah et al., 2015; Safari et al., 2011; Safari et al., 2012; Ogonda et al., 2014). However fish and fish by-products have not been utilized to their full capacity in scientific research work in Kenya (Owaga et al., 2010). Microbiology and microbial culturing is a very important aspect of most molecular biology and biotechnology work. The commercial media used not only in microbial culturing are usually expensive but necessary for culturing therefore there

is need for establishing an alternative media that can be used to culture microorganisms but also as efficient in microbial growth and maintenance of required properties of the organisms being grown (Duffose et al., 1997; Aspmo et al., 2005; Ovissipour et al., 2011). Since Dagaa is inexpensive and can be used to prepare protein and lipid rich fish hydrolysate, this study aims to compare the efficiency of Dagaa protein hydrolysate in the growth of microorganisms with the commercial medium such as Tryptone and Peptone currently in use. The purpose of this study is therefore to come up with an alternative and efficient microbial culture media using Dagaa protein hydrolysate.

1.3 Justification of study

Whole fish or fish by-products are rich in macronutrients required for growth of most organisms such as lipids and proteins (Gordon and Ratliff, 1992; Ogonda et al., 2014). Therefore, enzymatic hydrolysis of Dagaa to produce hydrolysate for microbial growth would be an added advantage in science due to its low cost and value addition to the otherwise less valued Dagaa fish (Owaga et al., 2010). Proximate analysis studies done on fish hydrolysate showed that fish of different types are rich in proteins, lipids and minerals (Ramakrishnan et al., 2013; Nurnadia et al., 2011). These macro and micronutrients are important for microbial growth and therefore the fish hydrolysate can be used as a nutritional component in media; this has been done with other fish species such as Yellow Fin Tuna, Yellow Stripe Trevally and Silver carp (Fallah et al., 2015; Safari et al., 2011; Klompong et al., 2009; Ovissipour et al., 2011). Other studies where the nitrogenous compound in culture media has been substituted with fish protein hydrolysate (FPH) have also been carried out and results indicated that the FPH as a nitrogen source in culture media gives equal or even better results than the traditional nitrogen sources such as peptone/tryptone in microbial culture media (Herpandi et al., 2010). During the rainy seasons, post harvest losses of Dagaa are very high due to the inability to dry Dagaa in the sun, the most effective method of preservation for the fish (Ofulla et al., 2007). If the Dagaa is converted to protein hydrolysate, these losses can be minimized, since no drying is required before hydrolysate preparation. Therefore since preparation of fish hydrolysate is cheap and Dagaa is easily available, it would help save costs of microbial culturing to come up with a protein source for microbial

media using Dagaa protein hydrolysate. Most of the commercial media in use for microbial growth are usually expensive and sometimes not readily available especially to resource poor economies of the world.

1.4 Hypothesis

Dagaa protein hydrolysate is a potential nutritional source for microbial culture of bacterial and fungal species.

1.5 Objectives

1.5.1 General objective

To establish the efficiency of Dagaa Protein Hydrolysate as a nitrogenous source in microbial culture media.

1.5.2 Specific objectives

1. To determine the nutritional and mineral profile for Dagaa fish and its hydrolysate derivatives.
2. To determine the potential of Dagaa Protein Hydrolysate formulated media as a nitrogenous source in culturing selected microbes.
3. To investigate the potential of Dagaa Protein Hydrolysate formulated media in production of industrial products.

CHAPTER TWO
2.0 LITERATURE REVIEW

2.1 Components of microbial culture media

Among microorganisms, prokaryotes are the most abundant life forms in the planet. They can survive in extreme temperatures as well as at high temperatures or high salt concentrations (DasSarmar and DasSarmar, 2012). The metabolic diversity of prokaryotes is also very diverse among themselves than in eukaryotes (Nair, 2008).

Bacterial, yeast and fungal populations have been manipulated in several ways to produce enzymes, antibiotics, chemicals, bio-chemicals and even utilized in factories to prepare beverages (Howard et al., 2004; Nair, 2008). Microbial cultures from *Bacillus* strains of bacteria have been utilized in several studies to produce enzymes such as amylase. Amylase is an important enzyme that is utilized in the breakdown of starch to give glucose units that are easily utilized by organisms as an energy source as compared to starch (Cuevas et al., 2015).

The chemical composition environment of microbes is very important for their growth. This mostly entails the nutritional conditions while its physical conditions of growth are dictated by its physical environment and factors such as pH and temperature (Nair, 2008). The nutritional demands of microbes help to provide an energy source for the microbes and these energy sources include, carbon, nitrogen, trace elements and growth factors (Oberhardt et al., 2015; Nair, 2008). These nutritional demands in combination with physical environmental demands are important for the growth of microbes in a laboratory microbial culturing situation.

The carbon source is very important as it is the most preferred primary source of energy for most microbes. It can be in form of simple sugars such as glucose, lactose and sucrose or polysaccharides such as starch, glycogen, cellulose or molasses (Nair, 2008; Mandelbaum and Hadar, 1990). Some fungi such as entomogenous *Metarhizium anisopliae* and filamentous fungi have the ability to produce enzyme amylase that they use to breakdown starch in the absence of dextrose which is its preferred carbon source of energy (Rosato et al., 1981; Hasan, 2015). Additionally, bacteria such as *Bacillus* strains also produce amylase among other enzymes that

they require in order to get the carbon source of energy as they grow (Thippeswamy et al., 2006).

Nitrogen sources usually are available in media as ammonium salts, urea, animal tissue extract, amino acids mixture and plant tissue extracts. It is important as a building block for enzymes and structural components in microbes (Nair, 2008). Several fish species protein hydrolysates have been utilized as a nitrogenous source in microbial culture media (Klompong et al., 2009; Fallah et al., 2015). Trace elements are very important in growth of microbes but they are only required in minute amounts. Some of the mineral elements that are important in the growth of microbes include sodium, magnesium, phosphorus and iron (Nair, 2008). Trace elements act as co-factors ensuring that the enzymes from the microbes work effectively. Halophytic bacteria have been shown to require magnesium in form of magnesium sulphate (Crisler et al., 2012).

Growth factors such as certain organic compounds are essential for growth of microbes (Nair, 2008). Yeast extract and vitamins are very important growth factors usually utilized in microbial culturing especially while using nutrient media (Tang et al., 2015). Some of the vitamins required for bacterial growth include folic acid that is used for the transfer of carbon units, synthesis of purine bases, serine and methionine and Vitamin K is also used by bacteria for electron transport processes (Todar, 2006). Water usually acts as a base for culture media. Usually while culturing in a laboratory situation it is important to note that distilled or double distilled water is always the preferred base of the culture media (Nair, 2008).

2.2 Fish protein hydrolysate and methods of its preparation

Fish hydrolysate (FH) can be defined as solubilised fish that can be obtained by hydrolysis of whole fish or fish by-products using acids, organic solvents, autolysis or exogenous enzymes (Maqueda et al., 2013).

2.2.1 Use of some exogenous and endogenous enzymes in protein hydrolysis

Exogenous enzyme hydrolysis of fish has been shown to give the best results in the production of fish hydrolysate, with protein concentration of 82.66% using Alcalase

enzyme and 73.51% using Protamex enzyme (Muzaifa et al., 2012). Endogenous enzymes are available within the fish and hence are usually a cheaper alternative than the exogenous enzymes (Guerard et al., 2002). Endogenous enzymes however cause autolysis of the proteins in the fish that cannot be controlled as it is a process that takes place as an organism undergoes post-mortem changes such as rotting or decomposition (Guerard et al., 2002). These enzymes are thus preferred because when used in hydrolysis of fish to attain a protein hydrolysate, the process can be controlled to achieve a hydrolysate with a good degree of hydrolysis for it to be applicable in various aspects such as microbial culturing (Guerard et al., 2002).

Various studies conducted on different fish species and mostly on pelagic fish have shown through proximate analysis that, fish hydrolysate contains proteins and lipids (Ogonda et al., 2014; Aitken et al., 2001; Ayuba and Iorkohol, 2013). However, most of the studies have focused on fish protein hydrolysate (FPH) because proteins constitute the highest concentration of macronutrients followed by lipids in fish species (Kristinsson and Rasco, 2000). Fish protein hydrolysate from different types of fish have been prepared and tested for their potential as nitrogenous sources for microbial culturing. Some of the fish studied are Tuna, Yellow Fin Tuna, Yellow Stripe Trevally and the Silver Carp among many (Safari et al., 2011; Klompong et al., 2009; Fallah et al., 2015; Guerard et al., 2002). The Silver Carp filleting by-product and head have been investigated for nitrogenous content and had a protein content of 92.9% and 20.7%, respectively when hydrolysed using Alcalase enzyme (Safari et al., 2011). In both cases the fish hydrolysate was found to be an effective source of nitrogenous content for microbial culturing (Fallah et al., 2015; Safari et al., 2011). The Yellow Fin Tuna stomach was found to have a protein content that was effective enough in growing microbial organisms as the only nitrogenous source (Guerard et al., 2002).

The most preferred method of acquiring FH from fish species is by use of exogenous enzymes because chemical methods of hydrolysis do not produce the best yields due to uncontrolled degree of hydrolysis, high salt retention in the hydrolysate and denaturation of proteins (Abdulazeez et al., 2013; Guerard et al., 2002). Some of the enzymes available in the market that are used in hydrolysis include Alcalase, Protamex and Flavourzyme (Abdulazeez et al., 2013). Alcalase has been shown to

give the best yields of protein as compared to the others and therefore is the most preferred enzyme when it comes to isolating fish protein hydrolysate (Muzaifa et al., 2012).

In addition to this property, Alcalase has been shown to give the same yield of proteins as Flavourzyme in the FH (Muzaifa et al., 2012). It has also been shown that there is no significant difference in protein yield using either Alcalase or Flavourzyme in hydrolysis of fish species; however Alcalase gives higher yield of protein when used in hydrolysis as compared to the use of Protamex (Ovissipour et al., 2009). Protamex has shown the best degree of hydrolysis of proteins from white croaker fish as compared to Alcalase and Flavourzyme (Silva et al., 2014). This could be an indication that the enzymes can have differing degree of hydrolysis based on the species of the fish. Enzymes can aid in the extraction of proteins in several ways including attacking the cell wall to increase protein yield by liberating more proteins from the matrix source (Tang et al., 2002). Enzymes used can be proteases, either used alone or in combination and are used to partially hydrolyze proteins to peptides increasing their solubility and making them more easily available.

Aqueous enzymatic protein hydrolysis is an environmentally friendly and safe alternative to extract oils and proteins (Latif and Anwar, 2009; De Moura et al., 2011). It avoids damage to the proteins produced by the refining steps, improving their nutritional and functional properties; it is also a non-toxic procedure (Moure et al., 2000). However, there are some limitations to this procedure, in that it can be time consuming and the high costs of enzymes can make it uneconomical. The use of immobilized enzyme in order to reduce overall cost by allowing the reuse of enzymes is an alternative to using free enzymes (Maqueda et al., 2013).

2.2.2 Use of organic solvents for protein extraction

Use of organic solvents in extraction of proteins from food sources involves the use of alcohols and acetone mostly. Organic solvents have extreme pH and negative charge that can denature the proteins along with precipitation of the protein arresting their activity during hydrolysis hence giving a low protein yield after hydrolysis. Proteins that are recovered by hydrolysis using organic solvents are also difficult to

re-dissolve (Nandakumar et al., 2003). Organic solvents have a high clean up capacity and low tendency to dissolve polysaccharides and nucleic acids. However, it is not the case for proteins in which organic solvents have a strong solvent action for and they can also affect protein functionality. Use of organic solvents can also be toxic and time consuming than other sample precipitation procedures (Maqueda et al., 2013)

2.2.3 Use of alkaline or acid pH in protein precipitation

Aqueous solutions in extraction of proteins and other nutrients from food material is gaining popularity due to the growing environmental concerns on the use of organic solvents for the same purpose. Aqueous solutions such as water are also non-flammable, non-toxic and non-explosive as compared to organic solvents such as alcohol (Maqueda et al., 2013). Production of protein concentrates and isolates commercially, consists of an aqueous solubilisation of proteins and carbohydrates at acidic, neutral or alkaline pH and the selective recovery of the solubilised protein, separation washing and neutralization before drying (Tan et al., 2011). In this case protein yield recovered is dependent on factors such as pH, salts concentration, ionic strength of medium, net charge and electrostatic repulsions (Tan et al., 2011).

High pH conditions affect protein yield in case of alkali hydrolysis and very low pH conditions also have the same effect on proteins, in this cases proteins are denatured. It is also of concern that protein quality can be altered by alkaline processing due to undesirable reactions involving racemization of amino acids, formation of toxic compounds such as lysinoalanine, reduction of digestibility, loss of essential amino acids and decrease in nutritive value (Maqueda et al., 2013). Additionally, washing sample after hydrolysis to remove left over acid or alkali which presents challenges of losing some of the final product (Sereewatthanawut et al., 2008).

2.2.4 Use of subcritical water as solvent in protein extraction

Recent studies have also focused on the use of subcritical water as a solvent in hydrolysis of foodstuff to produce proteins and other important bio-molecules. Subcritical water is an environmentally friendly reaction medium (Maqueda et al., 2013). It is water that maintains its liquid state in the temperature range of 100-374

°C under pressurized conditions. It has unique properties such as a lower relative dielectric constant and a higher ion product than ambient water. These properties make it a promising extraction solvent for various compounds including proteins (Hata et al., 2008). Studies of protein extraction from rice bran and soybean meal using subcritical water have indicated that the yield of proteins was high and of good functional properties of the proteins were noted (Watchararuji et al., 2008; Fabian et al., 2011).

2.3 Proximate composition analysis for food macronutrient content determination

Proximate analysis involves the method for quantitative analysis of different macronutrients in feed; it is based on the Weende analysis that was developed in 1860 by Henneberg and Stohmann in Germany. It is not a nutrient analysis rather it is a partitioning of both nutrients and non-nutrients into categories based on common chemical properties (Abdulazeez et al., 2013). It involves partitioning of compounds in a feed into six categories based on the chemical properties of the compounds. These include; moisture, ash, crude protein (kjeldahl protein), crude lipid, crude fibre and nitrogen free extract (digestible carbohydrates) (Ogonda et al., 2014).

Proximate analysis of fish focuses on the water, fat, protein and ash contents. Carbohydrates and non-protein compounds are present in negligible amount and are usually ignored for routine analysis in fish (Cui and Wootton, 1988). However, it has been determined that fish are rich in proteins with amino acid composition that is well suited for human dietary requirements. It compares favourably to egg, milk and meat in nutritional value of its protein (Olomu, 1995). The nutritional value of fish has not just been exploited in the human dietary industry but also in the animal feeds and microbiology industries. Dagaa fish has been used as a fish meal in animal feeds in East Africa apart from being used as a supplement in relief food as well as a source of protein to the poor and middle class (Ogonda et. al, 2014; Bille and Shemkai, 2006).

2.4 Mineral analysis and importance of trace elements in microbial culture media

Minerals are usually considered trace elements and are mostly not required in large quantities. Their main importance is that they act as cofactors for enzymes, for example nickel which is a cofactor for enzyme urease or magnesium that is a cofactor for hexokinase enzyme in bacteria (Nair, 2008). Magnesium and potassium are involved in action potential within the body and sodium is important for maintenance of cell structure (Crisler et al., 2012). Different entities within an environment have differing mineral concentration of different elements. To be able to determine the mineral content of an entity and then its mineral composition studies can be performed on soil, water and even animal feeds (Tang et al., 2015).

Several fish species have been investigated for the presence of minerals such as catfish, *Plotosus lineatus*, *Arius maculate* and many other fish and some of the mineral elements identified include calcium, magnesium, iron, sodium and zinc (Manikandarajan et al., 2014, Tenyang et al., 2014).

2.5 Incorporation of fish hydrolysate as a nutrient source in microbial media

The use of fish materials as a source of nutrients for microbes was reported as early as 1949 (Tarr and Deas, 1949). FH has been incorporated into culture media mostly as a source of nitrogen (Duffose et al., 2001). This is because in microbial culturing, the most expensive component of the media is usually the nitrogen source (Green et al., 1977). The nitrogen source is obtained from meat or milk sources from animals that are usually expensive and at times might be contaminated with pathogens since these animals are usually susceptible to diseases (Duffose et al., 2001). There is a need to develop cheaper alternative sources of nitrogen when making culture media (Aspmo et al., 2005).

Recent studies indicate that fish are rich in proteins and these proteins are easily obtained through exogenous enzymatic hydrolysis of whole fish or fish by-products (Ramakrishnan et al., 2013). Peptones achieved from these studies were incorporated in media as nitrogen source and most results have shown that the microorganisms grown on media that have fish peptone as nitrogen source show

better if not equal microbial growth titres as microorganisms grown on standard media (Ovissipour et al., 2009). Fish protein hydrolysate from hake filleting waste appeared to be of sufficient nutritional value to support growth of bacteria and archaea (Martone et al., 2005). Fish peptones from tuna, cod, red salmon, and unspecified fish were compared to one made with Casein using a new method based on Gompertz modeling of microbial growth (Duffose et al., 2001; Sathivel et al., 2003). Cumulative results obtained from 6 species of bacteria, yeasts, and fungi showed that in most cases, the fish peptones were very effective as a source of nitrogenous compound in microbial culturing (Dufosse et al., 2001).

2.6 Microorganisms to be used in determining efficacy of DPH formulated media.

Microorganisms or microbes are living creatures that are too small to be seen with the naked eye. They can only be observed under a microscope. There are 3 categories of microbes; bacteria, fungi and viruses (Teitzel, 2016).

2.6.1 Bacteria

Bacteria are unicellular creatures that usually do not have a nucleus or membranes around their organelles. They might have genomic DNA only or both genomic and plasmid DNA. Some of the bacteria that were used in this study are *Escherichia coli*, *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Rhodobacter capsulatus*.

2.6.1.1 *Escherichia coli*

Escherichia coli is a gram-negative, facultative anaerobic, rod shaped bacterium (Sweeney, 1996). It typically colonizes the lower gastrointestinal tract, precisely the colon of human infants' hours after birth. It is known to have a symbiotic relationship with its host unless the host is immune-compromised and has peritonitis allowing the bacteria access into the blood stream (Kaper et al., 2004). Some strains of *Escherichia coli* that are known to be pathogenic include entero-pathogenic, entero-invasive, entero-hemorrhagic and diffusely adherent *E. coli*. Some of the diseases they may cause are enteric diarrhoeal disease, urinary tract infections and sepsis or meningitis (Nataro and Kaper, 1998).

2.6.1.2 *Bacillus subtilis*

Bacillus subtilis is a gram-positive bacterium found mostly dwelling in soil and capable of producing endospores (Earl et al., 2008). It is one of the strains of *Bacillus* species that is known to produce amylase, protease and several other exo-enzymes (Van Dijl and Hecker, 2013). Amylase purified from *B. subtilis* is known to work best at pH 7.0 and optimal temperatures of 30°C (Raed and Shimaa, 2014). It has also been found to be one of the bacterium that can degrade oil with an efficiency of up to 74.3% degradation of crude oil (Raed and Shimaa, 2014). In another study *B. subtilis* has been seen to degrade petroleum and uses it as a source of carbon and energy (Samah et al., 2014).

2.6.1.3 *Pseudomonas aeruginosa*

It is a gram-negative bacterium that is known to cause a wide range of severe opportunistic infections in immune-compromised individuals such as those affected by cystic fibrosis or neutropenia (Lyczak et al., 2000). It is found mostly in soil and surfaces of aqueous environments. Infections caused by *P. aeruginosa* are mostly nosocomial and lead to severe neutrophilic response that leads to the destruction of tissues and they are also usually antibiotic resistant (Lyczak et al., 2000). The *P. aeruginosa* has the ability to form a bio-film and this makes the treatment of its infections even more difficult. It has virulence factors that can counteract host defense systems and cause direct damage to host tissue or increase the bacterium's competitiveness (Gellatly and Hancook, 2013). It has also been documented as one of the bacteria that can degrade crude oil with 76.7% efficiency in degradation (Raed and Shimaa, 2014).

2.6.1.4 *Rhodobacter capsulatus*

It is a gram-negative, facultative phototroph, purple non-sulfur photosynthetic bacterium that can produce carotenoids (Kranz et al., 1997). It has been widely studied for its photosynthesis and nitrogen fixation ability. It grows chemotrophically in the presence of abundant oxygen and induces intra-cytoplasmic photosynthetic membrane system in low oxygen conditions. Photosynthetic induction in this microbe triggers the expression of genes encoding pigment protein

complexes such as photochemical reaction centre and two light harvesting antennae. Carotenoids and bacterio-chlorophyll are produced here in these complexes (Krulwich et al., 1983).

2.6.2 Fungi

Fungi can be single celled or multi-cellular organisms that dwell mostly on land rather than water. On land, they are found in soil or plant material. They rarely infect animals but can do so if the conditions are favorable for growth. The fungi used in this study are also used as bio-pesticides: *Metarhizium anisopliae* and *Beauveria bassiana*.

2.6.1.1 *Metarhizium anisopliae*

Metarhizium anisopliae is an entomogenous fungus that is mostly used as a bio-pesticide. It usually produces green conidia and is found in soil (Sun et al., 2011). When cultured *in-vitro,* it grows best in the dark and takes 21 days to fully mature. It has been used as a bio-pesticide to kill aphids and moths as well as cockroaches and termites. It is a safer option for bio-pesticides because it does not affect the plant but kills the pests that harm plants. It has a high quick sporulation rate and overall low total sporulation as compared to its counterpart *Beauveria bassiana*. This gives it an upper hand as compared to *B. bassiana* in destroying pests (Sun et al., 2011). It produces a secondary metabolite Destruxin B that has some anti-cancer activity and is able to induce apoptosis in cancer infected cells hence killing them (Wu et al., 2013).

2.6.2.2 *Beauveria bassiana*

Beauveria bassiana is an entomogenous fungus that is mostly used as a bio-pesticide. It produces white spores and is found in soil just like *M. anisopliae*. It grows best in darkness when cultured *in-vitro* and takes about 21 days to fully mature. It has a low initial sporulation and a high total sporulation (Sun et al., 2011). Research has been done on genetically modified strains of *B. bassiana* as well as strain CBS110.25 and it was found that they produce secondary metabolites such as tenellin, that can be used to inhibit cancer growth (Yakasai, 2011).

CHAPTER THREE

3.0 METHODOLOGY

3.1 Preparation of Dagaa hydrolysate derivatives, DPH formulated media, LB and SDB media

3.1.1 Preparation of DFH and DPH from Dagaa

The Dagaa used in the study was bought from Nairobi City Market. Fish hydrolysate was prepared by use of a commercial exogenous protease Alcalase® (Sigma Aldrich, USA). Alcalase is a serine protease enzyme from *Bacillus licheniformis*. The DFH was prepared using the protocol as described by Ogonda et al., (2014). The hydrolysate was then mixed with hexane (Sigma Aldrich, USA) to extract lipids to yield Dagaa Protein Hydrolysate (DPH) composed mostly of peptides from the hydrolysed Dagaa. DPH was then transferred into 15 ml falcon tubes and centrifuged using a refrigerated centrifuge (Hanil Science Industrial, Korea) at 15922 x g for 20 minutes at 4 ^{o}C. The DPH was then stored at -20 ^{o}C until the time for the proximate, mineral and formulation of culture media.

3.1.2 Preparation of Luria Bertani (LB) media

LB samples were prepared according to Myers et al., (2009). Into 1 L of distilled water, 5 g of Yeast extract (Sigma Aldrich, USA), 10 g of Tryptone (Sigma Aldrich, USA) and 10 g of NaCl (Sigma Aldrich, USA) were dissolved. This was then autoclaved at 121 °C, 15 psi for 15 minutes using an autoclave (Tuttnauer, USA) then cooled and was ready for use.

3.1.3 Preparation of Sabouraud's Dextrose (SDB) media

Sabouraud's dextrose media was prepared according to Saigal et al., (2011). To 1 L of distilled water, 10 g of Mycological Peptone (Sigma Aldrich, USA) and 40 g of Dextrose (Sigma Aldrich, USA) were dissolved. In cases where Sabouraud's dextrose agar was required 15g of Agar (Sigma Aldrich, USA) was added. The mixture was then autoclaved at 121 °C, 15 psi for 15 minutes then cooled and was ready for use. The media with agar was poured into Petri dishes before it solidified.

3.1.4 Preparation of Dagaa protein hydrolysate-formulated (DPH-FM) media for bacterial growth.

DPH-FM was used for culturing of bacteria as an alternative to the standard LB media. It was the experimental media in this study. It was determined that 100 ml of LB prepared as described in section 3.1.2 had a protein concentration of 1584 mg/ml. To prepare the DPH-FM media, an equivalent of the same amount of protein concentration was required. It was determined that 100ml DPH contains 5212 mg/ml of protein, therefore, to prepare DPH-FM with protein concentration of 1584 mg/ml, 30ml of DPH was mixed with 70 ml distilled water. Therefore DPH-FM media was made up of 10 g NaCl, 300 ml of DPH and 5 g of Yeast Extract per liter. Sodium mineral is in very minute concentrations in DPH yet it is important for microbial growth, hence the supplementation using NaCl. It is important to note that 300 ml of DPH is equivalent to 15 g of DPH powder. DPH powder was prepared by freeze drying the liquid DPH at -10 °C for 3 days (Mason Technology, Ireland).

3.1.5 Preparation of Dagaa protein hydrolysate-Dextrose (DPH-Dextrose) for fungal growth.

It was determined that the concentration of protein in 100 ml of the standard media for culturing fungi Sabouraud's dextrose broth (SDB) had a concentration of 1391.4 mg/ml. To prepare the DPH-Dextrose media an equivalent of the same amount of protein concentration was required. To have a protein concentration of 1391.4 mg/ml in DPH-Dextrose media for culturing fungi, 30 ml of DPH was used and topped up to 100 ml with distilled water. Therefore the formulated DPH-Dextrose media for fungi was made up of 300 ml of DPH and 40 g of Dextrose per liter with dextrose being added as DPH does not contain any. 300 ml of DPH is equivalent to 15 g of DPH powder.

3.2 Proximate analysis of Dagaa, Dagaa hydrolysate derivatives, LB and SDB media

In this study proximate analysis of moisture content, ash minerals and macronutrients proteins and lipids content were performed. Proximate analysis

studies are best conducted in triplicates and all the studies below were done in triplicates (Aberoumand, 2012).

3.2.1 Determination of moisture/dry weight content

Crucibles were cleaned and dried in an oven (Memmert, Germany), cooled and weighed on a weighing balance (Mettler Toledo, Switzerland). The dry Dagaa was homogenized using a homogenizer (Sumeet Research and Holdings Company, India) and placed into the dried crucibles and weighed in triplicates. These were then investigated for moisture content using air oven method according to Association of Analytical Chemist (AOAC) method 950.46 (AOAC, 1995). Crucibles with the dry Dagaa sample were oven dried at 105 ^{o}C overnight using an oven (Memmert, Germany). Samples were then left in a desiccator (Thermo Fisher Scientific, U.S.A.) for 30 minutes to cool before being reweighed. The same procedure was repeated for the following samples which were also analyzed for dry weight content DFH, DPH, LB and SDB. Dry weight was estimated by the use of the formula;

$$\% \textbf{ Dry weight} = \left(\frac{\textbf{The weight of dried sample}}{\textbf{The original wet weight}}\right) * \mathbf{100}$$

3.2.2 Determination of ash content

Ash content was determined according to AOAC method 950.153 (AOAC, 1995). A muffle furnace (Baird and Tatlock, England) was preheated at 550 ^{o}C. Dried Dagaa fish samples, DFH, DPH, LB and SDB samples from moisture determination were then labelled and left to burn in furnace for 6 hours. The ash was cooled in a desiccator before being weighed. Ash content of the Dagaa, DFH, DPH, LB and SDB was determined according to the formula;

$$\% \textbf{ Ash content} = \frac{(\textbf{The weight of ash})}{(\textbf{The original wet weight})} * \mathbf{100}$$

3.2.3 Determination of crude protein content

Biuret's method was used to analyse the protein concentration in the Dagaa, DFH, DPH, LB and SDB samples (Keppy et al., 2009). Dagaa (2 g) were homogenized using a homogenizer in triplicate and transferred into 250 ml Erlenmeyer flasks. To

the ground Dagaa, 20 ml of 0.5 N NaOH (Sigma Aldrich, USA) was added and mixture heated for 10 minutes over a water bath (Memmert, Germany). The mixture was then cooled and filtered to remove the fat. The filtrate was mixed with Petroleum Ether (Sigma Aldrich, USA) to remove fat. Centrifugation was then performed to extract protein at 1769 x *g*. The protein extract was collected for protein analysis. The LB, SDB, DFH and DPH were prepared as mentioned earlier. A stock solution of 10 mg/ml Bovine Serum Albumin (BSA) (Sigma Aldrich, USA) was then prepared and used to make a Protein Standard curve for determining the protein content of Dagaa, LB, SDB, DFH and DPH samples. The sample, Biuret's reagent (Sigma Aldrich, USA) and sterile distilled water were then mixed as shown in Table 3.1.

Table 3.1: Biuret's assay sample preparation.

Test tube number	BSA concentration (mg/ml)	Sterile distilled water (µl)	BSA stock (µl)	Biuret's reagent (ml)
1	0	1000	0	2
2	1	900	100	2
3	2	800	200	2
4	3	700	300	2
5	4	600	400	2
6	5	500	500	2
7	6	400	600	2
8	7	300	700	2
9	8	200	800	2
10	9	100	900	2
11	10	0	1000	2
Dagaa 1(50 µl)	0	950	0	2
Dagaa 2 (50 µl)	0	950	0	2
Dagaa 3 (50 µl)	0	950	0	2
DFH 1 (50 µl)	0	950	0	2
DFH 2 (50 µl)	0	950	0	2
DFH 3(50 µl)	0	950	0	2
DPH 1 (50 µl)	0	950	0	2
DPH 2 (50 µl)	0	950	0	2
DPH 3 (50 µl)	0	950	0	2
LB 1 (50 µl)	0	950	0	2
LB 2 (50 µl)	0	950	0	2
LB 3 (50 µl)	0	950	0	2
SDB 1 (50 µl)	0	950	0	2
SDB 2 (50 µl)	0	950	0	2
SDB 3 (50 µl)	0	950	0	2

The mixtures were then incubated at room temperature for 15 minutes. Protein content for all the samples was determined by reading the absorbance at 550 nm. The absorbance readings were used to obtain protein content from the standard curve created using the optical densities and concentration of BSA.

3.2.4 Determination of crude lipid content

Total crude lipid content was determined using the modified Dyer and Bligh method of lipid extraction (Bligh and Dyer, 1959; Iverson et al., 2001). In this method 10 g of ground Dagaa were weighed in triplicate, into falcon tubes. To this, 10 ml of distilled water, 20 ml of chloroform (Sigma Aldrich, U.S.A) and 40ml of methanol (Sigma Aldrich, U.S.A) was added then homogenised for 2 minutes. Another 20 ml of chloroform was then added and homogenized for 30 seconds. To the homogenate, 20ml distilled water was then added and homogenized for additional 30 seconds. The resultant homogenate was then centrifuged for 10 minutes at 15922 x g. Organic phase containing chloroform was then pipetted off to a glass centrifuge tube. The weight of empty tube before addition of chloroform was determined and the weight of tube after addition of 1ml of chloroform was determined too. The glass tube with the chloroform was then placed in the oven at 70 ^{o}C. After evaporation of the chloroform; the tube was then cooled to room temperature and reweighed. Lipid content was determined by calculating the weight of lipid in tube in the presence of chloroform over the amount of sample weighed after the chloroform evaporation. The formula is illustrated below.

$$\frac{\textbf{Lipid}}{\textbf{100 g sample}} = \frac{\textbf{Lipid in tube} * (\textbf{volume of chloroform in total}) * \textbf{100 g}}{\textbf{Amount of sample weighed in}(\textbf{g}) * \textbf{ml chloroform evapourated}}$$

3.3 Mineral analysis of Dagaa, Dagaa hydrolysate derivatives, LB and SDB media

Mineral elemental concentration was determined using Atomic Absorption Spectrophotometry (AAS) (Shimadzu, Japan) (AOAC, 1975) and flame photometry (Sherwood Scientific, U.K.) (Thomas and Skujins, 2011). The mineral elements were investigated in Dagaa, LB, SDB, DFH and DPH and included; calcium, sodium, potassium, magnesium, iron, zinc, nickel and copper which have been

found in most fish from previous studies (Fawole et al., 2007; Younis et al., 2011). Magnesium, zinc, iron, nickel and copper were analyzed in the samples mentioned using AAS while sodium, potassium and calcium was analyzed using Flame Photometry.

3.3.1 Preparation of samples for mineral analysis

Dagaa, LB, DFH, DPH and SDB samples were prepared in triplicate. Each sample was then dissolved in 5 ml nitric acid (Sigma Aldrich, USA) and made up to 20 ml with distilled water. The mixture was then heated on a hot plate (Stuart, U.K.) until a third of the total volume was achieved. The solution was then filtered with Whatman filter paper 1 (Cole-Parmer, U.S.A) and analysed for minerals using AAS as described by Steiner-Asiedu et al., (1991) and Atta et al., (1997) and flame photometry for minerals in section 3.3. Appropriate dilutions were then made to enable all sample readings to fit in the calibration curve where necessary. The dilutions were finally incorporated in the calculation of the final mineral concentration.

3.3.2 Mineral analysis by AAS

3.3.2.1 Magnesium (Mg) mineral analysis

Samples were prepared as mentioned in section 3.3.1. The AAS machine was warmed up and wavelength aligned after the Mg lamp had been detected. Wavelength used to analyze samples was 285.17 nm. Original Dagaa samples were diluted 5000 X, LB samples were also diluted 10 X, DFH, DPH and SDB samples were not diluted. Standards of Mg were then read after blanking the machine with a blank prepared the same way as samples but lacking constituents of the samples (mixture of distilled water and concentrated nitric acid). The samples were then read and results recorded. The concentrations of the standards were in ppm as follows 0.2, 0.4, 0.6, 0.8, 1 and 2. These standards were used to prepare a standard curve that was used to extrapolate the concentration of Mg in Dagaa, LB, DFH, DPH and SDB.

3.3.2.2 Zinc (Zn) mineral analysis

All protocols for the mineral analysis of Zinc in Dagaa and LB were carried out as mentioned in section 3.3.2.1. However the Zn standards used had the following concentrations; 1 ppm, 2 ppm, 4 ppm and 6 ppm. The wavelength used was 213.52 nm. Original Dagaa samples were diluted 50 X while LB, DFH, DPH and SDB samples were not diluted.

3.3.2.3 Iron (Fe) mineral analysis

All protocols for the mineral analysis of Dagaa and LB as mentioned in section 3.3.2.1. However, the Fe standards had the following concentrations; 1 ppm, 2 ppm, 3 ppm, 4 ppm and 5 ppm. The wavelength used was 248.23 nm. The original Dagaa sample was diluted 5X while the LB, DFH, DPH and SDB samples were not diluted.

3.3.2.4 Nickel (Ni) mineral analysis

All protocols for the mineral analysis of Dagaa and LB as mentioned in section 3.3.2.1. However, the Ni standards had the following concentrations; 0.5 ppm, 1 ppm, 2 ppm, 3 ppm, 4 ppm and 5 ppm. The wavelength used was 232.10 nm. The original Dagaa, LB, DFH, DPH and SDB samples were not diluted.

3.3.2.5 Copper (Cu) mineral analysis

All protocols for the mineral analysis of Dagaa and LB as mentioned in section 3.3.2.1. However, the Cu standards used had the following concentrations; 0.25 ppm, 0.5 ppm, 1 ppm and 2 ppm. The wavelength used was 324.67 nm. The Dagaa, LB, DFH, DPH and SDB samples were not diluted.

3.3.3 Mineral analysis by flame photometry

3.3.3.1 Sodium (Na) mineral analysis

Na concentration in Dagaa, LB, DFH, DPH and SDB was analyzed using Flame photometry. The Dagaa, LB, DFH, and DPH samples were diluted 5000 X, 1000 X, 14 X and 14 X, respectively. SDB samples were not diluted. The flame photometer was blanked and calibrated before the Na standards were analyzed. Na standards had

the following concentrations; 1 ppm, 2 ppm, 4 ppm, 6 ppm, 8 ppm and 10 ppm. The emission values achieved after analyzing for the standards were used to create a standard curve. All samples were then analyzed and their emission level determined. The concentration of Sodium in all the samples was then extrapolated from the standard curve. The wavelength used could not be determined from the flame photometry machine (Overman and Davis, 1947).

3.3.3.2 Potassium (K) mineral analysis

All protocols for the analysis of K in Dagaa, DFH, DPH, LB and SDB were as described for Na in section 3.3.3.1 for Na mineral analysis. Standards for K used had the following concentrations; 1 ppm, 2 ppm, 4 ppm and 10 ppm. The dilution factor used in this case for Dagaa, LB, DFH and DPH was 1000 X, 1000 X, 14 X, and 14 X respectively. SDB was not diluted.

3.3.3.3 Calcium (Ca) mineral analysis

Ca mineral analysis was done using flame photometry just as Na and K mineral analysis were performed. However, for Ca which tends to naturally bind to phosphate groups and is not readily available, there was a need to break this calcium phosphate bonds using Lanthanum chloride. The Lanthanum (La) easily absorbs the phosphates bond to the Ca causing a release of free Ca which can then be analyzed for Ca mineral analysis (Thomas and Skujins, 2011). All the samples Dagaa, LB, DFH, DPH and SDB were treated the same as explained and then analyzed for Ca mineral content as mentioned in section 3.3.3.1. The standards used for Ca mineral analysis had the following concentration; 10 ppm, 20 ppm, 40 ppm, 60 ppm, 80 ppm and 100 ppm. Samples were not diluted.

3.4 Culture of microorganisms using DPH formulated media and standard media

3.4.1 Culture of bacteria to estimate growth titres

Four bacterial microorganisms were cultured: *Escherichia coli, Bacillus subtilis, Pseudomonas aeroginosa* and *Rhodobacter capsulatus*. All these microorganisms were acquired from Jomo Kenyatta University of Agriculture and Technology

laboratory. For the microbial culturing of *E. coli, B. subtilis* and *P. aeroginosa,* the media used was LB as the standard and DPH-FM served as the experimental media. LB media was prepared as in section 3.1.2 while DPH formulated media was prepared as in section 3.1.4. One hundred ml of each media was prepared in 250 ml Erlenmeyer flask. In a biological safety cabinet level 3 (Heraeus, Germany), 200 µl of each of the three microorganisms was inoculated in a separate 100 ml of the two different media. The bio-safety cabinet was sterile and was important in the prevention of contamination of study samples. There was no use of antibiotics. The microorganisms were then placed in an incubated orbital shaker (Gerhardt, Germany) at the speed of 120 rpm and temperature of 37 °C. Samples were picked at different intervals of 0, 0.5, 2, 5, 7, 10, 25 and 26 hours for optical density tests and biomass studies as mentioned in section 3.4.1.1 and 3.4.1.2 respectively. For the *R. capsulatus,* the standard and formulated media had specific adjustments made on them to accommodate the growth of this microorganism (Kambura, 2011). The protein content of DPH-FM media was equated to that of LB standard media for bacterial growth. Both media had their pH adjusted to 10. The readings from optical density and biomass were then recorded and used to draw growth curves to compare the growth of these bacteria in the two media.

3.4.1.1 Growth estimation of microorganisms by optical density

For all microorganisms cultured, optical density studies were obtained to compare the growth of the microorganisms in standard media and formulated media. Once the inoculation was done, the samples were placed in an incubated orbital shaker to control speed and temperature at which the microorganisms grew; 5ml of samples were then picked at intervals of 0.5, 2, 5, 7, 10, 25 and 26 hours. Of the 5 ml, 3 ml was used to read optical densities at 600 nm at specified time intervals with the blank being the media without an inoculum. The 3 ml was then put back into the falcon tube together with the remaining 2 ml and the total 5 ml was then used to conduct the biomass studies, the values of optical density for growth of microorganisms were used to draw the microbial growth curves based on optical density.

3.4.1.2 Estimating growth of microorganisms by biomass

The same 5ml picked from every sample grown in the incubated orbital shaker was used for optical density and biomass growth studies. A volume of 5 ml of each sample was placed into a pre-weighed falcon tube and centrifuged at 15922 x *g* for 10 minutes to achieve a pellet. The supernatant was poured out and the pellet dried overnight at 105 °C in an oven (Memmert, Germany). The falcon tube was then re-weighed and the previous weight of the empty tube subtracted from new weight to achieve the weight of the pellet. The weight of the pellet for each sample was used to generate the microbial growth curve based on biomass.

3.4.2 Culturing of fungi to estimate growth titres.

Two fungi were cultured in this study; *Metarhizium anisopliae* and *Beauveria bassiana.* Both microorganisms were grown on SDB standard media and formulated DPH-Dextrose media. SDB media was prepared as mentioned in section 3.1.3 while formulated DPH-Dextrose media was prepared as mentioned in section 3.1.5. After both media were prepared, pH was adjusted to 5.5 before autoclaving to accommodate the growth of both microorganisms. One hundred ml of each media was then placed into different 250 ml Erlenmeyer flasks at 100 ml each. Pre-grown cultures of 200 µl for both fungi were then inoculated into different media in a biological safety cabinet level 3 and sterile conditions were maintained. There was no use of antibiotics. The samples were then placed in an incubated orbital shaker at a speed of 180 rpm and temperature of 25 °C. The samples were then picked, 5ml at time intervals of 0 hour that is immediately after inoculation and after every 2 days during 21 days of growth. The 5 ml of the sample picked was used in optical density and biomass studies (Tarrs and Deas, 1949; Green et al., 1973; Beuchat, 1974; Green et al., 1977).

3.5 Potential of DPH-Starch media to maintain bacterial enzyme production and fungal sporulation

The microorganisms used in this study included *Bacillus subtilis*, *Rhodobacter capsulatus and Metarhizium anisopliae.*

3.5.1 Extracellular amylase enzyme production by *Bacillus subtilis* and *Rhodobacter capsulatus*

Both microorganisms were tested for amylase enzyme production. Modified Horikoshi's agar media (10 g of Starch, 5 g peptone (Sigma Aldrich, USA), 5 g Yeast Extract, 1 g Potassium dihydrogen phosphate (Sigma Aldrich, USA), 0.2 g Magnesium sulphate (Sigma Aldrich, USA) and 15g of Agar in 1litre of water) and DPH-Starch formulated agar media (10 g of Starch, 5 g Yeast Extract, 300 ml of DPH, 1 g of Potassium dihydrogen phosphate, 0.2 g of Magnesium sulphate and 15 g of Agar) were used as standard and experimental media, respectively (Horikoshi, 1971). For the culturing of *R. capsulatus,* the two media were adjusted to pH 10 but no pH adjustment was done for *B. subtilis* growth media (Kambura, 2011). The media was then autoclaved at 121 °C 15 psi for 15 minutes. After autoclaving the mixture was then poured into Petri dishes in a laminar flow at 20 ml / Petri dish after cooling the mixture to 50 °C. The mixture in the Petri dishes was left to solidify before spotting at the centre with the appropriate bacteria. Negative control plate of the Modified Horikoshi media and the DPH-Starch media were not spotted. All plates were labelled for each microorganism and incubated for 24 hours at 37 °C in an incubator (LEEC, U.K.). After 24 hours each plate for each microorganism was stained with 1% iodine stain (Sigma Aldrich, U.S.A.). Observations were then noted for the presence or absence of halos and the size of the halo determined.

3.5.2 Determination of amylase enzyme production via Dinitrosalicylic acid (DNS) assay

After determination of the extracellular production of amylase by *B. subtilis* and *R. capsulatus*, broths of the two media were prepared as mentioned in section 3.5.1 without agar. Each of the 200 µl of bacteria was inoculated, into separate 250 ml Erlenmeyer flasks with 100ml of the two different media and separately grown for intervals of 24, 48 and 72 hours. After every time interval, 10 ml of sample was removed and centrifuged at 15922 x *g* for 20 minutes in a refrigerated centrifuge. The supernatant was kept and the pellet discarded. Dinitrosalicylic acid (DNS) reagent (Sigma Aldrich) and Rochelle's reagent (made using Sodium Potassium Tartate (Sigma Aldrich, USA) were then prepared to prepare the DNS assay that would determine rate of glucose formation after breakdown of starch to glucose that

was catalysed by amylase (Miller, 1959; Malhow et al., 2016). A glucose standard curve using 10 mg/ml stock glucose solution was also prepared. The reagents for the preparation of glucose standard curve and the test samples were prepared as shown in Table 3.2.

Table 3.2: Preparation of glucose standard curve.

Sample (ml)	Distilled water (ml)	DNS reagent (ml)	Rochelle reagent (ml)
Glucose Standard concentration			
0.0	1.0	3.0	1.0
0.1	0.9	3.0	1.0
0.3	0.7	3.0	1.0
0.6	0.4	3.0	1.0
0.7	0.3	3.0	1.0
0.8	0.2	3.0	1.0
1.0	0.0	3.0	1.0
B. subtilis supernatant 1	1.0	3.0	1.0
B. subtilis supernatant 2	1.0	3.0	1.0
B. subtilis supernatant 3	1.0	3.0	1.0
R. capsulatus supernatant 1	1.0	3.0	1.0
R. capsulatus supernatant 2	1.0	3.0	1.0
R. capsulatus supernatant 3	1.0	3.0	1.0

After the mixtures were prepared absorbance was read at 540 nm. The rate of glucose formation was then determined from the glucose standard curve prepared.

3.5.3 Sporulation rates of *Metarhizium anisopliae*

Sporulation rates of *M. anisopliae* were compared in both the standard media (Sabouraud's Dextrose) and in the formulated media (DPH- Dextrose). This was done by preparing Sabouraud's Dextrose Agar (SDA) media and DPH-Dextrose Agar media, 20 ml of each media was then poured into separate Petri dishes. Previously cultured *M. anisopliae* (200 µg) was then spotted on both plates and the plates incubated at 25 °C for 11 days. Controls were however not inoculated. On the 11th day of growth, the conidia were harvested under sterile conditions whereby the plates were flooded with 3 ml distilled water that was then swirled to ensure the water picked up the spores on all corners. The conidia was then washed and swirled in 0.2 ml of 0.05% Tween 80 for 20 minutes. The mixtures were then diluted 3 X

and 5 X. The mixtures of different dilutions with the conidia were then filtered using a Whatman filter paper 1. Concentration of conidia on each of the filtrate was determined on a haemocytometer (Precicolor HGB, Germany) under a light microscope at X 40 magnification (Sun et al., 2011).

The formula below was used to determine the conidia concentration of *M. anisopliae*.

$$\textbf{Conidia concentration}\left(\frac{\textbf{conidia}}{\textbf{ml}}\right) = \frac{(\textbf{total conidia count})}{\textbf{number of squares}} * \textbf{10}\wedge\textbf{4}$$

3.6 Statistical analysis

Data from proximate composition analysis, mineral analysis, efficacy of DPH formulated media in growth of microbes as well as maintenance of exo-enzyme production and sporulation in fungi were analysed by one-way analysis of variance (ANOVA) using statistical software SPSS version 19 (SPSS, 2010). The means from all the studies were separated by Tukey's HSD multiple comparison test at α = 0.05. Media were compared based on their effect on growth and biomass of individual microorganisms. All conclusions are based on experiments that were done in triplicates to ensure reproducibility and reliability of results.

CHAPTER FOUR

4.0 RESULTS

4.1 Proximate analysis of Dagaa, Dagaa hydrolysate derivatives, LB and SDB media

Proximate analysis studies were done to analyze for moisture, ash, protein and lipid content in Dagaa, LB, DFH, DPH and SDB. SDB had the highest dry weight content while Dagaa had the second highest dry weight content and LB had the least dry weight content (Table 4.1). SDB, Dagaa and LB were all analysed on wet weight basis. DFH and DPH were analysed on wet weight basis and DFH had slightly higher moisture content than DPH (Table 4.1).

Table 4.1: Proximate analysis of Dagaa, LB, DFH, DPH and SDB showing the moisture/ dry weight, ash, protein and lipid content.

Sample	Dry weight/Moisture content (%)	Ash content (%)	Protein content (mg/0.05ml)	Lipid content (%)
Dagaa $_{dwb}$	95.00±0.00[a]	13.45±0.39[a]	5.23±0.05[a]	2.36±0.17[a]
LB $_{dwb}$	94.13±0.23[a]	44.00±0.00[a]	0.79±0.04[aa]	0.00±0.00[aa]
DFH $_{wwb}$	9.69±0.43[aa]	0.87±0.03[aa]	4.28±0.04[a]	1.02±0.15[a]
DPH $_{wwb}$	9.11±0.28[aa]	0.70±0.00[aa]	2.60±0.01[a]	0.00±0.00[aa]
SDB $_{dwb}$	95.87±0.12[a]	5.67±0.58[a]	0.70±0.11[aa]	0.00±0.00[aa]

Key: the abbreviation $_{dwb}$ indicates that the sample was analysed on dry weight basis while $_{wwb}$ indicates that the sample was analysed on wet weight basis. Values are means ± standard deviation (SD) for triplicate analysis of samples mentioned. Values with single letter indicate significant difference to the rest of the samples while those with double letters indicate no significant difference to other samples by analysis using one way analysis of variance (ANOVA).

The statistical analysis revealed that the dry weight/moisture content of Dagaa, LB, DFH, DPH and SDB was significantly different ($p<0.05$) (Appendix 2). Ash content of Dagaa, LB, DFH, DPH and SDB was significantly different ($p<0.05$) (Appendix 5). Protein content of Dagaa, LB, DFH, DPH and SDB was significantly different

($p<0.05$) (Appendix 8). Lipid content of Dagaa, LB, DFH, DPH and SDB was significantly different ($p<0.05$) (Appendix 11).

Multiple comparison using post hoc analysis Tukey's HSD revealed that the moisture content of Dagaa was significantly different ($p<0.05$) from LB, DFH, DPH and SDB moisture content (Appendix 3). LB moisture content was significantly different ($p<0.05$) from DFH, DPH and SDB moisture content (Appendix 3). DFH moisture content was not significantly different ($p>0.05$) from DPH moisture content. DFH moisture content was significantly different ($p<0.05$) from SDB moisture content (Appendix 3). DPH moisture content was significantly different ($p<0.05$) from SDB moisture content (Appendix 3).

Multiple comparison using post hoc analysis Tukey's HSD revealed that the ash content of Dagaa was significantly different ($p<0.05$) from LB, DFH, DPH and SDB ash content (Appendix 6). LB ash content was significantly different ($p<0.05$) from DFH, DPH and SDB ash content (Appendix 6). DFH ash content was not significantly different ($p>0.05$) from DPH ash content. DFH ash content was significantly different ($p<0.05$) from SDB ash content (Appendix 6). DPH ash content is significantly different ($p<0.05$) from SDB ash content (Appendix 6).

Multiple comparison using post hoc analysis Tukey's HSD revealed that protein content of Dagaa was significantly different ($p<0.05$) from LB, DFH, DPH and SDB protein content (Appendix 9). LB protein content was significantly different ($p<0.05$) from DFH, DPH and SDB protein content (Appendix 9). DFH protein content was not significantly different ($p>0.05$) from DPH protein content. DFH protein content was significantly different ($p<0.05$) from SDB protein content (Appendix 9). DPH protein content is significantly different ($p<0.05$) from SDB protein content (Appendix 9).

Multiple comparison using post hoc analysis Tukey's HSD revealed that the lipid content of Dagaa was significantly different ($p<0.05$) from LB, DFH, DPH and SDB lipid content (Appendix 12). LB lipid content was significantly different ($p<0.05$) from DFH lipid content (Appendix 12). LB lipid content was not significantly different ($p>0.05$) from DPH and SDB lipid content (Appendix 12). DFH lipid content was significantly different ($p<0.05$) from DPH and SDB lipid content

(Appendix 12). DPH lipid content is not significantly different ($p>0.05$) from SDB lipid content (Appendix 12).

4.2 Mineral analysis of Dagaa, Dagaa hydrolysate derivatives, LB and SDB media

Mineral analysis of Dagaa, LB, DFH, DPH and SDB was performed on the following minerals magnesium, zinc, iron, nickel and copper using AAS while sodium, potassium and calcium using Flame photometry. Mineral analysis generally showed that they are very minute levels of minerals in the Dagaa, LB, DFH, DPH and SDB samples. The highest level of the minerals Magnesium, Zinc, Iron, Nickel, Copper, Sodium and Potassium was in Dagaa while Calcium levels were highest in LB. Dagaa had 0.5 mg/ml, 0.01 mg/ml, 0.01 mg/ml, 22.74 mg/ml, 4.91 mg/ml and 0.02 mg/ml of Mg, Zn, Fe, Na, K and Ca (Table 4.2). LB had 4.93 mg/ml and 0.25 mg/ml of Na and Ca (Table 4.2). DFH had 0.1 mg/ml, 0.1 mg/ml and 0.01 mg/ml of Na, K and Ca (Table 4.2). DPH had 0.1 mg/ml, 0.08 mg/ml and 0.01 mg/ml of Na, K and Ca (Table 4.2). SDB had 0.02 mg/ml of Ca (Table 4.2). Dagaa had the highest mineral content of all samples analysed (Table 4.2). LB had the second highest mineral content while DFH and DPH had the same amount of mineral content (Table 4.2). SDB had the least mineral content (Table 4.2).

Table 4.2: Mineral analysis of Mg, Zn, Fe, Ni, Cu, Na, K and Ca content in Dagaa, LB, DFH, DPH and SDB.

Sample	**Concentration in mg/ml of specific mineral**							
	Mg	Zn	Fe	Ni	Cu	Na	K	Ca
Dagaa	0.50±0[a]	0.01±0[a]	0.01±0[a]	0.00±0[aa]	0.00±0[aa]	22.74±1[a]	4.91±1[a]	0.02±0[a]
LB	0.00±0[aa]	0.00±0[aa]	0.00±0[aa]	0.00±0[aa]	0.00±0[aa]	4.93±1[a]	0.00±0[aa]	0.25±0[a]
DFH	0.00±0[aa]	0.00±0[aa]	0.00±0[aa]	0.00±0[aa]	0.00±0[aa]	0.10±0[a]	0.10±0[a]	0.01±0[a]
DPH	0.00±0[aa]	0.00±0[aa]	0.00±0[aa]	0.00±0[aa]	0.00±0[aa]	0.10±0[a]	0.08±0[a]	0.01±0[a]
SDB	0.00±0[aa]	0.00±0[aa]	0.00±0[aa]	0.00±0[aa]	0.00±0[aa]	0.00±0[aa]	0.00±0[aa]	0.02±0[a]

Key: values indicate means ± standard deviations (SD) for triplicate analysis of samples mentioned. Values with single letter indicate significant difference to the rest of the samples while those with double letters indicate no significant difference to other samples by analysis using one way analysis of variance (ANOVA).

There was significant difference ($p<0.05$) in the content of Mg, Zn, Fe, Ni, Cu, Na, K and Ca minerals in Dagaa, LB, DFH, DPH and SDB (Appendix 22).

Multiple comparison by post hoc analysis Tukey's HSD revealed that the mineral content of Mg, Zn, Fe, Ni, Cu, Na, K and Ca minerals in Dagaa was significantly different ($p<0.05$) from the mineral content of LB, DFH, DPH and SDB (Appendix 23). Mineral content of Mg, Zn, Fe, Ni, Cu, Na, K and Ca minerals in LB was not significantly different ($p>0.05$) from the mineral content of DFH, DPH and SDB (Appendix 23). Mineral content of Mg, Zn, Fe, Ni, Cu, Na, K and Ca minerals in DFH was not significantly different ($p>0.05$) from mineral content of DPH and SDB (Appendix 23). Mineral content of Mg, Zn, Fe, Ni, Cu, Na, K and Ca minerals in DPH was not significantly different ($p>0.05$) from mineral content of SDB (Appendix 23).

4.3 Growth curves of microbes in standard media and formulated DPH media

Bacterial and fungal cultures were grown in both their standard media and the DPH-FM media. Growth estimation studies in both media were done using growth curves by optical density and biomass. Comparisons were made statistically to determine the efficacy of DPH-FM the standard media used for microbial growth.

4.3.1 Bacterial growth curves by optical density and biomass

4.3.1.1 *Escherichia coli* growth curves by optical density and biomass in LB and DPH-FM media

Escherichia coli were grown in LB and DPH-FM to compare growth in both media. Growth was determined by measuring changes in optical density and biomass over time. The growth of *E. coli* in LB was faster than in DPH-FM. The lag phase of growth for *E. coli* was fast in both LB and DPH. The log and stationary phase of growth was faster in LB than in DPH-FM media. The biomass was seen to have a similar growth pattern as that seen with the optical density (Figure 4.1).

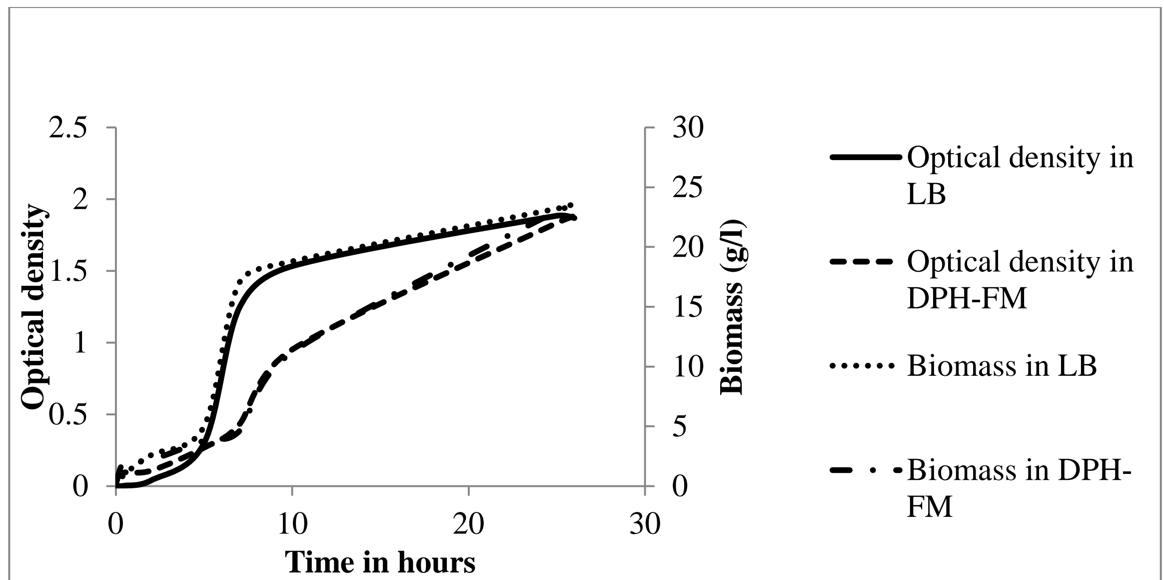


Figure 4.1: *Escherichia coli* growth curve by optical density and biomass.

4.3.1.2 *Bacillus subtilis* growth curve by optical density and biomass in LB and DPH-FM media

Bacillus subtilis were grown in two types of media LB and DPH-FM for comparison of their growth in the two media. The growth was determined by measurement of changes in optical density and biomass. The growth of *B. subtilis* was faster in LB than in DPH-FM. The lag and log phase of growth was faster in both media and only took 10 hours. The stationary growth phase was achieved faster in LB than in DPH-FM. The biomass growth pattern was similar to that of the optical density (Figure 4.2).

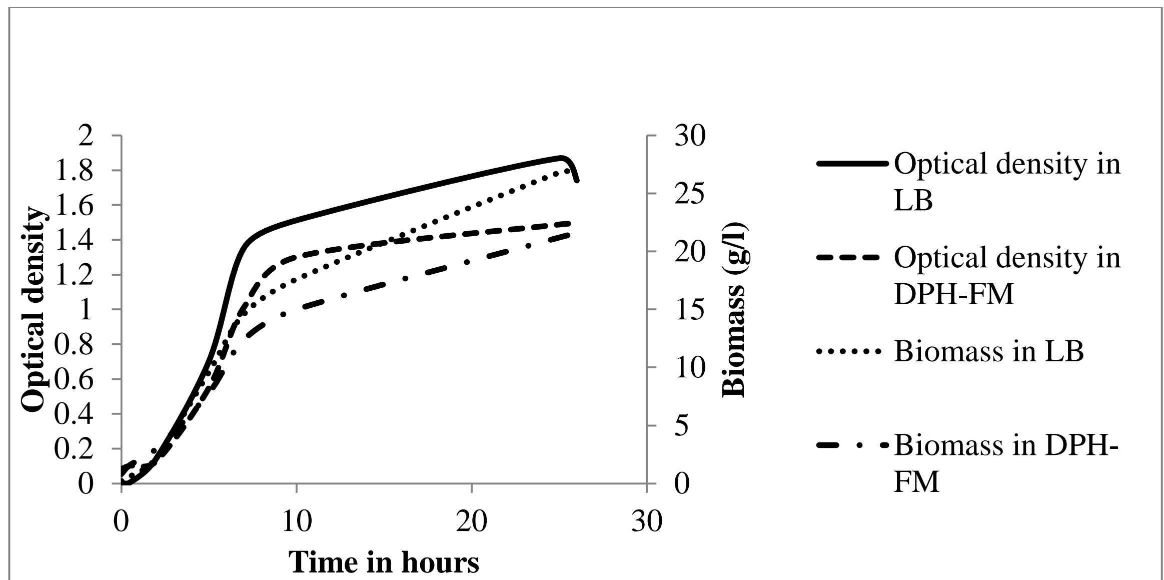


Figure 4.2: *Bacillus subtilis* growth curve by optical density and biomass.

4.3.1.3 *Pseudomonas aeruginosa* growth curve by optical density and biomass in LB and DPH-FM media

Pseudomonas aeruginosa were grown in two types of media LB and DPH-FM for comparison of their growth in the two media. The growth was determined by measurement of changes in optical density and biomass over time. The growth of *P. aeruginosa* was faster in LB than in DPH-FM. The lag and log phase of growth was similar in both LB and DPH-FM and only took 10 hours in both cases. The stationary phase of growth was faster in LB than in DPH-FM. Biomass growth curves were similar to those of the optical density study (Figure 4.3).

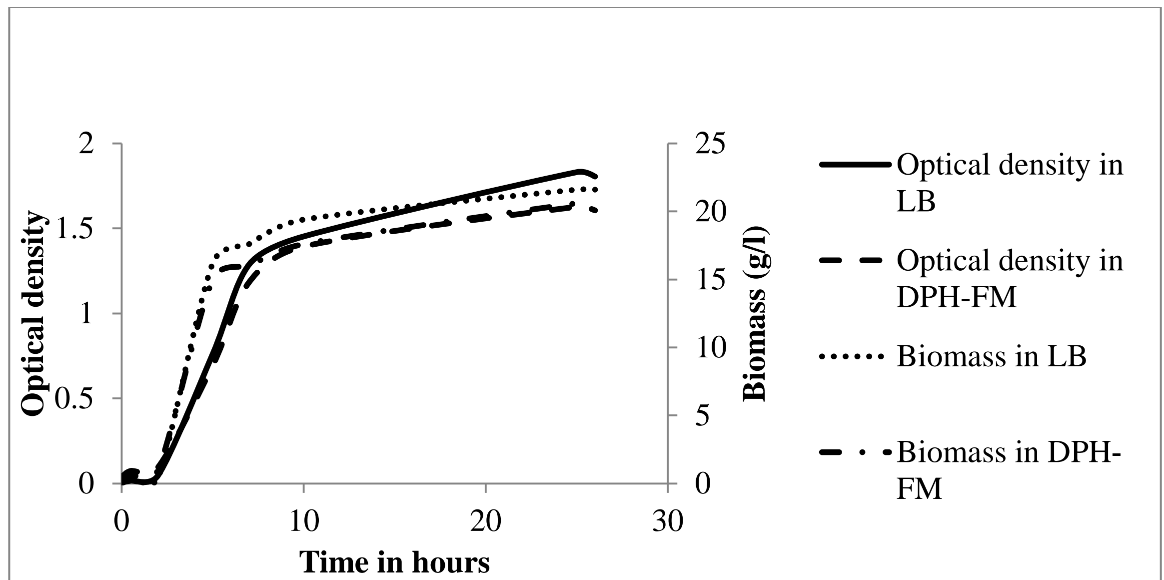


Figure 4.3: *Pseudomonas aeruginosa* growth curves by optical density and biomass

4.3.1.4 *Rhodobacter capsulatus* growth curve by optical density and biomass in LB and DPH-FM media

Rhodobacter capsulatus was grown in two types of media, LB and DPH-FM for comparison of their growth in the two media. Growth was measured through changes in optical density and biomass over time. The growth of *R. capsulatus* was almost identical in both LB and DPH-FM at all stages of growth by optical density and biomass (Figure 4.4).

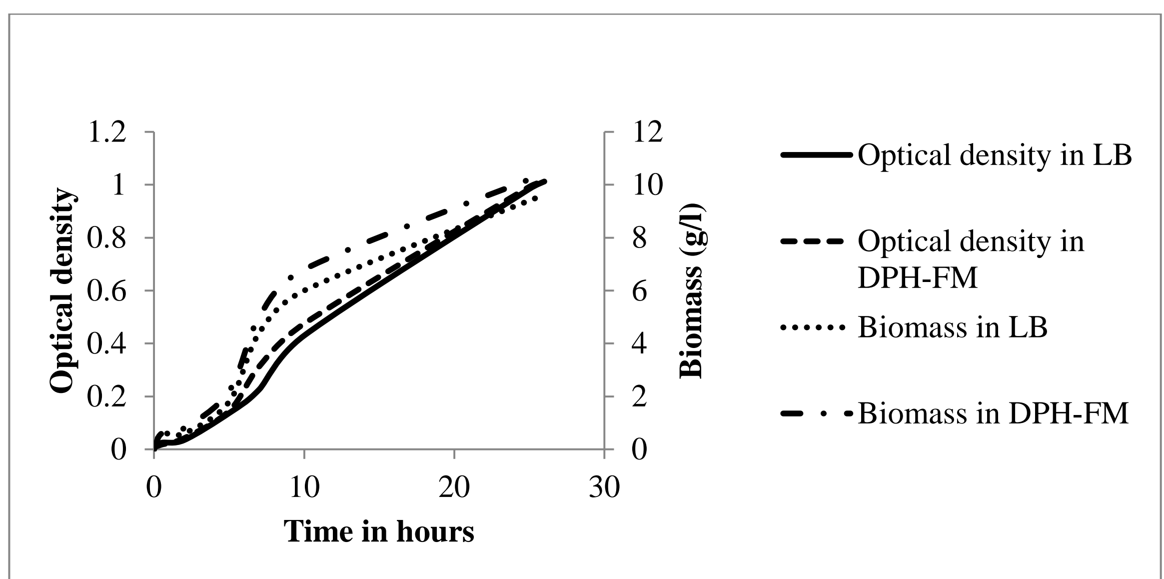


Figure 4.4: *Rhodobacter capsulatus* growth curve by optical density and biomass.

The results revealed that the growth of *E. coli*, *B. subtilis*, *P. aeruginosa* and *R. capsulatus* in LB and DPH-FM media was not significantly different (p>0.05) by optical density (Appendix 24) or by biomass (Appendix 25).

4.3.2 Fungal growth curves by optical density and biomass in SDB and DPH-Dextrose media

4.3.2.1 *Metarhizium anisopliae* growth curves by optical density and biomass in SDB and DPH-Dextrose media

Metarhizium anisopliae were grown in two types of media namely SDB and DPH-Dextrose for comparison of their growth in the two media. The growth was determined by measurement of changes in optical density and biomass over time. The growth of *M. anisopliae* was irregular and did not form a traditional sigmoid growth curve. Growth in SDB was faster than in DPH-Dextrose media. Biomass and optical density growth curves were not similar (Figure 4.5).

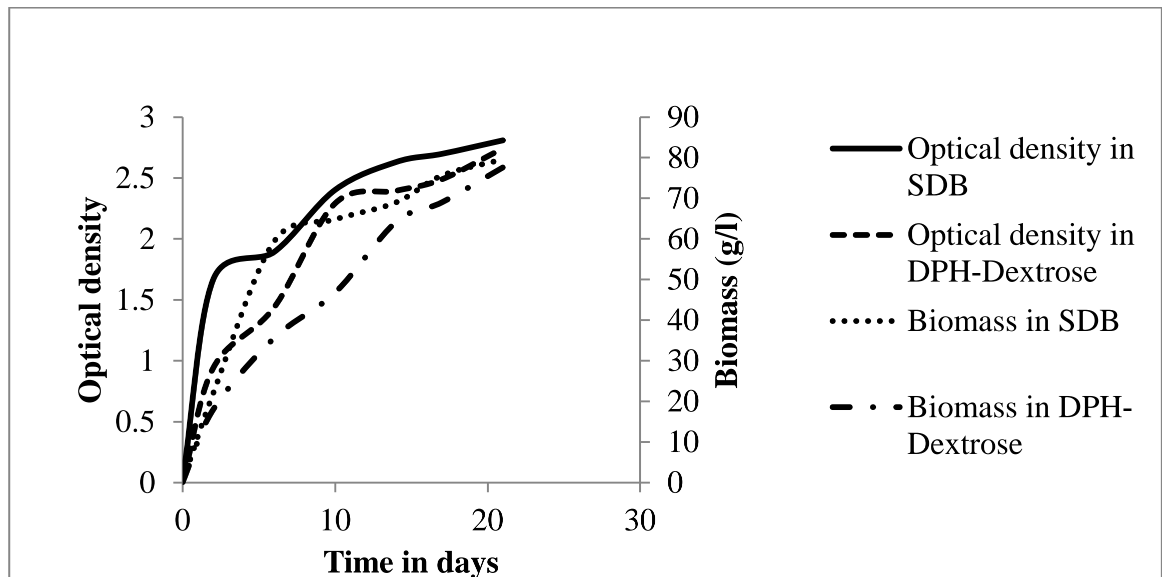


Figure 4.5: *Metarhizium anisopliae* growth curves by optical density and biomass.

4.3.2.2 *Beauveria bassiana* growth curves by optical density and biomass in SDB and DPH-Dextrose media

Beauveria bassiana was grown in two types of media SDB and DPH-Dextrose for comparison of their growth in the two media. The growth was determined by measurement of changes in optical density and biomass over time. The growth of *B.*

bassiana was also irregular and did not form a traditional sigmoid curve. The growth was faster in SDB than DPH-Dextrose in the first 6 days but grew faster towards day 17 to 21 in DPH-Dextrose than in SDB. Biomass and optical density growth curves were not similar (Figure 4.6)

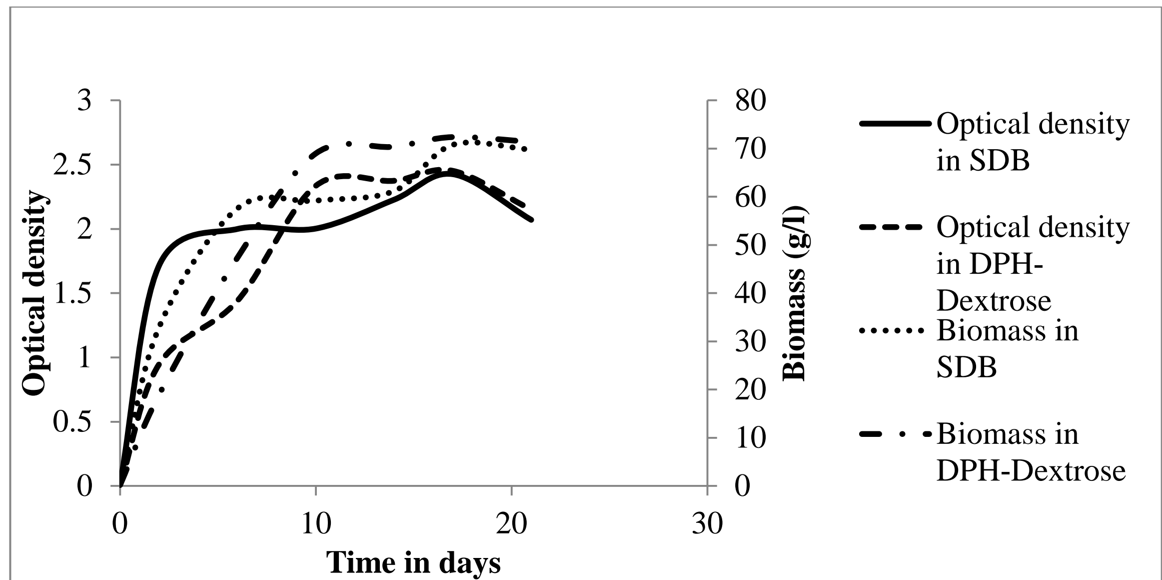


Figure 4.6: *Beauveria bassiana* growth curve by optical density and biomass.

Statistical analysis by one way ANOVA revealed that the growth of *M. anisopliae* and *B. bassiana* in SDB and DPH-Dextrose media was not significantly different ($p>0.05$) by optical density (Appendix 26) or by biomass (Appendix 27).

4.4 Formulated DPH-Starch media for bacterial enzyme production and fungal sporulation

4.4.1 Ability of DPH-Starch media to maintain amylase enzyme production in *Bacillus subtilis* and *Rhodobacter capsulatus*

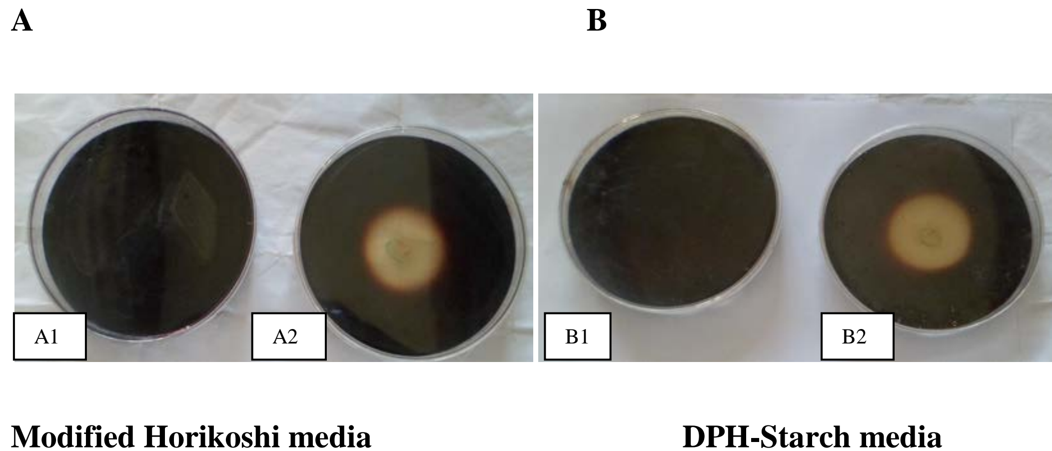
4.4.1.1 Biochemical test for amylase enzyme production in *Bacillus subtilis* and *Rhodobacter capsulatus*

The ability of the two micro-organisms to hydrolyze starch using their extracellularly secreted amylase enzyme was analyzed using biochemical test for amylase hydrolysis of starch. *B. subtilis* and *R. capsulatus* were cultured in the standard media, Modified Horikoshi media and in DPH-Starch formulated media plates. The microorganisms grew in all tested media except the negative control

(Figure 4.7 and Figure 4.8, respectively). The halo is an indication of starch hydrolysis by the extracellular amylase enzyme produced by the bacteria.

Bacillus subtilis was cultured in Modified Horikoshi media and in DPH-Starch formulated media and controls were included as well (Figure 4.7).

A **B**



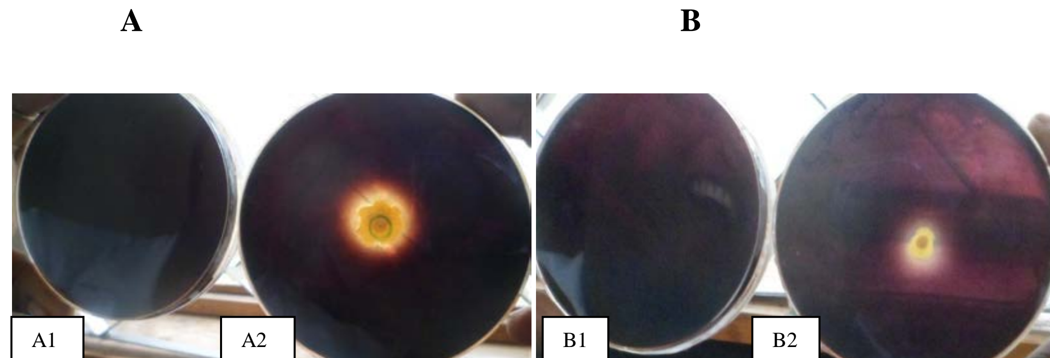
Modified Horikoshi media **DPH-Starch media**

Figure 4.7: Biochemical test for starch hydrolysis by amylase enzyme produced by *B. subtilis*.

Figure 4.7 A: Modified Horikoshi media plates; A1-negative control, A2-plate with a halo area (positive for starch hydrolysis by *B. subtilis* amylase). Figure 4.7 B: DPH-Starch media; B1-negative control, B2-plate with a halo area (positive for starch hydrolysis by *B. subtilis* amylase). The halo appeared on 1% Iodine staining of the plates. Negative controls retained the color of iodine with no halo as there was no breakdown of starch. The diameter of the halo in the Petri dish containing Modified Horikoshi media was 3 cm while the that containing DPH-Starch media was 3.3 cm (Figure 4.7). This indicated that the rate of hydrolysis of starch by amylase produced by *B. subtilis* was the almost same in Modified Horikoshi and DPH-Starch.

Rhodobacter capsulatus was cultured in Modified Horikoshi media and in DPH-Starch formulated media and controls were included as well (Figure 4.8).

A **B**



Modified Horikoshi media **DPH-Starch media**

Figure 4.8: Biochemical test for starch hydrolysis by amylase produced by *Rhodobacter capsulatus*.

Figure 4.8 A: Modified Horikoshi media plates; A1-negative control, A2-plate with a halo area (positive for starch hydrolysis by *R. capsulatus* amylase). Figure 4.8 B: DPH-Starch media; B1-negative control, B2-plate with a halo area (positive for starch hydrolysis by *R. capsulatus* amylase). The halo appeared on 1% Iodine staining of the plates. Negative controls retained the colour of iodine with no halo as there was no breakdown of starch. The diameter of the halo in the Petri dish containing Modified Horikoshi media was 2 cm while the that containing DPH-Starch media was 1.4 cm (Figure 4.8). This indicated that the rate of hydrolysis of starch by amylase produced by *R. capsulatus* was the slightly better in Modified Horikoshi than DPH-Starch.

4.4.1.2 Rate of glucose formation on hydrolysis of starch by *B. subtilis* and *R. capsulatus* amylases in Modified Horikoshi media and DPH-Starch media using DNS assay.

Rate of glucose formation was measured after performing the DNS assay test. The rate of glucose formation after the hydrolysis of starch by amylase produced by *B. subtilis* in Modified Horikoshi media and DPH-Starch media. The rate of glucose formation was higher in DPH-Starch media than in Modified Horikoshi media. In the Modified Horikoshi media rate of glucose formation increased over three days

while in DPH-Starch media the rate of glucose formation decreased over the three days (Figure 4.9).

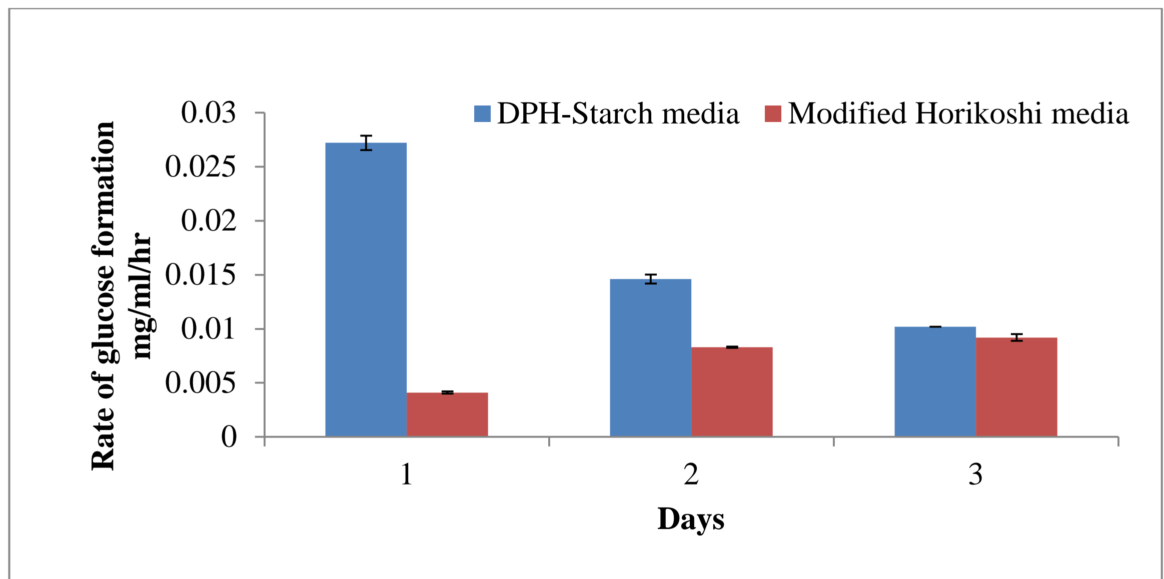


Figure 4.9: Rate of glucose formation due to breakdown of starch by *Bacillus subtilis* amylase enzyme over a 3-day period.

The rate of glucose formation after the hydrolysis of starch by amylase produced by *R. capsulatus* in both Modified Horikoshi media and DPH Starch media. The rate of glucose formation was higher in DPH-Starch media than in Modified Horikoshi media. The rate of glucose formation in Modified Horikoshi and DPH-Starch media increased for the first two days and then decreased on the third day. However, generally DPH-Starch media gave much higher glucose formation rate than Modified Horikoshi media (Figure 4.10).

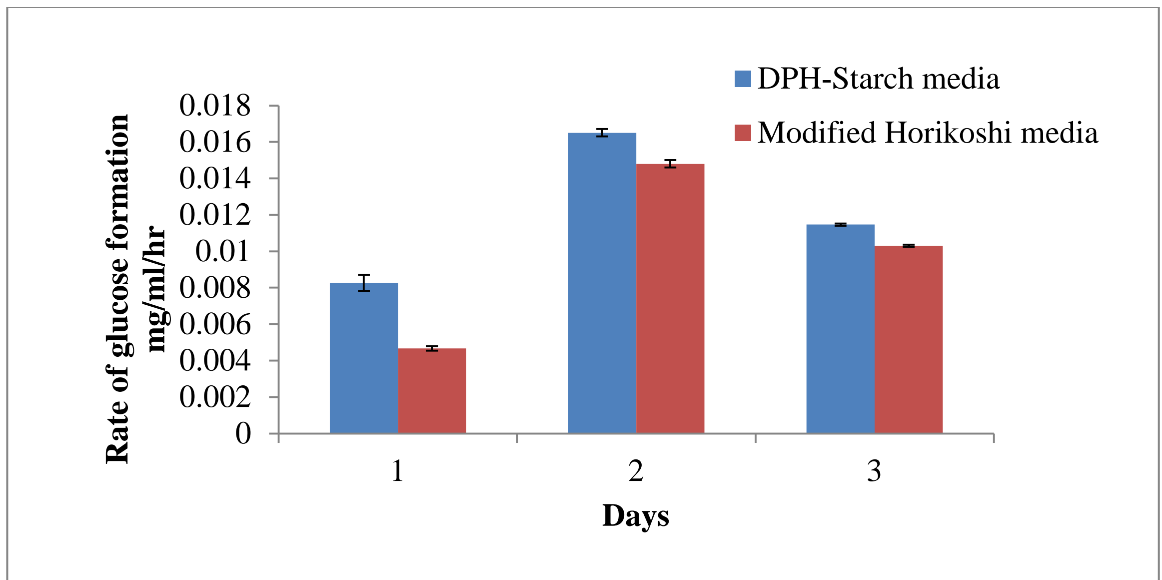


Figure 4.10: Rate of glucose formation due to breakdown of starch by *Rhodobacter capsulatus* amylase enzyme over a 3 day period.

The results from this study shows that the rate of glucose formation due to the production of extracellular enzyme amylase by *B. subtilis* and *R. capsulatus* in Modified Horikoshi and DPH-Starch media were significantly different ($p<0.05$) (Appendix 30). Multiple comparisons using post hoc analysis was not done as the comparison was only between the rate of glucose formation in Modified Horikoshi media and DPH-Starch media.

4.4.2 Sporulation of *Metarhizium anisopliae* in SDB and DPH-Dextrose media

Fungal sporulation rates were determined by establishing concentration of *M. anisopliae* conidia in the SDB and DPH media. SDB media had a slightly higher conidia concentration than DPH- Dextrose media (Table 4.3).

Table 4.3: Sporulation rates of *M. anisopliae* in SDB media and DPH-Dextrose media.

Microorganism	Media	Dilution factor	Conidia concentration (cell/ml)	Standard deviation	Standard error	Variance
Metarhizium anisopliae	SDB	10^3	$3.1000*10^9$	1.7900	1.0110	3.2100
	DPH-Dextrose	10^3	$2.9000*10^9$	1.7500	1.0110	3.0700
	SDB	10^5	$4.8000*10^7$	2.4990	1.4000	6.2000
	DPH-Dextrose	10^5	$4.7000*10^7$	2.5000	1.4000	6.1000

Statistics revealed that sporulation rates of *M. anisopliae* in SDB and DPH-Dextrose were not statistically difference ($p>0.05$) (Appendix 32).

CHAPTER FIVE
5.0 DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 Discussion

Dagaa protein hydrolysate contains lipids and is very rich in proteins (Ogonda et al., 2014; Owaga et al., 2010; Bille and Shemkai, 2006). This makes it a very good candidate for fortification of food for all organism including microbes. It is inexpensive to prepare and is easily available in Kenya, Uganda and Tanzania. It would add value to Dagaa fish if its hydrolysates are channelled into making microbial culture media.

Proximate analysis was carried out for moisture/dry weight, ash, protein and lipid content in Dagaa, LB, DFH, DPH and SDB. Dagaa, LB and SDB had the highest percentage dry weight and this is because they were all analyzed on dry weight basis (Table 4.1). DFH and DPH had the least dry weight content because they were analyzed on wet weight basis; hence more moisture was lost and also proving that there is less organic matter in these two samples (Table 4.1). This could be due to the fact that DPH and DFH are derivatives of Dagaa after an enzymatic hydrolysis process and during the hydrolysis there was loss of organic matter as Dagaa muscle protein was hydrolyzed to peptides and sludge with fats (Table 4.1).

LB had the highest ash content partly because it contained Sodium Chloride which did not ash at 600° C (Table 4.1). Dagaa and SDB had higher ash content than DFH and DPH as they were also analyzed based on dry basis (Table 4.1). DPH and DFH had the least ash content because they had very little inorganic matter as most of it was lost in the hydrolysis process (Table 4.1). DPH and DFH were analyzed on wet weight basis.

Dagaa had the highest protein content followed closely by DFH and DPH, the lower content of protein in the Dagaa derivatives could be due to the loss of organic material during the hydrolysis process (Table 4.1). LB and SDB both had lower protein content than the Dagaa or the variant hydrolysates of Dagaa that is DFH and DPH because both SDB and LB are commercialized in their protein content and have only a required set amount for microbial growth as compared to the Dagaa

derivatives (Table 4.1). It was noted that during the hydrolysis process most of the protein content was lost as Dagaa was converted to DFH by enzyme hydrolysis and as DFH was converted to DPH by the removal of fats through the use of hydrocarbon hexane. This could also be attributed to a low degree of hydrolysis of Dagaa leading to loss of protein content. This was noticeable when the proteins in Dagaa decreased from 5.2 mg/ 0.05 ml to 4.2 mg/ 0.05 ml in DFH and further reduced to 2.6 mg/ 0.05 ml in DPH (Table 4.1). DPH comprises only of soluble proteins, peptides and amino acids, this could account for the reduced protein content. DPH in every formulated media prepared, was used as a nitrogenous source. The protein source in media is usually broken down to amino acids that the microbe can utilize to make its own proteins as peptides and amino acids are easily broken down by the microbe for the nitrogen source than whole proteins (Todar, 2006).

LB and SDB do not contain lipids (Table 4.1). LB and SDB being standards for microbial growth did not contain lipids as lipids are not essential for microbial growth. DPH did not contain lipids as they had been extracted out from these samples in order to equate the lipid content of LB and SDB to DPH (Table 4.1). Lipids are not a preferred source of carbon for most microbes as they are more complicated to breakdown compared to simple sugars, peptides or amino acids (Todar, 2006). Lipids were therefore not present in LB and SDB as well as in DPH which was used to formulate microbial media. The lipid content in Dagaa was at 2.4% while in DFH was at 1.0% and absent in DPH indicating that further downstream applications on the original sample could lead to loss of macronutrient content (Table 4.1).

Enzyme Alcalase was utilized to hydrolyse Dagaa to its derivative hydrolysates DPH and DFH because it has been shown to be the best enzyme for preparing fish protein hydrolysate. Enzymes are preferred for hydrolysis process because they give higher yield of proteins (Muzaifa et al., 2012). For this study the degree of hydrolysis of Dagaa using Alcalase was not determined. Fish protein hydrolysates are best prepared using enzymes despite the fact that they can also be prepared using chemicals or solvents. This is because the protein hydrolysates produced using chemicals and solvents are of low nutritional value, poor functional ability and

cannot be utilized for commercial applications with most of the peptides being denatured (Abdulazeez et al., 2013).

Proximate analysis of Dagaa in this study was comparable with previous results of Dagaa proximate analysis in Kenya and Tanzania especially on the moisture/dry weight, ash, protein and lipid contents. The current study recorded a moisture content of 95%, ash content of 13.5%, crude protein content of 5.2mg/ml and crude lipid content of 2.4 % of Dagaa (Table 4.1). Dagaa protein content according to a study by Ogonda et al (2014) in Kenya was 21.7% while a study by Bille and Shemkai (2006) from Tanzania recorded 48.2% protein content in Dagaa. This is an indication that the protein content of Dagaa can depend on geographical regions of fish collection.

Other fish and fish by products like the king fish skin, mackerel, catfish, herring and tuna have been used to prepare fish protein hydrolysate (Abdulazeez et al., 2013; Guerard et al., 2002; Hoyle and Merritt, 1994; Kumar and Nazeer, 2011; Liceaga-Gesualdo and Li-Chan, 1999; Yin et al., 2010; Wu et al., 2003). The skin of the king fish, a fishery by product had a high crude protein content of 85.6%, an indication of the high potential not just from whole fish but also fishery by products as a source of high quality fish protein hydrolysate (Abdulazeez et al., 2013). Fish hydrolysates with the desired functional and nutritional values are being used in food industries in various forms such as milk replacers, stabilizers of beverages, flavor enhancers in confectionery products, protein supplements, animal feed and microbial media (Abdulazeez et al., 2013). They have also been found to have antioxidant, antihypertensive, immune-modulatory, neuroactive, antimicrobial and mineral or hormone regulating abilities (Abdulazeez et al., 2013; Jao and Ko et al., 2002; Ogonda et al., 2014). Therefore, the fish hydrolysate from Dagaa can be channeled into these applications if not into microbial culturing media industry.

Minerals are micronutrients and are only required in very small amounts for enzymatic activity in organisms (Prentice, 2005). There was very low mineral content in the DPH sample which then indicates that it is important to supplement DPH formulated media with minerals necessary for microbial growth (Table 4.2). The DPH had the lowest mineral content among the derivatives of Dagaa when compared to the Dagaa itself and DFH because of the loss of minerals during the

downstream processing (Table 4.2). Minerals are very important in the growth of microbes for their metabolism. They function as co-factors (Nair, 2008). Therefore, it is very important when using DPH formulated media as a microbial media that minerals are supplemented depending on the needs of the microbe being grown.

The DPH-FM supported the growth of *E. coli*, *B. subtilis*, *P. aeruginosa* and *R. capsulatus*. DPH-Dextrose supported the growth of *M. anisopliae* and *B. bassiana* after supplementation based on the proximate analysis and mineral content (Figure 4.1, 4.2, 4.3, 4.4, 4.5 and 4.6). Whatever extra micronutrient or macronutrient that lacked in the DPH was supplemented into the formulated media to ensure the growth of a specific microbe depending on its nutritional demands. The growth rate of the microbes in the standard and formulated media was found to be similar, an indication that the DPH-FM and DPH-Dextrose are equally good media for growth of these microbes. DPH was only found to be rich in proteins but lacking in important minerals such as nickel and also lacking in carbohydrates hence the need for supplementation depending on the preference of growth nutrients by a microbe to be cultured (Table 4.1 and 4.2).

DPH-FM growth of *E. coli*, *B. subtilis* and *P. aeruginosa* was slightly slower than in LB (Figure 4.1, 4.2 and 4.3). This could be attributed to the size of peptides in the LB media being smaller than in DPH-FM hence the microbe was able to easily use up the LB peptides and hence grew slightly faster than in DPH-FM. The stationary phase of growth for *E. coli* was attained faster in LB media than in DPH-FM an indication that there was an excess of nutrients in DPH-FM media that allowed the microbes to grow slowly or have a longer exponential phase of growth in DPH-FM than in LB (Figure 4.1). Death phase for *B. subtilis* was attained in LB media but was not in DPH-FM because there was a slight excess of nutrients in the DPH-media (Figure 4.2). The growth of *R. capsulatus* in LB and DPH-FM is almost identical hence these media can be interchanged for the growth of this microbe as well as all the other microbes (Figure 4.4).

The growth of *M. anisopliae* and *B. bassiana* in SDB and DPH-Dextrose media indicated that SDB supported slightly faster growth at the beginning but for *B. bassiana* the growth became slightly higher in DPH-Dextrose on the last days than in SDB (Fig 4.5 and 4.6). Overall, the growth in both media for these fungi was at

the same level and hence DPH-Dextrose can be used interchangeably with SDB media.

The results realized from this study concur with previous investigations on the potential of fish protein hydrolysate from Silver carp head peptone as microbial media that had no difference in growth of *Vibrio anguillarum* in comparison to the standard peptone media (Safari et al., 2011). Furthermore, some protein hydrolysate obtained from Silver carp filleting waste, yellow stripe Trevally and yellow fin Tuna have shown better activity as formulated microbial media than the standard media used in those studies (Fallah et al., 2015; Klompong et al., 2009; Ovissipour et al., 2011).

The extracellular amylase enzyme produced in DPH-Starch formulated media from *B. subtilis* had almost similar activity to that produced on Modified Horikoshi media plate with diameters of 3 cm and 3.3 cm respectively showing the area of hydrolysis (Figure 4.7). The amylase produced in Modified Horikoshi media from *R. capsulatus* indicated better hydrolysis of starch than in the DPH-Starch media with diameters of 2 cm and 1.4 cm respectively showing the area of hydrolysis (Figure 4.8). There was higher rate of glucose formation in DPH-Starch media as compared to Modified Horikoshi media for both microbes (Figure 4.9 and 4.10). This is an indication that amylase produced by *B. subtilis* and *R. capsulatus* in DPH-Starch media was more effective or in larger quantity that the amylase produced in Modified Horikoshi media for these microbes. Similar results were obtained from fish hydrolysates produced from fish wastes (viscera and chitinous material) tested as growth substrates for microbes that produce exo-enzymes; protease, lipase, chitinolytic and ligninolytic enzymes (Rebah and Mileb, 2013). This application of fish protein hydrolysates in exo-enzyme recovery from microbes saves on cost of microbial enzyme production and helps to eliminate environmental pollution brought about by improper disposal of fish waste (Rebah and Mileb, 2013).

The DPH-Dextrose formulated media also sustained sporulation of *M. anisopliae* species at the same rate as the standard media SDB media (Table 4.3). In this case, sporulation rate of *M. anisopliae* in DPH-Dextrose formulated media was as good as in SDB standard media. Hence, DPH-Dextrose can be easily substituted for SDB media in growth of *M. anisopliae*.

The cost of 1kg DPH production was estimated to be Kshs. 12, 500 which is about four times less than the cost of commercial nitrogenous source of culture media tryptone that goes at about Kshs. 48,500. DPH is therefore a more economical alternative source of nitrogen for culturing microbes (Sigma Aldrich, 2016).

5.2 Conclusion

1. DPH does not contain all macronutrients and micronutrients required for the proper growth of microbes. It is rich in proteins but lacks some important mineral elements as well as carbohydrate content required for microbial culturing.
2. DPH formulated media can be utilized to grow microbes such as bacteria and fungi. It is important to supplement the DPH formulated media with nutrients that it may lack according to the nutritional demands of the specific microorganisms to be grown.
3. DPH formulated media can be utilized for the growth of microorganisms that produce extracellular enzymes. It can also support the sporulation of fungi.

5.3 Recommendations

1. DPH can be analyzed for the specific amino acids it contains and investigated for the potential of its use in the human food fortification industry.
2. DPH formulated media should be utilized in the growth of clinical microbes and applied as a diagnostic media in hospitals.
3. DPH formulated media should be applied in industries that grow microbes for their extracellular enzymes or spores.

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7.0 APPENDICES

Appendix 1: Table for preparation and triplicate results of moisture content analysis of Dagaa, LB, DFH, DPH and SDB.

Sample	Original weight	Weight after moisture content analysis	% Dry weight
Dagaa 1	2g	1.9	95
Dagaa 2	2g	1.9	95
Dagaa 3	2g	1.9	95
LB 1	2.5g	2.36	94.4
LB 2	2.5g	2.35	94
LB 3	2.5g	2.35	94
DFH 1	10.73g	1.02	9.506
DFH 2	10.46g	0.98	9.369
DFH 3	10.02g	1.02	10.180
DPH 1	10.03g	0.9	8.973
DPH 2	10.07g	0.95	9.434
DPH 3	10.08g	0.9	8.929
SDB 1	5g	4.8	96
SDB 2	5g	4.79	95.8
SDB 3	5g	4.79	95.8

Appendix 2: Table for one way ANOVA results of moisture / dry weight content of Dagaa, LB, DFH, DPH and SDB.

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	26384.4200	4	6596.1050	98929.3060	0.0000
Within Groups	0.6670	10	0.0670		
Total	26385.0860	14			

Appendix 3: Table for post hoc statistical analysis of results for moisture / dry weight content analysis giving multiple comparisons.

	(I) VAR00001	(J) VAR00001	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Tukey HSD	Dagaa	LB	.86667*	.21083	.014	.1728	1.5605
		DFH	85.31500*	.21083	.000	84.6211	86.0089
		DPH	85.88800*	.21083	.000	85.1941	86.5819
		SDB	-.86667*	.21083	.014	-1.5605	-.1728
	LB	Dagaa	-.86667*	.21083	.014	-1.5605	-.1728
		DFH	84.44833*	.21083	.000	83.7545	85.1422
		DPH	85.02133*	.21083	.000	84.3275	85.7152
		SDB	-1.73333*	.21083	.000	-2.4272	-1.0395
	DFH	Dagaa	-85.31500*	.21083	.000	-86.0089	-84.6211
		LB	-84.44833*	.21083	.000	-85.1422	-83.7545
		DPH	.57300	.21083	.121	-.1209	1.2669
		SDB	-86.18167*	.21083	.000	-86.8755	-85.4878
	DPH	Dagaa	-85.88800*	.21083	.000	-86.5819	-85.1941
		LB	-85.02133*	.21083	.000	-85.7152	-84.3275
		DFH	-.57300	.21083	.121	-1.2669	.1209
		SDB	-86.75467*	.21083	.000	-87.4485	-86.0608
	SDB	Dagaa	.86667*	.21083	.014	.1728	1.5605
		LB	1.73333*	.21083	.000	1.0395	2.4272
		DFH	86.18167*	.21083	.000	85.4878	86.8755
		DPH	86.75467*	.21083	.000	86.0608	87.4485

*. The mean difference is significant at the 0.05 level.

Appendix 4: Table of sample preparation and triplicate results for ash content analysis of Dagaa, LB, DFH, DPH and SDB.

Sample	Original weight (g)	Weight after moisture content analysis (g)	Weight after ash content analysis (g)	% ash content.
Dagaa 1	2	1.9	0.26	13.68
Dagaa 2	2	1.9	0.26	13.68
Dagaa 3	2	2	0.26	13
LB 1	2.5	2.36	1.10	44
LB 2	2.5	2.35	1.10	44
LB 3	2.5	2.35	1.10	44
DFH 1	10.73g	1.02	0.09	0.839
DFH 2	10.46g	0.98	0.09	0.860
DFH 3	10.02g	1.02	0.09	0.898
DPH 1	10.03g	0.9	0.07	0.698
DPH 2	10.07g	0.95	0.07	0.695
DPH 3	10.08g	0.9	0.07	0.694
SDB 1	5	4.8	0.25	5
SDB 2	5	4.79	0.3	6
SDB 3	5	4.79	0.3	6

Appendix 5: Table for one way ANOVA results of ash content analysis of Dagaa, LB, DFH, DPH and SDB.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	3940.8050	4	985.2010	10086.7240	0.0000
Within Groups	0.9770	10	0.0980		
Total	3941.7820	14			

Appendix 6: Post hoc analysis results for ash content of Dagaa, LB, DFH, DPH and SDB giving multiple comparisons.

	(I) VAR00001	(J) VAR00001	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Tukey HSD	Dagaa	LB	-30.54667*	.25518	.000	-31.3865	-29.7069
		DFH	12.58767*	.25518	.000	11.7479	13.4275
		DPH	12.75767*	.25518	.000	11.9179	13.5975
		SDB	7.78667*	.25518	.000	6.9469	8.6265
	LB	Dagaa	30.54667*	.25518	.000	29.7069	31.3865
		DFH	43.13433*	.25518	.000	42.2945	43.9741
		DPH	43.30433*	.25518	.000	42.4645	44.1441
		SDB	38.33333*	.25518	.000	37.4935	39.1731
	DFH	Dagaa	-12.58767*	.25518	.000	-13.4275	-11.7479
		LB	-43.13433*	.25518	.000	-43.9741	-42.2945
		DPH	.17000	.25518	.959	-.6698	1.0098
		SDB	-4.80100*	.25518	.000	-5.6408	-3.9612
	DPH	Dagaa	-12.75767*	.25518	.000	-13.5975	-11.9179
		LB	-43.30433*	.25518	.000	-44.1441	-42.4645
		DFH	-.17000	.25518	.959	-1.0098	.6698
		SDB	-4.97100*	.25518	.000	-5.8108	-4.1312
	SDB	Dagaa	-7.78667*	.25518	.000	-8.6265	-6.9469
		LB	-38.33333*	.25518	.000	-39.1731	-37.4935
		DFH	4.80100*	.25518	.000	3.9612	5.6408
		DPH	4.97100*	.25518	.000	4.1312	5.8108

*. The mean difference is significant at the 0.05 level

Appendix 7: Table for sample preparation and triplicate results for protein content analysis in Dagaa, DFH, DPH and SDB.

Test tube number	BSA (mg/ml)	Sterile distilled water (µl)	BSA stock (µl)	Biuret's reagent (ml)	Absorbance	Proteins (mg/ml)
1	0	1000	0	2	0.0000	0.000
2	1	900	100	2	0.0840	1
3	2	800	200	2	0.1350	2
4	3	700	300	2	0.2587	3
5	4	600	400	2	0.3150	4
6	5	500	500	2	0.4077	5
7	6	400	600	2	0.5565	6
8	7	300	700	2	0.6320	7
9	8	200	800	2	0.6820	8
10	9	100	900	2	0.7507	9
11	10	0	1000	2	0.7800	10
Dagaa 1(50µl)	0	950	0	2	0.431	5.193
Dagaa 2 (50µl)	0	950	0	2	0.440	5.301
Dagaa 3 (50µl)	0	950	0	2	0.436	5.253
LB 1 (50µl)	0	950	0	2	0.062	0.747
LB 2 (50µl)	0	950	0	2	0.066	0.795
LB 3 (50µl)	0	950	0	2	0.069	0.831
DFH 1 (50µl)	0	950	0	2	0.352	4.241
DFH 2 (50µl)	0	950	0	2	0.359	4.325
DFH 3(50µl)	0	950	0	2	0.355	4.277
DPH 1 (50µl)	0	950	0	2	0.216	2.602
DPH 2 (50µl)	0	950	0	2	0.216	2.602
DPH 3 (50µl)	0	950	0	2	0.217	2.614
SDB 1 (50µl)	0	950	0	2	0.059	0.711
SDB 2 (50µl)	0	950	0	2	0.053	0.639
SDB 3 (50µl)	0	950	0	2	0.057	0.687

Appendix 8: Table for one way ANOVA results of protein content analysis of Dagaa, LB, DFH, DPH and SDB.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	49.9930	4	12.4980	9319.6830	0.0000
Within Groups	.0130	10	.0010		
Total	50.0070	14			

Appendix 9: Post hoc analysis for protein analysis in Dagaa, LB, DFH, DPH and SDB giving multiple comparisons.

	(I) VAR00001	(J) VAR00001	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Tukey HSD	Dagaa	LB	4.45800*	.02990	.000	4.3596	4.5564
		DFH	.96800*	.02990	.000	.8696	1.0664
		DPH	2.64300*	.02990	.000	2.5446	2.7414
		SDB	4.55333*	.02990	.000	4.4549	4.6517
	LB	Dagaa	-4.45800*	.02990	.000	-4.5564	-4.3596
		DFH	-3.49000*	.02990	.000	-3.5884	-3.3916
		DPH	-1.81500*	.02990	.000	-1.9134	-1.7166
		SDB	.09533	.02990	.059	-.0031	.1937
	DFH	Dagaa	-.96800*	.02990	.000	-1.0664	-.8696
		LB	3.49000*	.02990	.000	3.3916	3.5884
		DPH	1.67500*	.02990	.000	1.5766	1.7734
		SDB	3.58533*	.02990	.000	3.4869	3.6837
	DPH	Dagaa	-2.64300*	.02990	.000	-2.7414	-2.5446
		LB	1.81500*	.02990	.000	1.7166	1.9134
		DFH	-1.67500*	.02990	.000	-1.7734	-1.5766
		SDB	1.91033*	.02990	.000	1.8119	2.0087
	SDB	Dagaa	-4.55333*	.02990	.000	-4.6517	-4.4549
		LB	-.09533	.02990	.059	-.1937	.0031
		DFH	-3.58533*	.02990	.000	-3.6837	-3.4869
		DPH	-1.91033*	.02990	.000	-2.0087	-1.8119

*. The mean difference is significant at the 0.05 level.

Appendix 10: Table for sample preparation and triplicate results for lipid analysis.

Sample	Original amount of sample (g)	Weight of lipid in tube (g)	Lipid/100g sample (g)	% lipid content
Dagaa 1	10g	0.21	2.400	2.400
Dagaa 2	10g	0.19	2.174	2.174
Dagaa 3	10g	0.22	2.514	2.514
LB 1	10ml	0.00	0	0
LB 2	10ml	0.00	0	0
LB 3	10ml	0.00	0	0
DFH 1	10ml	0.07	0.933	0.933
DFH 2	10ml	0.07	0.933	0.933
DFH 3	10ml	0.09	1.200	1.200
DPH 1	10ml	0	0	0
DPH 2	10ml	0	0	0
DPH 3	10ml	0	0	0
SDB	10ml	0	0	0
SDB	10ml	0	0	0
SDB	10ml	0	0	0

Appendix 11: Table for one way ANOVA results of lipid content analysis of Dagaa, LB, DFH, DPH and SDB.

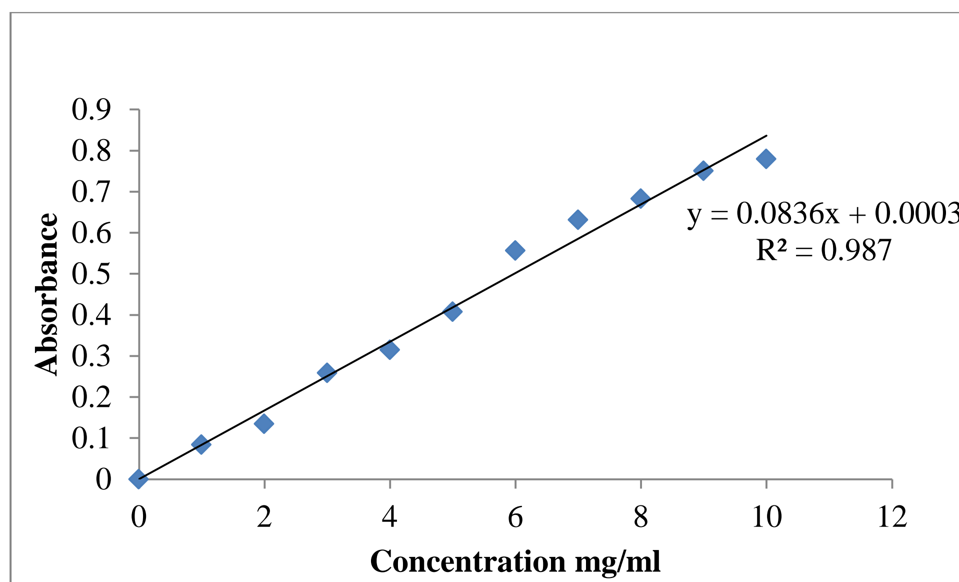
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	13.0060	4	3.2520	302.7100	0.0000
Within Groups	.1070	10	.0110		
Total	13.1140	14			

Appendix 12: Post hoc table for lipid analysis of Dagaa, LB, DFH, DPH and SDB giving multiple comparisons.

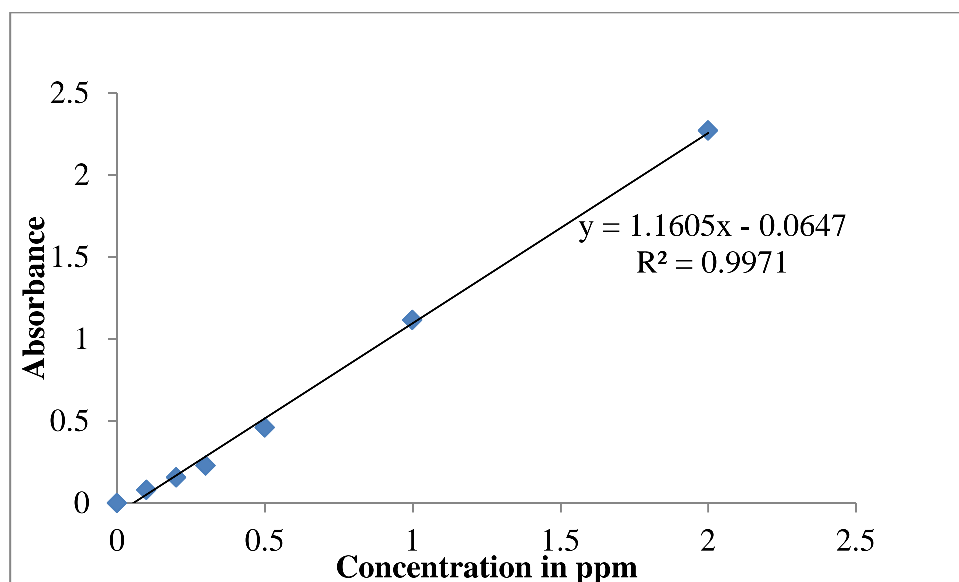
	(I) VAR00001	(J) VAR00001	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Tukey HSD	Dagaa	LB	2.36267*	.08462	.000	2.0842	2.6412
		DFH	1.34067*	.08462	.000	1.0622	1.6192
		DPH	2.36267*	.08462	.000	2.0842	2.6412
		SDB	2.36267*	.08462	.000	2.0842	2.6412
	LB	Dagaa	-2.36267*	.08462	.000	-2.6412	-2.0842
		DFH	-1.02200*	.08462	.000	-1.3005	-.7435
		DPH	.00000	.08462	1.000	-.2785	.2785
		SDB	.00000	.08462	1.000	-.2785	.2785
	DFH	Dagaa	-1.34067*	.08462	.000	-1.6192	-1.0622
		LB	1.02200*	.08462	.000	.7435	1.3005
		DPH	1.02200*	.08462	.000	.7435	1.3005
		SDB	1.02200*	.08462	.000	.7435	1.3005
	DPH	Dagaa	-2.36267*	.08462	.000	-2.6412	-2.0842
		LB	.00000	.08462	1.000	-.2785	.2785
		DFH	-1.02200*	.08462	.000	-1.3005	-.7435
		SDB	.00000	.08462	1.000	-.2785	.2785
	SDB	Dagaa	-2.36267*	.08462	.000	-2.6412	-2.0842
		LB	.00000	.08462	1.000	-.2785	.2785
		DFH	-1.02200*	.08462	.000	-1.3005	-.7435
		DPH	.00000	.08462	1.000	-.2785	.2785

*. The mean difference is significant at the 0.05 level.

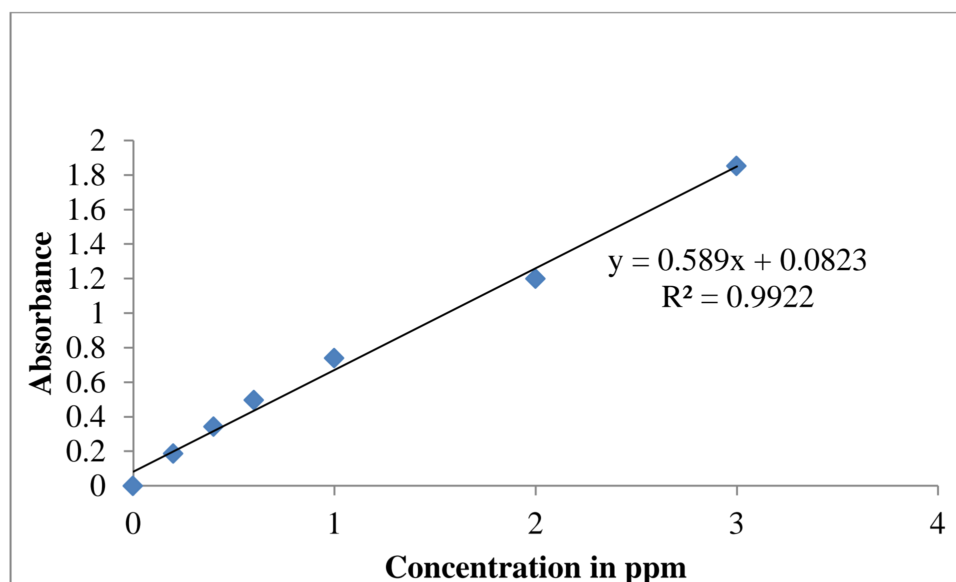
Appendix 13: Protein standard curve.



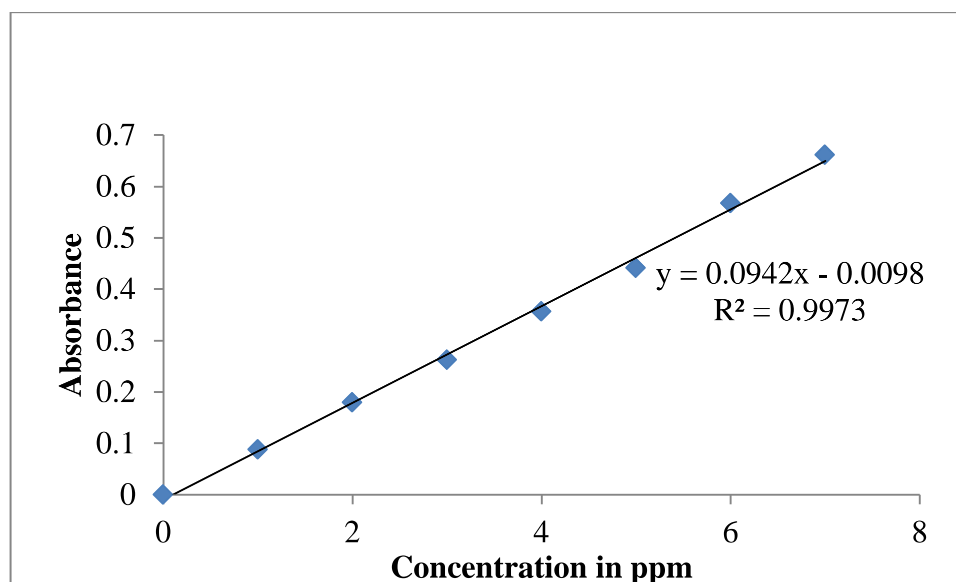
Appendix 14: Magnesium standard curve.



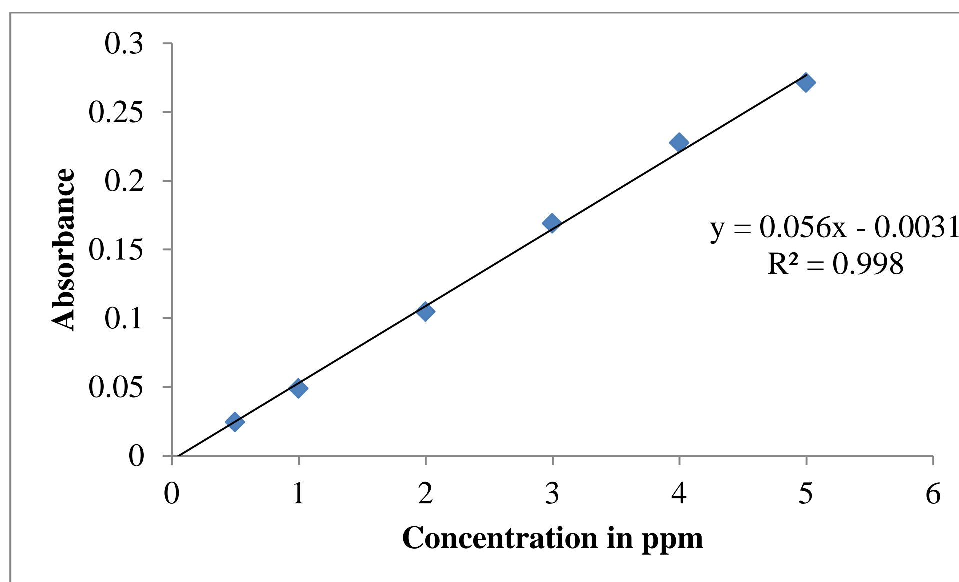
Appendix 15: Zinc standard curve.



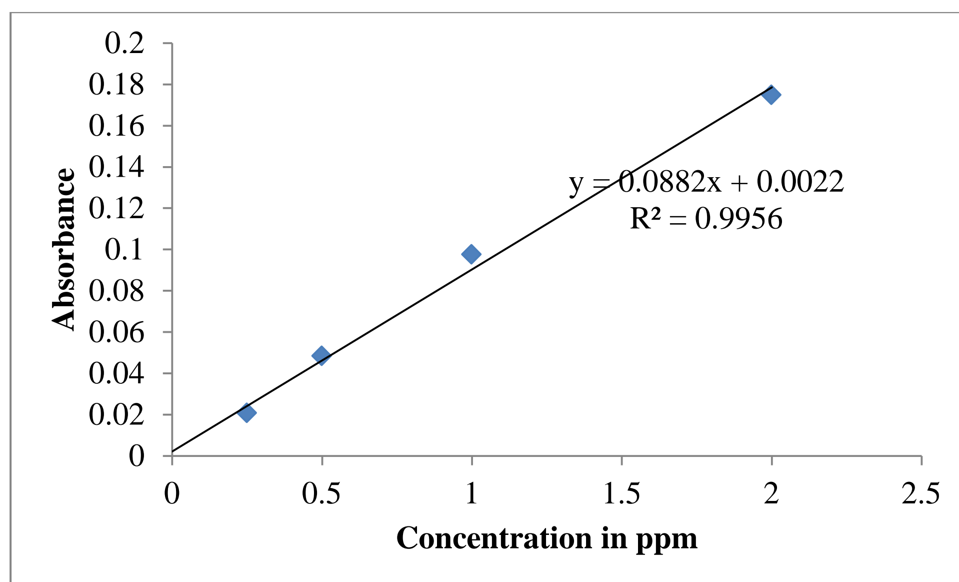
Appendix 16: Iron standard curve.



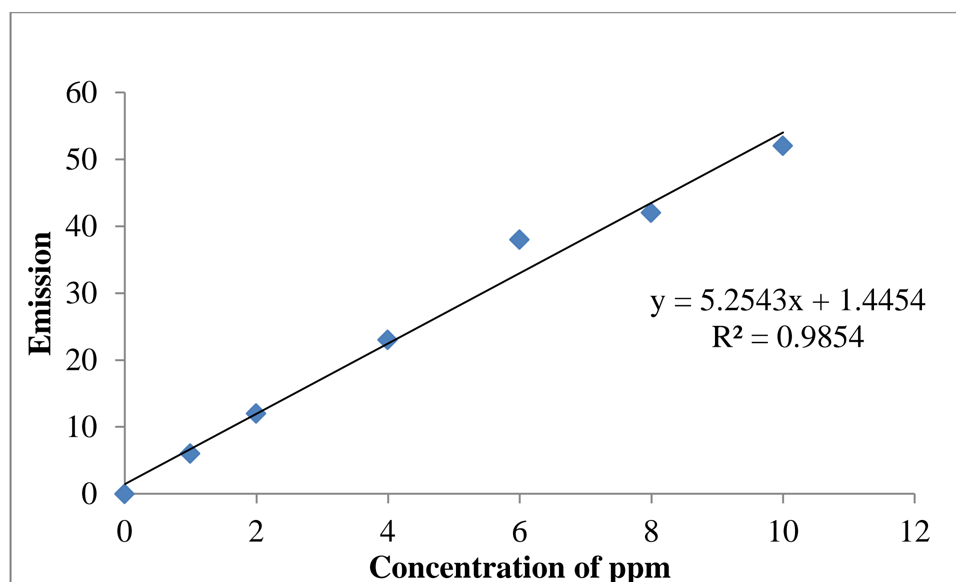
Appendix 17: Nickel standard curve.



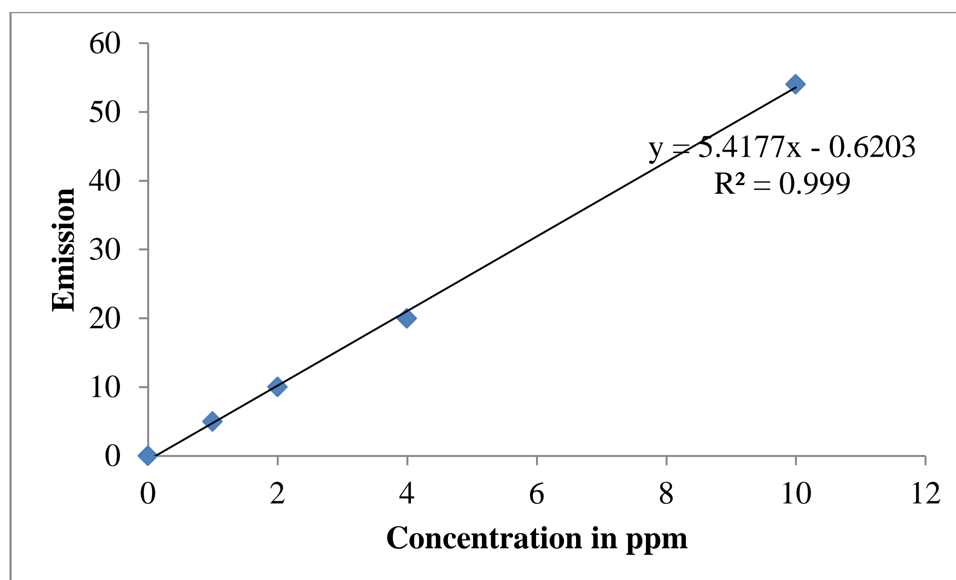
Appendix 18: Copper standard curve.



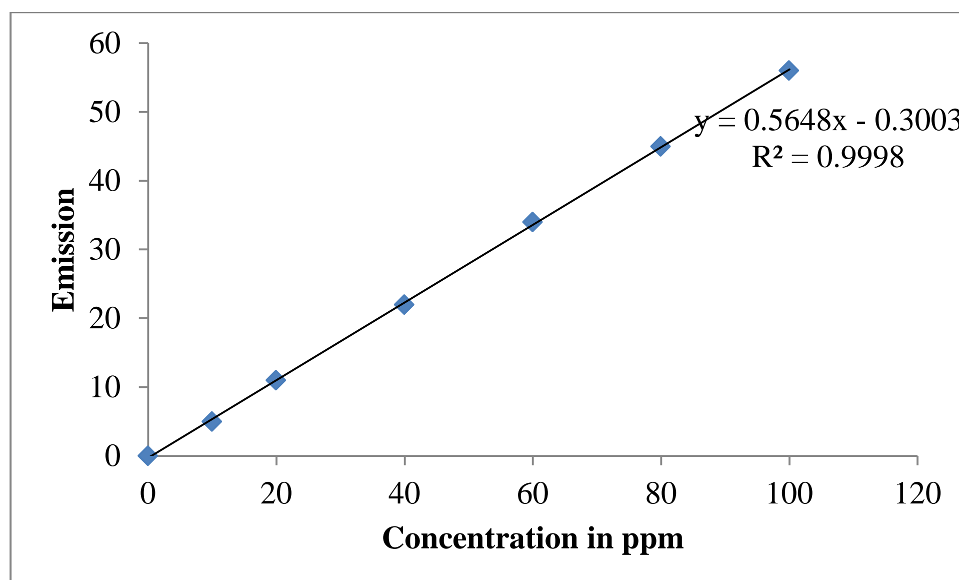
Appendix 19: Sodium standard curve.



Appendix 20: Potassium standard curve.



Appendix 21: Calcium standard curve.



Appendix 22: Table for one way ANOVA results of mineral content analysis in Dagaa, LB, DFH, DPH and SDB.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	224.191	4	56.048	4.618	.002
Within Groups	1395.677	115	12.136		
Total	1619.868	119			

Appendix 23: Post hoc analysis for mineral analysis of Magnesium, Zinc, Iron, Nickel, Copper, Sodium, Potassium and Calcium in the Dagaa, LB, DFH, DPH and SDB giving multiple comparisons.

	(I) Sample	(J) Sample	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Tukey HSD	Dagaa	LB	2.90868096*	1.00566417	.036	.1214477	5.6959143
		DFH	3.51297296*	1.00566417	.006	.7257397	6.3002063
		DPH	3.51593980*	1.00566417	.006	.7287065	6.3031731
		SDB	3.52543099*	1.00566417	.006	.7381977	6.3126643
	LB	Dagaa	-2.90868096*	1.00566417	.036	-5.6959143	-.1214477
		DFH	.60429200	1.00566417	.975	-2.1829413	3.3915253
		DPH	.60725884	1.00566417	.974	-2.1799745	3.3944921
		SDB	.61675003	1.00566417	.973	-2.1704833	3.4039833
	DFH	Dagaa	-3.51297296*	1.00566417	.006	-6.3002063	-.7257397
		LB	-.60429200	1.00566417	.975	-3.3915253	2.1829413
		DPH	.00296684	1.00566417	1.000	-2.7842665	2.7902001
		SDB	.01245803	1.00566417	1.000	-2.7747753	2.7996913
	DPH	Dagaa	-3.51593980*	1.00566417	.006	-6.3031731	-.7287065
		LB	-.60725884	1.00566417	.974	-3.3944921	2.1799745
		DFH	-.00296684	1.00566417	1.000	-2.7902001	2.7842665
		SDB	.00949119	1.00566417	1.000	-2.7777421	2.7967245
	SDB	Dagaa	-3.52543099*	1.00566417	.006	-6.3126643	-.7381977
		LB	-.61675003	1.00566417	.973	-3.4039833	2.1704833
		DFH	-.01245803	1.00566417	1.000	-2.7996913	2.7747753
		DPH	-.00949119	1.00566417	1.000	-2.7967245	2.7777421

*. The mean difference is significant at the 0.05 level.

Appendix 24: Table for one-way ANOVA for growth curves of *E. coli*, *B. subtilis*, *P. aeruginosa* and *R. capsulatus* in LB and DPH-FM media by optical density.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.358	1	.358	.767	.382
Within Groups	88.698	190	.467		
Total	89.056	191			

Appendix 25: Table for one way ANOVA for growth curves of *E. coli*, *B. subtilis*, *P. aeruginosa* and *R. capsulatus* in LB and DPH-FM media by biomass.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.002	1	.002	.872	.351
Within Groups	.363	190	.002		
Total	.365	191			

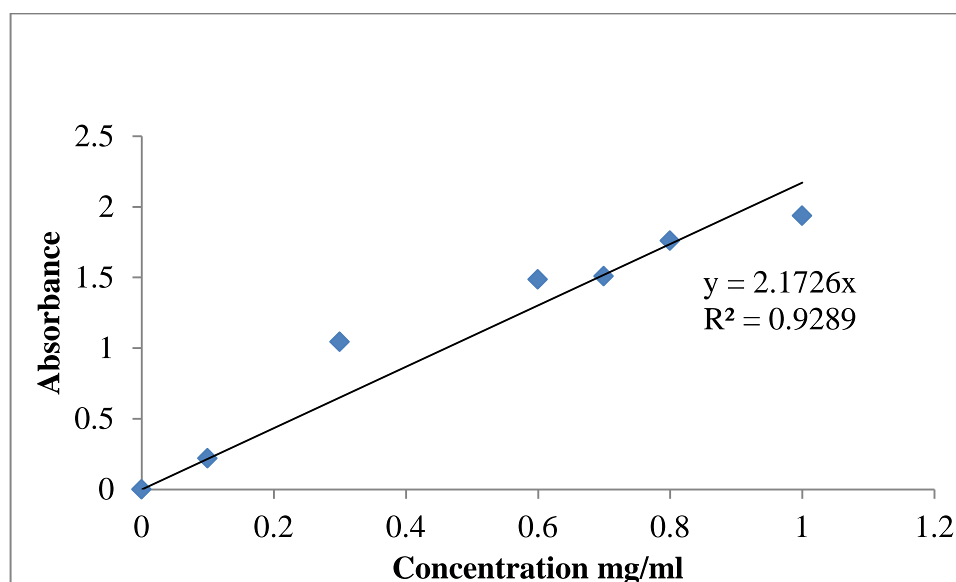
Appendix 26: Table for one way ANOVA for growth curves of *Metarhizium anisopliae* and *Beauveria bassiana* in SDB and DPH-Dextrose media by optical density.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.788	1	1.788	2.661	.107
Within Groups	55.088	82	.672		
Total	56.876	83			

Appendix 27: Table for one way ANOVA for growth curves of *Metarhizium anisopliae* and *Beauveria bassiana* in SDB and DPH-Dextrose media by biomass.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.545	1	.545	.703	.404
Within Groups	63.579	82	.775		
Total	64.124	83			

Appendix 28: Glucose standard curve



Appendix 29: Table for preparation of samples and triplicate results for the rate of glucose formation in DPH-Starch media and Modified Horikoshi media.

Microorganism	Media type	Day	Optical density	Glucose concentration (mg/ml)	Rate of glucose formation (mg/ml/ hr)
Rhodobacter capsulatus	DPH-Starch	1	0.405	0.1865	0.0078
		1	0.432	0.1989	0.0083
		1	0.452	0.2079	0.0087
		2	1.724	0.7937	0.0165
		2	1.703	0.7841	0.0163
		2	1.744	0.8029	0.0167
		3	1.789	0.8237	0.0114
		3	1.801	0.8292	0.0115
		3	1.792	0.8250	0.0115
Rhodobacter capsulatus	Horikoshi's Media	1	0.251	0.1156	0.0048
		1	0.240	0.1105	0.0046
		1	0.239	0.1100	0.0046
		2	1.543	0.7104	0.0148
		2	1.564	0.7201	0.0150
		2	1.523	0.7012	0.0146
		3	1.624	0.7477	0.0104
		3	1.604	0.7385	0.0103
		3	1.632	0.7514	0.0104
Bacillus subtilis	DPH-Starch	1	1.458	0.6713	0.0280
		1	1.400	0.6446	0.0269
		1	1.398	0.6436	0.0268
		2	1.567	0.7210	0.0150
		2	1.542	0.7099	0.0147
		2	1.480	0.6814	0.0142
		3	1.602	0.7376	0.0102
		3	1.596	0.7348	0.0102
		3	1.588	0.7311	0.0102
Bacillus subtilis	Horikoshi's	1	0.218	0.1004	0.0042
		1	0.209	0.0962	0.0040
		1	0.214	0.0985	0.0041
		2	0.856	0.3941	0.0082
		2	0.879	0.4047	0.0083
		2	0.863	0.3973	0.0083
		3	1.424	0.6556	0.0091
		3	1.396	0.6427	0.0089
		3	1.493	0.6874	0.0095

Appendix 30: Table for one way ANOVA for rate of glucose formation after breakdown of starch by amylase produced by *B. subtilis* and *R. capsulatus* over 3 days.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.000	1	.000	12.435	.001
Within Groups	.001	34	.000		
Total	.001	35			

Appendix 31: Table for triplicate results for sporulation rates of *Metarhizium anisopliae* in SDB and DPH-Dextrose media.

Microorganism	Media	Dilution factor	Conidia concentration (cell/ml)
Metarhizium anisopliae	SDB	10^3	$2.8*10^9$
	SDB	10^3	$3.1*10^9$
	SDB	10^3	$3.4*10^9$
	DPH-Dextrose	10^3	$2.5*10^9$
	DPH-Dextrose	10^3	$2.8*10^9$
	DPH-Dextrose	10^3	$3.3*10^9$
	Media		
	SDB	10^5	$4.7*10^7$
	SDB	10^5	$4.8*10^7$
	SDB	10^5	$4.8*10^7$
	DPH-Dextrose	10^5	$4.8*10^7$
	DPH-Dextrose	10^5	$4.7*10^7$
	DPH-Dextrose	10^5	$4.7*10^7$

Appendix 32: Table for one way ANOVA for *Metarhizium anisopliae* sporulation rates in SDB media and DPH-Dextrose media.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	4.095E18	1	4.095E18	.016	.903
Within Groups	2.640E21	10	2.640E20		
Total	2.645E21	11			