# THE USE OF FUNGI IN THE PRODUCTION OF SIGLE CELL PROTEIN FROM HYDROLYZED PINEAPPLE (*Ananas comosus*) PEEL WASTES

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**FACULTY OF BIOLOGICAL SCIENCES**

**UNIVERSITY OF NIGERIA, NSUKKA**

**MAY, 2018.**

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**SUPERVISOR: DR. NWOKORO O.**

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# TITLE PAGE

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**MAY, 2018.**

## CERTIFICATION

This is to certify that, **Clement, Patience Ndidiamaka**, a postgraduate student in the Department of Microbiology, has satisfactorily completed the requirement for research work for the award of Masters of Science degree (M.Sc.) in Industrial Microbiology.

The work embodied in this thesis is original and has not been submitted in part or full for any other diploma or degree of this university or any other university.

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## DEDICATION

This dissertation is dedicated to God Almighty and my lovely dad, Late Chief Clement Sunday Onoyima for all he went through for me even though he was not here at the completion of this work.

## ACKNOWLEDGEMENT

I give thanks and praises to Almighty God who has been my strength during this project research.

My sincere appreciation goes to my able supervisor, Dr. Nwokoro, O. for his guidance, encouragement and support.

I am most grateful to Dr. Eze, C. N. who helped me o through my work and stood in for my supervisor when he was on leave.

I express my unflinching gratitude to my mum Mrs. Regina-Violet Onoyima, for giving me strength, also to my siblings; Rose and her husband David, Kelvin and his wife Blessing, Martha, Juliana, Daniel, Levi and Charity for their encouragement, moral and financial support.

My sincere appreciation goes to my Uncle and Aunt; Mr. Lazarus Nwodo and Mrs. Juliana Nwodo and my Cousins; Paschal, Uriel, Ernest and Victor for everything they did for me throughout my stay in Nsukka for the course of my study. Thank you for your understanding, love and care. God bless you all.

I acknowledge in a very special way my best friends; Nzekwe Charles and Awodiran Idunnu for their inspiration, strength and assistance given to me throughout the course of this research work.

I cannot but appreciate my friends; Paschaline, Basirat, Temidayo, Abiodun, Sanmi and all my classmates in 2014 PG class of Microbiology Department for their words of encouragement that kept me going.

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## ABSTRACT

The growing shortage of protein rich food supplies has stimulated the effort in searching alternate source of protein rich foods. Therefore, sources mainly microorganisms named single cell protein (SCP) was coined to describe the protein produced from microbial biomass. Single cell protein production on hydrolyzed pineapple peel waste by fungi was investigated. *Trichoderma viride* was selected based on its high cellulase activity; diameter of clear zone on CMCagar (7.4 cm) and activity on carboxymethylcellulose (4.64 mg glucose/ml), filter paper (3.76 mg glucose/ml) and cotton wool (4.12 mg glucose/ml). Samples of pineapple peel were hydrolyzed with the solutions of HCl, $H_2SO_4$ and NaOH at 0.5% concentration.The NaOH hydrolysates (138mg/ml, 298mg/ml and 9.44mg/ml) contained higher reducing sugar, soluble sugar and protein content than $H_2SO_4$ (129mg/ml, 206mg/ml and 6.28mg/ml) and HCl hydrolysates (131mg/ml, 279mg/ml and 7.32mg/ml) respectively. The culture of *Trichoderma viride* was used in fermentation of hydrolyzed pineapple peels. The protein yield produced in 0.5% NaOH hydrolysates (27.35 mg/ml) was significantly ($p \leq 0.05$) higher than that of $H_2SO_4$ hydrolysate (18.32 mg/ml) and HCl hydrolysate (16.48 mg/ml) after 7 days of incubation. The unhydrolyzed samples which served as control produced the lowest protein. Several nitrogen sources were added to the samples, the media supplemented with ammonium oxalate [$(NH_4)_2C_2O_4$] gave the highest protein of 55.44 mg/ml for NaOH hydrolysate. Different concentrations (2%, 4%, 6%, 8% and 10%) of glucose were also added to the extracts and the protein yield increased. At day 7, the protein content of NaOH hydrolysate with 10% glucose (42.95 mg/ml) was the highest. The maximum weight of biomass produced after drying of biomass was 0.66g/100ml. This study demonstrated the potential of pineapple peel waste as a substrate for by- product recovery and waste management.

# CHAPTER ONE

## 1.0 INTRODUCTION

The growing shortage of protein and other protein rich food supplies has stimulated the effort in searching new and alternate source of protein rich food and feed (Khan and Dahot, 2010). For this reason, sources mainly yeast, fungi, bacteria and algae named single cell protein (SCP) as coined to describe the protein production from biomass, originating from different microbial sources and has been used as protein sources (Parajo *et al*., 1995). Single cell protein (SCP) represents microbial cells (primary) grown in mass culture and harvested for use as protein sources in foods or animal feeds (Dhanasekaran *et al.,* 2011). The protein obtained from microorganisms such as algae, fungi, yeast and bacteria is cheap and competes well with other sources of protein and may provide good nutritive value depending, however, on the amino acid composition (Dhanasekaran *et al.,* 2011). The single cell protein (SCP) is a dehydrated cell consisting of mixture of proteins, lipids, carbohydrates, nucleic acids, inorganic compounds and a variety of other non- protein nitrogenous compounds such as vitamins (Dhanasekaran *et al.*, 2011).

In animal feeding and nutrition, single cell protein has application as fattening of calves, poultry, pigs and fish breeding. In food also, it is used as aroma carriers, vitamins, emulsifying agents and improved the nutritional value of food. There have been studies as well as efforts to improve the protein quantity and quality of finished food products by augmenting protein rich cheaper ingredient in food formation (Nasir and Butt, 2011). Although, animals are considered to be the best quality of protein, microbial protein also known as single cell protein (SCP) growth on agricultural wastes is one of the important optional proteins because of higher protein content

and very short growth cycle of microorganism, thereby leading to rapid biomass production (Bacha *et al*., 2011).

Since 2500BC, yeast has been used in bread and beverage production. In 1781, it was processed for preparing highly concentrated forms of yeast as biomass that is very rich in protein (Adoki, 2008). Single Cell Protein (SCP) are seen as microbial organism which are allowed to grow on waste products especially agricultural wastes as well as agro-based industries and they produce large quantity of protein and store them in their cell bodies. Interest in microbial production is increased because micro-organisms can utilize the waste materials that cause pollution problems and or sanitary hazards. With the present alarming danger of food shortage, the world will soon be unable to feed its population because of inadequate and shortage of food supply (mainly protein rich food). Therefore, the development of novel food production independent of agricultural land use is thus becoming imperative (Anupama and Ravindera, 2000).

Microorganisms can utilize a variety of substrates like agricultural wastes and effluents, industrial wastes, natural gas like methane, etc. so also help in decomposing pollutants (Huang and Kinsella, 1986). Agricultural wastes are useful substrates for production of microbial protein, but must meet the following criteria: it should be non-toxic, abundant, totally regenerable, non- exotic, cheap and able to support rapid growth and multiplication of the organisms resulting in high quality biomass (Dhanasekaran *et al.,* 2011). Several studies have been conducted using agricultural waste as a substrate including mango kernel meal (Diarra and Usman, 2008), Hyacinth bean (*Lablab purpureus)* (Rasha *et al*., 2007), Leaf meal (*Ipomeoeaasarifolia)* (Madubuike and Ekenyem, 2006), Breadfruit (*Treculia africana)* hulls (Nwabueze and Otunwa, 2006), Papaya (*Carica papaya L.)* (Ojokoh and Uzeh, 2005), Rice bran (Oshoma and Ikenebomeh, 2005), Banana waste peel (Sankar *et al*., 2011) and Pineapple waste

(Dhanasekaran *et al.*, 2011). The use of such a cheap and readily available substrate is desirable to lower the cost of production, reduce waste disposal and management problems, conserve natural resources and provide feed for livestock purpose (Sanker *et al*., 2011).

The pineapple (*Ananas comosus*) is one of the most important fruits in the world and is the leading edible member of the family *Bromeliaceae*. This fruit juice is the third most preferred worldwide after orange and apple juices (Cabrera *et al*., 2000). It is short, having a stout stump with narrow, fibrous and spiny leaves. It is an herbaceous perennial plant which grows from 1.0 to 1.5 m tall with 30 or more trough-shaped and pointed leaves, 30 cm long, surrounding a thick stem. It is a multiple fruit, forming what appears to be a single fleshy fruit (Idise, 2012).The plant develops to a cone-shaped juicy and fleshy fruit with crown at the top (Tran, 2006). According to FAO online data base, the area under pineapple plantation in 2007 was almost 920,349 ha with an estimated production of more than 18 million tons (FAO, 2007). Commercially, it is mainly produced as canned fruits and consumed worldwide (Tran, 2006). Besides, it is also processed as juices, concentrates, and jams. Pineapple slices have also been preserved after freezing (Larrauri *et al*., 2009). Furthermore, bromelain, the proteolytic enzyme present in the stem of pineapple, is finding wide applications in pharmaceutical and food uses (Hebbar *et al.,* 2008).

Until recently, about 80% of pineapple produced in Nigeria came from small scale farms managed under mixed cropping systems and current production figures shows that Nigeria is the 6th largest producer of pineapple in the world (FAO/World bank, 1999; Fawole, 2008). The skin waste was found to contain both carbohydrate and protein nutrients that are suitable and favourable for the growth of microorganisms, (Dhanasekaran *et al*., 2008).

## 1.1 STATEMENT OF PROBLEM

- ✓ There is deficit in protein production from conventional sources;
- ✓ There is increase in pollution due to lack of proper waste disposal;
- ✓ The deficiency of protein in human food and animal feed is well recognized due to the rapid growth of population and as such pressure is exacted on the food industry to produce enough protein to meet the region's nutritional requirement;
- ✓ Consequently, the cost of protein secondary use as food for livestock has also risen.

## 1.2 AIM OF STUDY

To produce single cell protein from pineapple peels using fungi isolated from pineapple.

## 1.3 RESEARCH OBJECTIVES

- ✓ To isolate fungi from pineapple, select the best fungal strain base on cellulase activity and identify the selected fungal strain
- ✓ To hydrolyze the pineapple peels using acids and alkaline and analyze or determine the carbohydrate and protein composition of the hydrolyzed pineapple extract
- ✓ To establish the fermentation and bioconversion of the pineapple substrates into single cell protein (SCP) using various glucose concentration (2%, 4%, 6%, 8%, and 10%) w/v and nitrogen sources (sodium nitrate, potassium nitrate, ammonium nitrate, sodium nitrite and ammonium oxalate) by fungi.
- ✓ To compare the effects of various glucose concentration and nitrogen sources on the growth and protein yield of fungi.

## 1.4 LITERATURE REVIEW

## 1.5 DEFINITION OF SINGLE-CELL PROTEIN (SCP)

Single-cell protein (SCP) typically refers to sources of mixed protein extracted from pure or mixed cultures of algae, yeasts, fungi or bacteria (grown on agricultural wastes) used as a substitute for protein-rich foods, in human and animal feeds (Nangul and Bhatia, 2013). It can also be defined as a protein that is produced by micro-organisms fermenting in liquid or gaseous petroleum fractions or other organic substances used as food supplement (abbreviated SCP). A variety of microorganisms and substrate are used to produce single cell proteins. Yeast is suitable for single cell protein production because of its superior nutritional quality .The supplementation of cereals with single cell proteins, especially yeast, makes them as good as animal protein (Huang and Kinsella, 1986).

The necessary factor considered for use of SCP is the demonstration of the absence of toxic and carcinogenic compounds originated from the substrates, biosynthesized by the microorganisms or formed during processing. High nucleic acid content and low cell wall digestibility are two of the most important factors limiting nutritional and toxicological value of yeast for animal or human consumption (Alvarez and Enriquez, 1998). As constituents of nucleic acid, purine compounds in human diet mostly metabolized to yield uric acid whose high concentration may lead to gout or renal stones. However, nucleic acid is not a toxic component and it causes only physiological effects at higher levels like any other essential dietary ingredients taken in larger amounts. It has been calculated that 100 lbs. of yeast will produce 250 tons of proteins in 24 hr.

Algae grown in ponds can produce 20 tons (dry weight) of protein, per acre, per year. Bacteria are usually high in protein (50 to 80%) and have a rapid growth rate. In the case of algae it has to be stressed that, due to technical and economic reasons, it is not the general intention to isolate

and utilize the sole protein, but to propagate the whole algal biomass. So, the term SCP is not quite correct, because the micro-algal material is definitely more than just protein. The annual world production of all microalgae species is estimated to about 10,000 tons year-1 (Becker, 2007).

**1.6 HISTORY OF SINGLE CELL PROTEIN**

A survey of history of microorganisms for human consumption indicates three major trends: (a) microbes as a source of enzymes in food industry (baking, brewing, distilling, wine making, cheese production) has at present a new aspect-the use of immobilized enzymes of microbial origin, (b) microbes as producers of nutritive substances (amino acids, nucleotides, vitamins, organic acids, sugars, aromatizers) and (c) direct utilization of microbial biomass as foodstuff.

Since 2500 BC, yeasts have been used in bread and beverage production. In 1781, processes for preparing highly concentrated forms of yeast were established (Marx, 2001). In 1919, *Endomyces vernalis* yielded fats from sulphite liquor (from paper manufacture), and similarly in 1941 employing *Geotrichum*. In the 1960s, researchers at British Petroleum developed what they called "proteins-from-oil process": a technology for producing single-cell protein by yeast fed by waxy n-paraffin, a product produced by oil refineries (Bamberg, 2000). The term SCP was coined in 1966 by Carol L. Wilson. The primary use of the product was as poultry and cattle feed. The Soviets were particularly enthusiastic, opening large "BVK" (*belkovo-vitaminny kontsentrat*, i.e. “protein-vitamin concentrate") plants next to their oil refineries (Shabad, 1973). The Soviet Ministry of Microbiological Industry had eight plants of this kind by 1989, when, pressured by the environmentalist movements, the government decided to close them down, or convert to some other microbiological processes (Johnson, 2002).

## 1.7 MICROORGANISMS USED AS SINGLE CELL PROTEIN

Various bacteria, mold, yeast and algae have been employed for the production of single cell proteins. The desired microorganisms are cultured on the medium under sterile condition. Organisms to be cultured must have the following properties which are: it should be non-pathogenic to plants human and animals, it must have good nutritional values, it must be usable as food and feed, should not contain toxic compounds and production cost should be low (Adedayo *et al.,* 2011). *Cyanobacterium spirulina* is an example of food supplement used as food source in Africa and sold in US health food stores as dried cake or powdered product. Probiotic microbes (primarily *Lactobacillus acidophilus)* when grown as single cell protein can be applied to decrease *E. coli* occurrence in beef cattle (Prescott, 2008; Shweta *et al.,* 2016).

- **BACTERIA**

The characteristics that made bacteria suitable for Single cell protein production include rapid growth of bacteria, short generation time and can double their cell mass in 20minutes to 2hours. They are also capable of growing on a variety of raw materials that range from carbohydrates such as starch and sugars to gaseous and liquid hydrocarbons which include methane and petroleum fractions to petrochemicals such as methanol and ethanol, nitrogen sources which are useful for bacterial growth include ammonia, ammonium salts, urea, nitrates, and the organic nitrogen in wastes, also it is suggested to add mineral nutrient supplement to the bacterial culture medium to fulfill deficiency of nutrients that may be absent in natural waters in concentration sufficient to support growth. Potential phototrophic bacterial strains are recommended for single cell protein production. Generation time of *Methylophilus* is about 2 hours is used in animal feed and in general produces a more favourable protein composition than yeast or fungi. Therefore the large quantities of single cell protein animal feed can be produced using bacteria like

*Brevibacterium* (Adedayo *et al.*,2011) *Methylophilus methylitropous*, *Acromobacter delvaevate*, *Acinetobacter calcoacenticus, Aeromonas hydrophilla, Bacillus megaterium, Bacillus subtilis* (Gomashe *et al*,2014), *Lactobacillus species, Cellulomonas spp, Methylomonas methylotrophus, Pseudomonas fluorescens, Flavobacterium spp, Thermomonospora fusca* and others (Dhanasekaran *et al.,* 2011).

- **ALGAE**

Since ancient times, *Spirulina* was cultivated by people near Lake Chad in Africa and the Aztecs near Texcoco in Mexico. They used it as a food after drying it. *Spirulina* is the most widely used algae so much that even astronauts take it to space during their space travel. Similarly, biomass obtained from *Chlorella* has been harvested and used as source of food by tribal communities in certain parts of the world. Algae are used as a food in many different ways and its advantages include simple cultivation, effective utilization of solar energy, faster growth and high protein content. The algae *Spirulina* has been considered for use as a supplementary protein (Raja *et al.,* 2008). It is a blue green algae having strong antioxidant activity and provokes a free radical scavenging enzyme system. *Spirulina maxima* prevent fatty liver development induced by carbon tetrachloride. The use of *Spirulina* should be encouraged in patients suffering from malnutrition, immune suppression, hepatic and neural compromise, etc. although further investigations on the antiviral effects of these algae and its clinical implications are strongly needed. Single cell protein (SCP) production by five strains of *Chlorella* species, isolated from different habitats, andwas studied under the influence of eight environmental factors (Mahasneh, 2005). Some other algae used are *Chondrus crispus, Scenedesmus acutus,* and *Porphyrium sp* (Marx, 2001).

- **YEAST**

Yeast single-cell protein (SCP) is a high nutrient feed substitute (Burgents *et al.*, 2004). Among these, most popular are yeast species *Candida*, *Hansenula, Pitchia, Torulopsis* and *Saccharomyces*. The production of single cell protein using *Saccharomyces cerevisiae* has been grown on various fruit waste (Tanveer, 2010). Cucumber and orange peels were evaluated for the production of single cell protein using *Saccharomyces cerevisiae* by submerged fermentation (Sengupta *et al.,* 2006). The typical oily yeasts genera include *Yarrowia, Candida, Rhodotorula, Rhodosporidium, Cryptococcus, Trichosporon, Lipomyces* and Yeasts such as *Candida utilis* (Torula yeast), *Candida lipolytica, Candida tropicalis, Candida novellas* and *Candida intermedia* are all among the various yeast that have been used for the production of SCP (Bhalla *et al*., 2007).

- **FUNGI**

Many fungal species are used as a source of protein rich food (Bhalla *et al.*, 2007). Many other filamentous species are also used as source of single cell protein. In 1973, in second international conference convened at MIT, it was reported that *Actinomycetes* and filamentous fungi produced protein from various substrates. During the World War II, trials were made to utilize the cultures of *Fusarium* and *Rhizopus* (Yousuf, 2012) grown in fermentation as a source of protein food. The inoculums of *Aspergillus oryzae* (Oshoma, 2005*)* or *Rhizopus arrhizus* were selected because of their nontoxic nature. Saprophytic fungi grow on complex organic compounds and convert them into simple structures. High amount of fungal biomass is produced as a result of growth. Mycelia yield vary greatly which depends upon organisms and substrates. There are some species of molds, for example, *Aspergillus niger* (Yabaya and Ado, 2008)*, A. fumigates*, *Fusarium graminearum* which are very dangerous to human, therefore, such fungi, must not be

used or toxicological evaluations should be done before recommending to use as SCP. Very recently, SCP technology is using fungal species for bioconversion of lignocellulosic wastes (Lenihan *et al.*, 2010). Other filamentous fungi that have been used include *Chaetomium celluloliticum*, *Cephalosporium cichhorniae, Penicillium cyclopium, Rhizopus chinensis*, *Scytalidium aciduphlium, Trichoderma viride, Trichoderma alba* and *Paecilomyces varioti* (Bhalla *et al.*, 2007).

**1.8 CHOICE OF FUNGI IN THE PRODUCTION OF SINGLE CELL PROTEIN**

The increasing popularity of fungi used in the production of single cell protein is because of the following reasons:-

- Fungi grow as fast as most of the single celled organism;
- The finished product of fungi is fibrous in nature and can be easily converted into various textured foods. In comparison, protein is extracted from other single celled organisms and spun into fibrous form;
- Filamentous fungi have a greater retention time in the digestive system than other single celled organisms;
- Protein content can be as high as 35-50 per cent with comparatively less nucleic acid than single celled organism;
- Digestibility and net protein utilization without any pretreatment is higher than other single celled organisms;
- The overall cost of protein production from filamentous fungi is more economical as compared to that of other single celled organism;
- Filamentous fungi have greater penetrating power into insoluble substrates and are therefore, more suitable for solid state fermentation of lignocellulosic materials;

- Most filamentous fungi have a faint mushroom-like odor and taste which may be more readily acceptable as a new source of food than the yeast odor and green color associated with yeasts and algae respectively;
- The biomass produced by filamentous fungi can be used as such without any further processing because it provides carbohydrates, lipids, minerals, vitamins and proteins. In addition, a nucleic acid content of fungal protein is lower than that of yeast and bacteria (Kavanagh, 2005).

## 1.9 NUTRITIONAL COMPOSITION OF FUNGI USED IN SCP PRODUCTION

Average nutritional compositions (% dry weight) of fungi are;

Protein (30-45%), Fat (4-8 %), Carbohydrate (2-5%), Ash (9-14%) and Nucleic acid (7-10%). The remaining percentage includes a wide variety of minerals and vitamins, particularly zinc and vitamin B 12 (Adedayo *et al*., 2011).

## 1.10 GROWTH CHARACTERISTICS OF FUNGI IN RELATION TO SCP PRODUCTION

The following factors affect the growth of fungi in single cell protein production:

- Carbon, nitrogen (C:N) ratio is required to be in the range of 5:1 to 15:1;
- Ammonium salts are used as a source of nitrogen in continuous culture and phospheric acid for phosphorus. Moreover, most fungi, for their growth require minerals, such as potassium, sulphur, magnesium, calcium, iron, manganese, zinc, copper and cobalt. Their concentration differs with respect to species;
- pH of growth medium ranges from 3.0 to 7.0 but pH 5-6 or below is desirable for most of fungi because bacterial contaminants do not grow;
- Temperature ranges from 25°C to 30°C with certain exceptions;

- Oxygen is required for good growth of fungi. During agitation, mycelial mat forms pellets.

**1.11 SAFETY OF FUNGI AS SCP**

As for any food or feed product, SCP needs to be safe to produce and use. Regulations exist in most regions to ensure that food or feed are safe for consumption (Bagchi, 2006). Regulations differ depending on the intended purpose of the product, and although SCP is expected to be either food or feed, some products may enter the market as additives (e.g., providing colour), rather than as SCP, even though protein is present in the product, limiting the extent to which they are added and their value as SCP. Authorization is required before sale of new feed or additives (Smedley, 2013). Key concerns are the RNA content, toxins produced by microbes (production hosts or contaminants), potential allergy symptoms, and harmful substances. Methods have been developed and are in industrial use to decrease the RNA content to acceptable levels. The challenge of toxins is overcome by carefully selecting the production organism, the process conditions, and the product formulation. Some fungi produce mycotoxins and this make them undesirable sources of SCP (Anupama and Ravindra, 2000). QuornTM mycoprotein underwent extensive testing for the presence of mycotoxins or other toxic compounds before being approved for human consumption (Wiebe, 2004). The particular strain of *Fusarium venenatum* does not produce mycotoxins under production conditions, but the process is still monitored to ensure none are present. Another fungus whose safety has been extensively assessed is *Yarrowia lipolytica*, demonstrating that it would be safe to use in a variety of food applications, including as SCP (Groenewald *et al*., 2014). Any product for human consumption which would be produced from biomass hydrolysates or waste streams would need to provide an equivalent safety record before finding approval. In addition to the safety

requirements associated with production of SCP, public perception and acceptance of waste-derived foods would be a key element to consider when implementing SCPs in human diets.

## 1.12 COMPOSITION OF THE MAIN GROUPS OF MICROORGANISMS

Four types of microorganisms are used to produce biomass: bacteria, yeasts, fungi and algae. The choice of a microorganism depends on numerous criteria, the most important of which is the nature of the raw material available. The other criteria are:

– Nutritional: energy value, protein content, amino acid balance;

– Technological: type of culture, nutritional requirements, type of separation;

– Toxicological.

The ideal microorganism should possess the following technological characteristics:

– High specific growth rate and biomass yield;

– High affinity for the substrate;

– Low nutritional requirements, i.e., few indispensable growth factors;

– Ability to use complex substrates;

– Ability to develop high cell density;

– Stability during multiplication;

– Capacity for genetic modification;

– Good tolerance to temperature and pH.

In addition, it must have a low nucleic acid content, good digestibility and be non-toxic (Nasseri *et al*., 2011). It should have a balanced carbohydrate, protein, lipid, nitrogen composition (Lichtfield, 1983).

## 1.13 POTENTIAL SUBSTRATES FOR SCP PRODUCTION

The choice of substrates that are normally abundant and in proximity to the production plant has determined the design and strategy of SCP processes. The most widespread and commonly used substrates for SCP production have been those where the carbon and energy source is derived from carbohydrates. This is due to the fact that their building blocks (mono and disaccharides) are natural microbial substrates, and that carbohydrates are a renewable resource which is widely distributed. Many raw materials have been considered as substrate for SCP production (Nasseri *et al.*, 2011). Conventional substrates such as starch, molasses, fruit and vegetable wastes have been used for SCP production, as well as unconventional ones such as petroleum by-products, natural gas, ethanol, methanol and lignocellulosic biomass (Bekatorou *et al.*, 2006). Molasses, the residual liquid obtained after crystallization of sugar from the concentrated sugar solution obtained from the milling of sugar cane or sugar beet, contains 45- 55 % sugars, namely sucrose, glucose, fructose, raffinos and galactose. It is estimated that for every 100kg of cane milled for sugar production, some 3.5 to 4.5 kg of molasses is obtained (Oura, 1983). The use of molasses for the production of SCP is determined by its availability and low cost, its composition and absence of toxic substances and fermentation inhibitors (Bekatorou *et al.*, 2006). The fact that molasses may be extracted from at least two sources of plant adapted to tropical and temperate climates permits the obtainment of molasses in a wide range of geographical locations. Besides its high sugar content, molasses contains minerals, organic compounds and vitamins which are valuable nutrients in fermentation processes. In fact, about 9% of the dry matter in yeast grown on molasses has been estimated to originate from substances other than sucrose (Olbrick, 1973). Nevertheless, biomass production from molasses requires supplementation with a suitable nitrogen source, the traditional nitrogen sources used are ammonia or ammonium salts, and

phosphorus can be added in the form of salts (Ugalde and Castrillo, 2002). Baker´s yeast was the first microorganism to be produced in aerobic stirred fermentation on molasses as it is still produced today (Chen and Chinger., 1985). However, this yeast has seldom been destined as food, but rather for baking purposes. A cheaper, more amenable SCP substrate of carbohydrate origin is starch. This very abundant carbohydrate may be obtained from bulb plants of tropical and temperate regions, or from rice, maize and cereals. In tropical countries, cassava has been proposed as a good source of starch for SCP production (Zhao and Zhang, 2009).

Single Cell Protein can be produced by a number of different substrates, often this is done to reduce biological oxygen demand of the effluent streams leaving various types of agricultural processing plants. The two main strategies with regard to substrate were to consider low grade waste material and very high quality of protein in it. The nature of the raw materials supplying substrates is very crucial for SCP production. The cost of raw material significantly influences the final cost of SCP. The most commonly used raw materials may be grouped in the following categories:

1. Carbohydrates.

2. Waste products e.g. molasses, whey, sewage, animal manures, straw.

3. Agricultural and forestry sources e.g. cellulose, lignin.

4. Carbon dioxide, the simplest carbon source.

**1.14 SINGLE CELL PROTEIN PRODUCTION**

The worldwide, large-scale development of SCP processes has contributed greatly to the advancement of present day biotechnology. Research and development of SCP processes has

involved work in the fields of microbiology, biochemistry, genetics, chemical and process engineering, food technology, agriculture, animal nutrition, ecology, toxicology, medicine and veterinary science and economics. In developing SCP processes new technical solutions for other related technologies in waste water treatment, production of alcohol, enzyme technology and nutritional science also improves. The future of SCP will be heavily dependent on reducing production costs and improving quality by fermentation, downstream processing and improvement in the producer organisms as a result of conventional applied genetics together with recombinant DNA technologies (Omar and Sabry, 1991). The production of single cell protein takes place in a fermentation process. The fermentation process requires a pure culture of the chosen organism that is in the correct physiological state, sterilization of the growth medium which is used for the organism, a production fermenter which is the equipment used for drawing the culture medium in the steady state, cell separation, collection of cell free supernatant, product purification and effluent treatment. Then the technical conditions of cultivation for the optimized strains are done and all metabolic pathways and cell structures are determined. Besides, process engineering and apparatus technology adapt the technical performance of the process in order to make the production ready for use on the large technical scale. Here is where the economic factors (energy, cost) come into play. Safety demands and environmental protection is also considered in the production of SCP in relation both to the process and to the product. Finally, safety and the protection of innovation throw up legal and controlled aspects, namely operating licenses, product authorizations for particular applications and the legal protection of new process and strains of microorganisms (Steinkraus, 1986). A fermenter is the instrument, which is set up to carry out the process of fermentation mainly the mass culture of plant or animal cells. Fermenters can vary in size from laboratory experimental models of one or two litres capacity, to

industrial models of several hundred litres capacity. A bioreactor is different from a fermenter as it used for the mass culture of microorganisms. The chemical compounds synthesized by these cultured cells such as therapeutic agents can be extracted easily from the cell biomass. The design engineering and operational parameters of both fermenters and bioreactors are identical. Fermenters and bioreactors are also equipped with an aerator, which supplies oxygen to aerobic processes also a stirrer is used to keep the concentration of the medium the same. A thermostat is used to regulate temperature and a pH detector and some other control devices, which keep all the different parameters needed for growth constant (Ferrianti and Fiechter, 1983; Sinclair and Cantero, 1990). Producing and harvesting of microbial proteins cost is a major problem. Such a production even in high rate causes dilute solutions usually less than 5% solids. There are many methods available for concentrating the solutions like filtration, precipitation, centrifugation and the use of semi-permeable membranes. The equipment used for these methods of de-watering is expensive and so would not be suitable for small scale productions and operations. The removal of the amount of water that is necessary to make the material stable for mass storage is not economically viable. Single cell proteins need to be dried to 10% moisture or they can be condensed and denatured to prevent spoilage (Sinclair and Cantero, 1990). Batch fermentations are clearly inadequate for the purpose of biomass production, since the conditions in the reaction medium change with time. Fed-batch fermentations are better suited for the purpose of biomass production, since they involve the control of the carbon source supply through feeding rates. However, as the biomass concentration increases, the oxygen demand of the culture reaches a level which cannot be met in engineering or economic terms. Fed-batch culture is still in use for baker's yeast production using well established and proven models. However, they have not been favoured for the production of SCP at a large industrial scale. Prolonging a microbial culture by

continuous addition of fresh medium with the simultaneous harvesting of product has been implemented successfully in industrial fermentations destined to biomass production. The most commonly used principle has been the chemostat: a perfectly mixed suspension of biomass into which medium is fed at a constant rate and the culture is harvested at the same rate so that the culture volume remains constant. The technical implications of chemostat culture are various and extremely relevant (Cooney, 1986). Production periods as long as six weeks have been implemented in many fungal and yeast (Forage and Righelato, 1979). A common problem of industrial fermentations is the profuse appearance of foam on the head space of the reactor, causing reactor pressurization, spillages and contamination hazard. Among the various designs which have been put to effect, the deep-jet fermenter and the air-lift fermenter have been the most successfully applied (Stanbury *et al*., 2000). The biomass from yeast fermentation processes is harvested normally by continuous centrifugation. Filamentous fungi are harvested by filtration .The biomass is then treated for RNA reduction and dried in steam drums of spray driers. Drying is expensive, but results in stabilized product with shelf lives of years. Solid State Fermentation (SSF) is growth of microorganisms on predominantly insoluble substrate where there is no free liquid. Generally, under combined conditions of low water activity and presence of intractable solid substrate, fungi show luxuriant growth. Hence, proper growth of fungi in SSF gives much higher concentration of the biomass and higher yield when compared to submerged fermentation. The advantage in SSF process is the unique possibility of efficient utilization of waste as the substrate to produce commercially viable products. The process does not need elaborate prearrangements for media preparation. The process of SSF initially concentrated on enzyme production. But presently, there is worldwide interest for SCP production due to the dwindling conventional food resources (Zadrazil and Puniya, 1995).

### 1.14.1 CULTIVATION METHODS OF SCP (FERMENTATION PROCESS)

The production of single cell protein takes place in a fermentation process (Chandrani-Wijeyaratne and Tayathilake, 2000). Process development begins with microbial screening, in which suitable production strains are obtained from samples of soil, water, air or from swabs of inorganic or biological materials and are subsequently optimized by selection, mutation, or other genetic methods. Single cell protein can be produced by fermentation processes, namely:

**SUBMERGED FERMENTATION**

In submerged process (Varavinit *et al.*, 1996), the substrate used for fermentation is always in liquid state which contains the nutrients needed for growth. The fermentor which contains the substrate is operated continuously and the product biomass is continuously harvested from the fermentor by using different techniques then the product is filtered or centrifuged and then dried. Aeration is an important operation in the cultivation, heat is generated during cultivation and it is removed by using a cooling device. The microbial biomass can be harvested by various methods (Kargi *et al.,* 2005).Filtration, precipitation, centrifugation and the use of semi-permeable membranes. Single cell organisms like yeast and bacteria are recovered by centrifugation while filamentous fungi are recovered by filtration. It is important to recover as much water as possible prior to final drying done under clean and hygienic conditions. Microorganism involved in this type of fermentation is *Saccharomyces cerevisiae* (Sengupta *et al.*, 2006).

**SEMISOLID FERMENTATION**

In semisolid fermentation (Adedayo *et al.,* 2011), the preparation of the substrate is not cleared and it is also more used in solid state e.g. cassava waste. Submerged culture fermentations require more capital investment and have high operating cost. The cultivation involves many operations which include stirring and mixing of a multiphase system, transport of oxygen from

the gas bubbles through the liquid phase to the microorganisms and the process of heat transfers from liquid phase to the surroundings (Anderson and Jorgensen, 2005). A special bioreactor is designed for identifying mass and energy transportation phenomena, called U-loop fermentor (Jorgensen, 2010). Production of single cell protein involves basic steps of preparation of suitable medium with suitable carbon source, prevention of the contamination of medium and the fermentor, production of microorganisms with desired properties and separation of synthesized biomass and its processing (Soland, 2005). Carbon source used can be n-alkenes, gaseous hydrocarbons, methanol and ethanol, renewable sources like carbon oxide molasses, polysaccharides and effluents of breweries (Talebnia, 2008).

**SOLID STATE FERMENTATION**

Solid state fermentation (SSF) has been extensively studied with thousands of publications describing various types of bioreactor designs, process conditions and microorganisms for the production of various value added products like SCP, feeds, enzymes, ethanol, organic acids, B-complex vitamins, pigments, flavours, (Singhania *et al.*,2009). This process consists of depositing a solid culture substrate, such as rice or wheat bran, on flatbeds after seeding it with microorganisms; the substrate is then left in a temperature-controlled room for several days. Liquid state fermentation is performed in tanks, which can reach 1,001to 2,500 square metres (10,770 to 26,910 sq. ft.) at an industrial scale. Liquid culture is ideal for the growing of unicellular organisms such as bacteria or yeasts. To achieve liquid aerobic fermentation, it is necessary to constantly supply the microorganism with oxygen, which is generally done via stirring the fermentation media. Accurately managing the synthesis of the desired metabolites requires regulating temperature, soluble oxygen, ionic strength and pH and control nutrients. Microorganism involved in this type of fermentation is *Asperigillus niger.*

### 1.14.2 FACTORS AFFECTING SINGLE CELL PROTEIN PRODUCTION INCLUDE:

- **AERATION REQUIREMENT AND ENERGY COST**

In order to achieve a suitable yield of single cell product (except from algae), a sufficient aeration with filtered air has to be applied. The running costs are 20% of the total production costs of SCP. Aeration costs contribute 60% of running costs. Therefore, aeration costs can be as much as 12% of the production costs of SCP. Power cost for aeration, fluid handling, and steam for cleaning, recovery, and drying the product are significant factors in the total energy costs (Lewis, 1976).

- **GROWTH RATE AND BIOMASS**

High growth rate is needed for high output (weight of biomass produced per unit of time). However, this will give also disadvantage of high RNA content in cell because nucleic acid content is proportional to the growth rate. The biomass yield coefficient is weight of cells produced per unit of substrate consumed. High yield coefficient will be a target to give high output in order to utilize as much as possible of substrate (especially if the substrate is expensive). The slower the growth rate, which means larger proportion of the total substrate consumed for maintenance, gives as a result less available substrate for biomass synthesis; consequently, the substrate yield coefficient is diminished.

- **STRESS TOLERANCE TO BROAD RANGE OF pH**

A pH of a medium tends to change during fermentation. Most often media are buffered, and the fermenter is fitted with pH control. Fungi, for example, grow at lower pH than bacteria. Therefore, cultivation can be at very low pH to prevent bacterial growth. That means than an

aseptic process will be less prone to contamination if aseptic procedures fail. *Saccharomyces cerevisiae*, a model organism in biology, grows better at acidic than at neutral or alkaline pH. Maintenance of an acidic environment is based primarily on the active proton extrusion mediated by its plasma membrane H+-ATPase, and this proton gradient critical for the uptake of different nutrients, including diverse cations. Therefore, sudden alkalinization of the environment represents a stress condition for this yeast, and to survive, *S. cerevisiae* must detect the change and react to it, triggering an adaptive response. The response of budding yeast to alkalinization of the environment has been characterized in some detail in the last few years, in many cases through the study of mutants sensitive to high pH conditions and/or the definition of the transcriptional adjustments after exposure to alkaline pH (Serrano, 2006).

- **MUTATION RATE**

Mutant is a strain which exhibits inheritably changed characteristic when a microbial cell divides, while a mutation is the process giving rise to it. Metabolism of an organism is controlled by its genome. Microbial products can be quantitatively enhanced by improving fermenter design and optimal culture conditions. But that needs genetic stability which has made genetic improvement of the organism fundamental in fermentation technology. The probability of a mutation occurring may be increased by exposing the culture to a mutagenic agent such as UV light, ionizing radiation, and various chemicals. Such an exposure usually involves subjecting the population to a mutagen dose which results in the death of the vast majority of the cells. The survivors of the mutagen exposure may then contain some mutants, the vast majority of which will produce lower levels of the desired product. Organisms for SCP

production require a high degree of genetic stability. In continuous process, which is often used for SCP production, the long growth period of several weeks can enhance the mutation.

### 1.14.3 PRODUCT SAFETY AND QUALITY

Some contaminants can produce mycotoxins. Some bacterial SCP has amino acid profiles different from animal proteins. Yeast and fungal proteins tend to be deficient in methionine. Microbial biomass has a high nucleic acid content, and levels must be limited in the diets of monogastric animals to <50 g per day. Ingestion of purine compounds arising from RNA breakdown leads to increased plasma levels of uric acid, which can cause gout and kidney stones. Uric acid can be converted to allantoin, which is excreted in urine. Nucleic acid removal is not necessary from animal feeds but is from human foods. A temperature hold at 64°C inactivates fungal proteases and allows RNases to hydrolyze RNA with release of nucleotides from cell to culture broth (Wikipedia 2012).

## 1.15 SINGLE CELL PROTEIN PROCESSING FOR FOOD

The effective use of microbial protein for human food requires some methods as discussed below:

### 1.15.1 METHODS OF CELL WALL DISRUPTION

The use of microorganism for refined SCP requires not only an adequate amount of specific organism but also an efficient means of disrupting the cell wall (Baldwin and Robinson, 1994). Mechanical integration of cell wall can be carried out either by crushing, crumbling, grinding, pressure homogenization or ultra sonification. Various enzymes or combination of enzymes can be used to digest and disrupt cell wall, either partially or completely. Enzymatic hydrolysis of cell wall is attractive in terms of its delicacy and specificity for only the cell wall structure. It may be used as an alternative to the mechanical disruption, especially for materials that can be

inactivated during the mechanical process and it can be performed by endogenous or exogenous enzyme from other microorganisms. However, extensive enzymatic lysis of cells is a very slow process compared to mechanical disruptions. It is possible to use two or more methods for cell disruptions. Combined mechanical and enzymatic degradation of yeast cell wall was tested (Asenjo and Dunnill 2001). In case of yeast cells they can first be mechanically broken and then incubated with a lytic enzyme (Damodaran and Kinsella, 1995). This resulted in the release of a substantial amount of protein mostly from organelles and cell walls. Baldwin and Robinson (1994) reported enhanced disruption of *Candida utilis* by enzymatic pretreatment and high pressure homogenization. Other methods employed for yeast cell breakage include: autolysis followed by enzymatic or alkali treatments (Benaiges *et al*., 1989), NaCl induced autolysis at different temperature, chemical disintegration using detergent such as sodium dodecyl -sulfate or Triton X-100, acid or solvent (Lee, 1996). The digestibility of yeast and microalgae can be greatly increased by drying at high temperature under certain conditions. However, the heat treatment needed to increase the digestibility of the cells also affects the protein quality and other valuable cell components (Hedenskog and Morgen, 1973).

### 1.15.2 REMOVAL OF NUCLEIC ACIDS

Several methods have been proposed to reduce nucleic acid levels in SCP. These methods involve chemical and enzymatic treatments. Each has disadvantages both in terms of cost and potential nutritional concern. In 1977, the extraction of nucleic acid by acidified alcohol, salt, acid and alkalis has been proposed. Alkaline extraction of microbial biomass at elevated temperature was also used in 1970. The process resulted in high protein yield with low nucleic acid. However, alkaline hydrolysis of nucleic acid at high temperature causes the formation of potentially toxic compounds such as lysinoalanine. It is an unusual amino acid involved in

crosslinking of alkaline protein. Lysinoalanine has been shown to reduce digestion and induce kidneys changes in rats (Damodaran and Kiinsella, 1995). It was also implicated in skin allergy in some persons consuming treated protein (Scrimshaw and Dillen, 1977). Chemical modification of yeast nucleoproteins with anhydrides has been used to reduce the nucleic acid levels. Yeast contains considerable amounts of endogenous ribonulease activity that is used to hydrolyze yeast RNA and that causes reduction of nucleic acid level in yeast protein. At the optimum conditions of ribonuclease activity, significant activation of endogenous protease also occurs. This results in proteolytic degradation of protein and thereby, decreases the yield of protein. Alternatively, nuclease has been added exogenously to reduce the nucleic acid content of SCP. Pancreatic ribonuclease (RNase A) and a fungal ribonuclease of *Aspergillus candidus* strain M16 has been used as the source of exogenous nuclease for the reduction of nucleic acid in the cells of yeast species allowing a substantial reduction of NA (Maul *et al*., 1970; Kunhi and Rao, 1995). Bacterial or pancreatic nucleases have also been studied for NA removal from yeast cells. Hydrolysis of NA has also been performed by using immobilized enzymes (Parajo *et al*., 1995).

**1.16 ACCEPTABILITY AND TOXICOLOGY OF SINGLE CELL PROTEIN**

The name of the raw materials used in SCP processes represents the main safety hazard. Toxicology testing of the final product must include short-term acute toxicity testing with several different laboratory animal species, followed by extensive and detailed long term studies. It represents a major scientific and financial investment. The acceptability of SCP when presented as a human food does not depend only on its safety and nutritional value. In addition to the general reluctance of people to consume material derived from microbes, the eating of food has many subtle psychological, sociological and religious implications (Nasseri *et al.*, 2011).

## 1.17 NUTRITIONAL VALUE OF SINGLE CELL PROTEINS

For the assessment of the nutritional value of SCP, factors such as nutrient composition, amino acid profile, vitamin and nucleic acid content as well as palatability, allergies and gastrointestinal effects should be taken into consideration. Also long term feeding trials should be undertaken for toxicological effects and carcinogenesis (Burgent *et al*., 2004). Nutritive and food values of SCP vary with the microorganisms used. The method of harvesting, drying and processing has an effect on the nutritive value of the finished product. Single cell protein basically comprises proteins, fats, carbohydrates, ash ingredients, water, and other elements such as phosphorus and potassium. The composition depends upon the organism and the substrate upon which it grows. Proteins not only provide a nutritional component in a food system but also perform a number of other functions (Mahajan and Dua, 1995). Some typical compositions are compared with soy meal and fish meal. It has high protein and low fat content and it is a good source of vitamins particularly B-complex, with good amino acid composition and it is furnished with thiamine, riboflavin, glutathione, folic acid and other amino acids but less in sulphur containing amino acids. SCP from yeast and fungi has up to about 50 – 55 **%** protein and it has high protein – carbohydrate ratio than forages. It is rich in lysine but poor in methionine and cysteine. It has also been noted for having good balance of amino acids and rich in B –complex vitamins and more suitable as poultry feed. SCP produced from bacteria has more than 80% protein although they are poor in sulphur containing amino acids and it has high nucleic acid content (Adedayo *et al.*, 2011). Yeast single-cell proteins (SCPs) are playing a greater role in the evolution of aquaculture diets. With excellent nutrient profiles and capacity to be mass produced economically, SCPs have been added to aquaculture diets as partial replacement for fishmeal (Olvera-Novoa *et al.*, 2002) and for HUFA-fortification of rotifer and *Artemia* (Zubi *et al*.,

2005). Some yeast strains with probiotic properties, such as *Saccharomyces cerevisiae* and *Debaryomyces hansenii*, boost larval survival either by colonizing the gut of fish larvae, thus triggering the early maturation of the pancreas, or via the immunostimulating glucans derived from the yeast cell wall (Campa-Cordova *et al*., 2002, Burgents *et al.*, 2004). However, many of these yeast supplements are deficient in sulfated amino acids, particularly methionine (Muzinic *et al.,* 2006) which restricts their extensive use as the sole protein source.

Aside from the nutritional values of SCP, it also has the benefits of the possibility of its production through-out the year since it is independent of seasonal as well as climatic conditions, (Ndihi, 2010). Waste materials are used as substrate for the production of these proteins therefore it reduces the environmental pollution and helps in recycling of materials. SCP organisms grow faster and produce large quantities of protein from relatively small area of land and time. These has proteins with required amino acids that can be easily selected by genetic engineering and finally during the production of SCP biomass, some organisms produce useful by-products such as organic acids and fats.

An accurate method to evaluate the quality of proteins is the determination of the Protein Efficiency Ratio (PER), expressed in terms of weight gain per unit of protein consumed by the test animal in short-term feeding trials. Estimation of the Biological Value (BV) is a measure of nitrogen retained for growth or maintenance. Another parameter, which reflects the quality of a protein, is the Digestibility Coefficient (DC). Finally, the Net Protein Utilization (NPU) equivalent to the calculation BV × DC is a measure of the digestibility of the protein and the biological value of the **amino acids** absorbed from the food. The SCP generally values in terms of Kjeldhal nitrogen × 6.25 (standard factor relating amino nitrogen to protein content).

However, about 10-15% of the total nitrogen in fungi and yeasts is in the form of **nucleic acids** (Mahajan *et al.*, 2010).

## 1.18 SINGLE CELL PROTEIN CONSUMPTION

SCP is normally considered as a source of protein. However, like any other biological material, it also contains nucleic acids, carbohydrate cell wall material, lipids, minerals and vitamins. Nevertheless, these contributions are given little importance by nutritionists, who generally value SCP in terms of Kjieldhal nitrogen x 6.25 (standard factor relating amino nitrogen to protein content). However, about 10-15 % of the total nitrogen in fungi and yeasts is in the form of nucleic acids. These are not metabolized in the same way as proteins but follow a different route. Amino N, represents approximately 80% of total microbial nitrogen, and is composed of all essential amino acids required for human growth and nutrition (Riviere, *et al*., 1977). With respect to egg albumin, which is considered a well-balanced source of essential amino acids for human nutrition, fungal SCP compares well, except that it is deficient in sulfur containing amino acids. However, they are relatively rich in lysine and threonine with respect to other traditional protein sources of agricultural origin, such as wheat (Riviere, 1977).

## 1.19 COMMERCIALLY AVAILABLE SINGLE-CELL PROTEINS

Some microorganisms are very important commercially these include;

***Spirulina–*** *Spirulina* is popular SCPs with 60-72% protein and is also a rich source of vitamin, amino acid, minerals, crude fiber etc. It is commonly used in supplemented diet for undernourished children. It is a part of diet for sportsmen and is used in baby foods as well. *Spirulina* is recommended for diabetes patients to control blood sugar level; it helps in maintaining healthy eyes and skin and is beneficial for lactating mothers. Being rich source of vitamin A and B it plays a key role in cosmetic products.

***Chlorella*** – Another commonly used SCP is *Chlorella*, single cell green algae. It contains around 45% protein, 20% fat, 20% carbohydrate and 10% minerals along with fibers and vitamins. It is promoted as a super food and is routinely used to provide health benefits. Apart from being a food supplement it is also used as an alternative medicine in several cases.

***Fusarium***– Fungi has been used since ancient times either as source of food or for its medicinal value. *Fusarium* is one such fungus with varied application. Quorn is the commercially available protein which is marketed in several supermarkets across Europe and North America as a high-protein, low-fat, and cholesterol free product. It contains traces of vitamin B and high dietary fiber and is used to control body mass and lower risk of heart disease.

Proteins extracted from yeast, fungi and bacteria are also available in market. They differ in protein and vitamin content and their applications may depend on the nutritive values as well as presence of certain amino acid.

**1.20 ECONOMIC IMPORTANCE OF SCP**

It has been calculated that 100 lbs of yeast will produce 250 tons of proteins in 24 hours. Algae grown in ponds can produce 20 tons (dry weight) of protein, per acre, per year. Bacteria are usually high in protein (50 to 80%) and have a rapid growth rate. Yeasts have advantages such as their larger size (easier to harvest), lower nucleic add content, high lysine content and ability to grow at acidic pH. However, the most important advantage is familiarity and acceptability because of the long history of its use in traditional fermentations. Disadvantages include lower growth rates, lower protein content (45 to 65%) and lower methionine content than in bacteria. Filamentous fungi have advantages in ease of harvesting, but have their limitations in lower growth rates, lower protein content and acceptability. Algae have disadvantages of having cellulosic cell walls which are not digested by human beings. Secondly, they also concentrate

heavy metals. In the case of algae it has to be stressed that, due to technical and economic reasons, it is not the general intention to isolate and utilize the sole protein, but to propagate the whole algal biomass. So, the term SCP is not quite correct, because the micro-algal material is definitely more than just protein. The annual world production of all microalgae species is estimated to about 10,000 tons year-1 (Richmond, 2004; Becker, 2007). More than 75% of the annual micro algal biomass production is used for the manufacture of powders, tablets, capsules, or pastilles as the predominant product in microalgal biotechnology (Radmer, 1996).

## 1.21 USES OF SINGLE CELL PROTEIN

### Ruminants

Single cell protein seems to be utilized better by ruminant animals. Feeding single cell protein to calves did not affect rate of gain, feed or dressing percentage. Up to 20 % of single cell protein in the diet had no detrimental effect on performance in calves. Single cell protein was demonstrated to be a viable supplemental protein source for sheep. It was found that up to 75 % of the total dietary protein can be provided by single cell protein while maintaining normal performance in lambs. Single cell protein was a suitable supplemental protein source for lactating dairy goats. Milk production and milk production efficiency was increased when single cell protein replaced groundnut meal in lactating goat diets.

### Pigs

Single cell protein was found to be able to replace up to 55 % of the fish meal and soybean meal in swine diets, while still maintaining satisfactory performance.

### Poultry

Similar performance occurred when single cell protein was fed to layers. When single cell protein was fed to layers no depression in egg production was observed and the nucleic acid

content in tissues and eggs were not affected. As the dietary level of single cell protein increases in broiler diets the gain, feed conversion, and feed intake decreased.

**Fish**

Single cell protein was found to be able to replace up to 40 % of fish meal in tilapia diets without affecting performance.

**Crustaceans**

When single cell protein was included in prawn diets there was an increase in gains and feed conversion. Increase levels of single cell protein resulted in increased uric acid in serum and urine, because of the nucleic acid content.

**Food**

Single cell protein can also be applied in food stuff areas which include: Vitamin carriers, Aroma carriers, Emulsifying acids, they also improve the nutritive value of baked products, soups, diet recipes and ready-to-serve meals.

**Cosmetics**

Single cell protein can be helpful in this area; Bio-lipsticks and herbal face cream production and they are also capable of replacing local dye based cosmetics.

**Waste to wealth**

Waste utilization in fruits and vegetable processing industries is one of the important and challengeable jobs around the world. It is anticipated that the discarded fruits as well as its waste materials could be utilized for further industrial purposes *viz*. fermentation, extraction of bioactive components, extraction of functional ingredients etc. Pertinent scientific and technological implications would produce better and more profitable markets for pineapple wastes and other fruit wastes.

## 1.22 PINEAPPLE PEEL WASTE

Pineapple (*Ananas comosus*) is one of the most important fruits in the world and is the leading edible member of the family *Bromeliaceae*. This fruit juice is the third most preferred worldwide after orange and apple juices (Cabrera *et al*., 2000). The plant can grow up to a height of 75-150 cm with a spread of 90-120 cm. It is short, having a stout stump with narrow, fibrous and spiny leaves. The plant develops to a cone-shaped juicy and fleshy fruit with crown at the top (Morton, 1987; Tran, 2006). According to FAO online data base, the area under pineapple plantation in 2007 was almost 920,349 ha with an estimated production of more than 18 million tons (FAO, 2007). Commercially, it is mainly produced as canned fruits and consumed worldwide (Tran, 2006). Besides, it is also processed as juices, concentrates, and jams. Pineapple slices have also been preserved after freezing (Larrauri *et al*., 1997). Furthermore, bromelain, the proteolytic enzyme present in the stem of pineapple, is finding wide applications in pharmaceutical and food uses (Hebbar *et al.*, 2008).

### Pineapple wastes

Tropical and subtropical fruits processing have considerably higher ratios of by-products than the temperate fruits (Schieber *et al*., 2001). Pineapple by-products are not exceptions and they consist basically of the residual pulp, peels, stem and leaves. The increasing production of pineapple processed items, results in massive waste generations. This is mainly due to selection and elimination of components unsuitable for human consumption. Besides, rough handling of fruits and exposure to adverse environmental conditions during transportation and storage can cause up to 55% of product waste (Nunes *et al*., 2009). These wastes are usually prone to microbial spoilage thus limiting further exploitation. Further, the drying, storage and shipment of these wastes is cost effective and hence efficient, inexpensive and eco-friendly utilization is

becoming more and more necessary. Compositional analysis of pineapple wastes have been carried out (Ban-Koffi and Han 1990; Bardiya *et al*., 1996; Rani and Nand, 2004).

Except for high quality fruits that are selected for shipment, most pineapples are consumed fresh or as canned products. However, low quality fruits do not fetch market and are left on farms. Besides, during pineapple processing, large amount of unusable waste material are generated (Tanaka *et al*., 1999). Reports have shown that 40-80% of pineapple fruit is discarded as waste having high biological oxygen demand (BOD) and chemical oxygen demand (COD) values (Ban-Koffi and Han, 1990).

**Physical and chemical constituents of pineapple waste**

Moisture 91.35%; Ash content 0.04 mg/100g; Total soluble solids 10.2%; Crude fibre 0.60 g/100g-fw; Total sugars 9.75%; Reducing sugars 8.2%; Non-reducing sugars 8.8%; Titratable acidity 1.86%; Ascorbic acid 26.5 mg/100g. (Hemalatha and Anbuselvi, 2013)

**Disposal**

Fruit residues may cause serious environmental problems, since it accumulates in agro-industrial yards without having any significant and commercial value. Since disposal of these wastes is expensive due to high costs of transportation and a limited availability of landfills they are unscrupulously disposed causing concern as environmental problems. Furthermore, the problem of disposing by-products is further aggravated by legal restrictions. A high level of BOD and COD in pineapple wastes add to further difficulties in disposal. Researcher have focused on co-digestion of pineapple waste along with several other fruit and vegetable wastes, manure, and slaughter house wastes to reduce volatile solids by 50 to 65% (Alvarez and Liden, 2007). Recently, composting of pineapple wastes using earthworm is reported (Mainoo *et al*., 2009). They have reported that vermicomposting rapidly decomposed about 99% of pineapple pulp wet

mass while peel had a loss in weight by almost 87%. The pH of the waste changed from acidic to a neutral to alkaline during composting. However, cost effectiveness is yet to be studied.

**Utilization of pineapple waste**

It is anticipated that discarded fruit as well as the waste material can be utilized for further industrial processes like fermentation, bioactive component extraction, etc. There has been numerous works on the utilization of waste obtained from fruit and vegetable, dairy and meat industries. In this regard, several efforts have been made in order to utilize pineapple wastes obtained from different sources. The wastes from pineapple canneries have been used as the substrate for bromelain, organic acids, ethanol, etc. since these are potential source of sugars, vitamins and growth factors (Larrauri *et al.*, 1997; Nigam, 1999a, b; Dacera *et al*., 2009). Several studies have been carried out since decades on trying to explore the possibility of using these wastes. In past, sugar has been obtained from pineapple effluent by ion exchange and further use it in syrup for canning pineapple slices (Beohner and Mindler, 1949).

**Bromelain:** Bromelain is probably the most valuable and the most studied component from the pineapple waste. It has been investigated since 1894 (Devakate *et. al.*, 2009) and was first identified in 1891 by Marcano (Balls *et al*., 1945).It is a crude extract of pineapple that contains, among other components, various closely related proteinases, demonstrating,*in vitro* and *in vivo*, antiedematous, anti-inflammatory, antithrombotic (Bhui *et al*., 2009), fibrinolytic activities and has potential as an anticancer agent (Chobotova *et al*., 2009). It is also used in food industry as meat tenderizer and as a dietary supplement (Maurer, 2001). Bromelain is primarily present in stem, known as stem bromelain (EC 3.4.22.32) and also in fruit (EC 3.4.22.33), however small amount of bromelain is also found in pineapple waste (Hebbar *et al.*, 2008). They used reverse

micellar systems to extract and purify bromelain from crude aqueous extract of pineapple wastes (core, peel, crown and extended stem).

**Ethanol:** Interest in the economic conversion of renewable resources into alcohol using low cost substrate, such as pineapple waste, has been increasing since the last decade. Waste from pineapple cannery has been examined for ethanol production. Organisms like *Saccharomyces cerevisiae* and *Zymomonas mobilis* were used for ethanol fermentation (Ban-Koffi and Han, 1990). However, they have claimed that fermentable sugars which included sucrose, glucose and fructose were relatively low and pretreatment of the substrate with enzymes like cellulase and hemi-cellulase were necessary for alcohol production. Both organisms were capable of producing about 8% ethanol from pineapple waste in 48 h after pretreating with enzymes cellulase and hemi-cellulase.

Nigam (1999b) has used respiration deficient strain *Saccharomyces cerevisiae* ATCC 24553 for continuous ethanol production from pressed juice of pineapple cannery waste. No pretreatment of juice was done and the liquid effluents collected from various stages of processing were added. At a dilution rate of 0.05 $h^{-1}$, the ethanol production was 92.5% of the theoretical value. Immobilization of the yeast in k-carrageenan increased the volumetric ethanol productivity by 11.5 times higher than yeast cells at a dilution rate of 1.5 $h^{-1}$ (Nigam, 2000). The other study used *Zymomonas mobilis* ATCC 10988 as fermenting organisms for ethanol production (Tanaka *et al*., 1999). The raw material used here was pineapple cannery waste as well as the juice of rotten or discarded fruit. Ethanol production was 59.0 g/l without supplementation and regulation in pH.

A new technique to produce ethanol from the extraction of liquid from the pineapple fruit and pineapple plant waste has been developed (ICIS, 2006). This technique allows the extraction of

ethanol from the plant waste without requiring the producer to choose between food or fuel uses as the end product.

**Phenolic antioxidant:** Search for new natural antioxidants has been increased dramatically over the past years and in this regard agro-industrial by-products are extensively being explored. The low cost of these residues, which otherwise would be discarded as waste in the environment, may be one of the reasons. Phytochemicals, especially phenolic, in fruits and vegetable are suggested to be the major bioactive compounds for the health benefits. Phenolic compounds from pineapple wastes (residual pulp, peels and skin) have been enhanced using certain bioprocesses (Correia *et al*., 2004a). Total phenolics were increased by two times when the fungus *Rhizopus oligosporus* was incubated for 12 days in 1:1 pineapple: soybean flour mixture. Another bioprocess where mixture of pineapple residue and soy flour (9:1 and 5:5) using *R. oligosporus* has revealed that extracts obtained after 2 days with 9:1 treatment showed potent α-amylase inhibition while the extract obtained after 10 days with 5:5 treatment exhibited *Helicobacter pylori* inhibition (Correia *et al*., 2004b). They have linked these activities with the phenolic compounds present in the system.

The ethanolic extract of pineapple leaves containing phenolics have shown to inhibit the increase in blood glucose in diabetic rats as well as inhibit the increase in postprandial triglycerides (Xie *et al*., 2005). The other report on the ethanolic extracts of pineapple leaves have shown to content high amount of phytochemicals including *p*-coumaric acid, 1-*o*-*p*-coumaroylglycerol, caffeic acid and 1-*o*-caffeoylglycerol (Xie *et al*., 2006). They reported that leaves extract application inhibited the development of insulin resistance in high-fat diet-fed and low-dose streptozotozin treated diabetic rats.

**Organic acid:** Organic acid production from fruit wastes in the search of low cost substrate has been a research of interest. In this regard, pineapple wastes have been utilized for the production of various organic acids particularly citric, lactic and ferulic acid using fermentation technology

**Citric acid:** This commercially valuable product is widely used in food, pharmaceutical and beverage industries as substrate to acidify and enhance flavor. Some researchers have investigated the production of citric acid by *A. niger* under solid state fermentation conditions using pineapple waste (from juice extractor) as substrates (Kumar *et al*., 2003). They also investigated the effect of methanol on the fermentation, which increased the yield from 37.8% to 54.2%. The other groups of researchers studied the production of citric acid by *Yarrowia lipolytica* under solid state fermentation conditions using pineapple waste (from local juice manufacturer) as the sole substrate (Imandi *et al*., 2008).

**Lactic acid:** Lactic acid has an important position in the family of carboxylic acids because of its application in both food and non-food industries. It is used as a preservative and acidulant in food industries. However, commercial production of lactic acid is costly due to the raw materials used (exploitation of biological waste). Some researchers have used pineapple syrup, a food processing waste, as low cost substrate for the production of lactic acid using *Lactobacillus lactis* and enzyme invertase to hydrolyze sucrose into glucose and fructose. They have reported the yield of 20 and 92 g/l from 20 and 100 g total sugars/l (Ueno *et al*., 2003).

**Ferulic acid:** Ferulic acid is the most abundant hydroxycinnamic acid found in plat cell walls. This phenolic antioxidant is widely used in the food and cosmetic industry. Pineapple peel has been used for the alkali extraction of ferulic acid (Tialy *et al*., 2008).

**Energy and carbon source:** Pineapple wastes generally comprise of organic substances and hence the disposal problem could be attenuated by anaerobic digestion and composting. Some of

these wastes could have industrial applications for gas generations (Mbuligwe and Kassenga, 2004).

The sugars contained in pineapple cannery effluent have been utilized for the production of single cell protein using continuous cultivation (Nigam, 1999b). The dilution rate had significant effect on biomass as well as protein content. There was an increase in biomass and protein content of *Candida utilis* with increasing dilution rate.

**Anti-dyeing agent :** Dyes used in textile industries have been a threat to environmental problem since these are visible in small quantities due to their brilliance when mixed and thrown with large volumes of waste water from different steps in the dyeing and finishing processes (Robinson *et al*., 2001; Babu *et al*., 2008). Some works on utilizing pineapple waste to remove the dyes have been reported. Pineapple stem is used as low-cost adsorbent to remove basic dye (methylene blue) from aqueous solution by adsorption (Hameed *et al*., 2009). In another report, pineapple leaf powder has been used as an unconventional bio-adsorbent of methylene blue from aqueous solution (Weng *et al*., 2009).

**Fiber*:*** Fibers from pineapple fruit has been reported by several researchers (Gorinstein *et al*, 1999). However, some studies have focused on utilizing fibers of pineapple wastes. Researchers have reported that dietary fiber powder prepared from pineapple shell has 70.6% total dietary fiber with better sensory properties than commercial dietary fibers from apple and citrus fruits (Larrauri *et al*., 1997). The pineapple leaves have been used to make coarse textiles and threads in some Southeast Asian countries (Tran, 2006). Alkaline pulping methods were found to be superior over semi-chemical mechanical pulping with yields below 40%. A yield of 2.1g fiber/100 g pineapple pulp waste has been reported (Sreenath *et al*., 1996). Furthermore, pineapple leaf fibers are investigated in making fiber-reinforced polymeric composites because

of high cellulosic content, abundance and inexpensiveness (Arib *et al*., 2006). They investigated the tensile, flexural, and impact behavior of pineapple leaf fiber-reinforced polyster composites as a function of fiber loading, fiber length, and fiber surface modification. They found that the mechanical properties of the composites are superior to other cellulose-based natural fiber composites.

**As animal feed/single cell protein*:*** Feed production has become a new industry. The utilization of agro-industrial wastes as animal feed seems to mitigate the difficulties of forage shortage during critical seasons. Several studies have focused on exploiting pineapple wastes as feed for ruminants. The outer peel or skin and core from the pineapple canning industries, called bran, and the leaves are being utilized as feed for ruminants (Tran, 2006). The nutritive value of pineapple peel has been reported (Negesse *et al*., 2009). In China, pineapple waste from the field or from the cannery are being used as dairy feed (Sruamisri, 2007). Cattle preferred fermented pineapple waste with higher acidity to fresh waste. Dried and ensiled pineapple waste can be used as supplemental roughage and could replace 50% roughage in the total mixed ration for dairy cattle (Sruamisri, 2007). Besides, researchers have also focused on the performance and the apparent digestibility of pineapple by-product when used as feed. On feeding twenty four cross bred local goats for 80 days, it was found that dehydrated pineapple by-products would increase the digestibility with increase in weight of the animals (Costa *et al*., 2007). Survey reports that in Nigeria, pineapple wastes are also used for feeding small ruminants and that they could be used after proper processing (Onwuka *et al.*, 1997). Another report on suitability of pineapple waste as animal feed and pulp for human consumption is also found (Cabrera *et al*., 2000). However, some researchers have reported that by-product of pineapple processing industry is not considered attractive as an animal feed because of high fiber content and soluble carbohydrates

with low protein content (Correia *et al*., 2004a). Several researchers have also reported the use of pineapple waste for production of single cell proteins (Daniyan *et al*., 2000; Dhanasekaran *et al*., 2011; Yousufi 2012; Omwangi *et al*., 2013).

## 1.23 WASTES AS RAW MATERIAL IN THE PRODUCTION OF SINGLE CELL PROTEIN.

The substances that form the waste materials should generally be recycled back and become the part of the ecosystem, e.g. food wastes, straw, citric acid, bagasse, molasses, olive and date wastes, whey, animal manures and sewage. The quantity of these waste materials can be very high locally and may consider as contributing factor in rising considerable pollution level in waterways. Thus, the exploitation of these materials in SCP practices provides two functions i.e. decrease in pollution level and formation of protein suitable for eating.

Though, the waste might not be appropriate for SCP or its dilution or composition may be so discrete that their transportation to a manufacturing centre may be unaffordable. SCP methods employing waste substrates have been performed on a large scale by means of a variety of microorganisms in complicated bioreactor organizations. Substrates utilized are however supplemented with nutrients while they are being used in the SCP production (Ali *et al*., 2017).

### Effect of Glucose as Carbon Source on Fermentation

While producing proteins by microbial sources, carbon source perform an imperative role. The striking characteristics to utilize the glucose as carbon source are their lesser cost (Zhao *et al*., 2010). SCP production higher than 8 % was obtained by cultivating the microorganisms with glucose. The concentration of glucose considerably influences the SCP production (Smith and Bull, 1976).

**Effect of Nitrogen Sources on Fermentation**

For the production of proteins, source of nitrogen is one of the essential factors because of the structural characteristics of proteins. While using yeast extract and tryptone, production of SCP using *Haloarcula Spp* IRU1 has been improved. It is not capable of producing SCP after growing in the medium containing peptone and $NH_4Cl$. Highest yield of SCP was gained when tryptone was complemented at 0.8 % w/v concentration (Barnett, 1981).

**Effect of Phosphorous Sources on Fermentation**

For producing SCP, $NaH_2PO_4$ and $K_2HPO_4$ were the most excellent phosphorous sources. The highest SCP production was attained by microbial cultures growing on the medium containing 0.016% (w/v) concentration of $NaH_2PO_4$. Elevated concentrations of phosphorous sources brought about an increase in the production of SCP in all experimentations (Brasen and Schonheit, 2004).

# CHAPTER TWO

## 2.0 MATERIALS AND METHODS

### 2.1 SOURCE OF SAMPLES

Fresh pineapple fruits were obtained from Ogige market, Nsukka, Enugu, Nigeria.

### 2.2 ISOLATION OF FUNGI FROM PINEAPPLE

Spoilt pineapple peels (10 g) were homogenized in a sterile mortar containing 20ml 0f distilled water. The homogenate was filtered with a Whatman No 1 filter and 1 ml transferred by a sterile pipette into a 9 ml 0.1% peptone water diluents and mixed. A serial dilution in 0.1% peptone water was prepared, a dilution of 5 tubes was attained with 0.1 ml each dilution of $10^{-3}$ to $10^{-4}$ spread onto Potato Dextrose Agar and incubated at 30 ± 2$^{o}$C for 3–4 days. Pure cultures were obtained by spreading on fresh PDA plates. The cultures was assigned arbitrary numbers and stored on agar slants at 4$^{o}$C.

### 2.3 FUNGAL SELECTION BASED ON CELLULASE ACTIVITY

#### 2.3.1 Fungal selection based on growth on carboxymethylcellulase agar (CMCagar) plates

The fungal isolates were grown on CMC agar containing (Distilled water 1000ml, $NH_4H_2PO_4$ 1g, KCl 0.2g, $MgSO_4.7H_2O$ 1g, Yeast Extract 1g, CMC 26g, and Agar 3g) the CMC agar plates were incubated at 30 ± 2$^{o}$C for 5 days to allow for the secretion of cellulase (Stewart *et al.*,1982). At the end of the incubation, to visualize the hydrolysis zone, the agar medium was flooded with an aqueous solution of Congo red (1% w/v) for 15 min. The Congo red solution was then poured off, and the plates were further treated by flooding with 1M NaCl for 15 min. To indicate the cellulase activity of the fungi, diameter of clear zones around colonies on CMC agar were measured. The ratio of the clear zone diameter to colony diameter was measured in order to select for the highest cellulase activity producers (Teather and Wood, 1982).

### 2.3.2 Fungal selection based on carboxymethylcellulase (CMCase) / filter paper / cotton wool activities.

Twenty isolates that gave the best cellulase activity on CMC agar were selected and subjected to a more quantitative method of cellulase activity test. According to the method of Nwokoro *et al*., (2013), a loopful of each fungal isolate was combined with 4 ml of 0.2 M sodium acetate buffer (pH 6.5) and supplemented with : (a) 1% (w/v) carboxymethyl cellulose for the carboxymethylcellulase (CMCase) activity test; (b) 50 mg Whatman No. 1 Filter paper strip (1 x 8 cm) for the filter paper activity (FPA) test and, (c) 50 mg of well-blended, high grade commercial raw cotton wool for the cotton wool activity (CWA) test and then incubated for: 60 min. at 50$^{o}$C (a) and 24 h at 50$^{o}$C (b and c). Reducing sugars in the medium after incubation was estimated by a modification of the dinitrosalicylic acid method of Miller (1959).The fungal isolate with the best cellulase activity was selected, identified and used for further works.

## 2.4 MACROSCOPIC/MICROSCOPIC MORPHOLOGY OF SELECTED ISOLATE

The morphological characteristics and cultural characteristics comprising growth rate, colour and colony appearance was examined. The microscopic examination of the shape, arrangement and development of condiophores or phialides, and conidia was made from slide preparations stained with lactophenol-cotton blue. A drop of lactophenol-cotton blue was placed on a microscopic slide. Using a sterile wire loop, a colony of the isolate was placed into the lactophenol-cotton blue on the slide. The preparation was covered with a cover slip and examined the under the x40 objective of a microscope. Characteristics observed were compared with reference materials of Smith (1994) and Shaiesta *et al*., (2012).

## 2.5 IDENTIFICATION OF SELECTED ISOLATE BY DNA SEQUENCING

**2.5.1 Primer used:** 27F.1 Forward 5'AGRGTTTGATCMTGGCTCAG 3' and 1492R reverse 5'GGTTACCTTGTTACGACTT 3'

**2.5.2 Molecular Analysis:**

Methodology was based on PCR and Sanger Sequencing analysis. DNA Extraction and Bioinformatics analysis of sequences was performed at Molecular Laboratory Services Division of Teddy & Thaddeus Nig. Co. Akoka, Lagos. Sequencing analysis was done at Inqaba Biotechnology Pty South Africa.

**2.5.3 DNA extraction**

DNA extraction was from a 24 hours growth of microbial isolates in BHI broth harvested by centrifugation at 14, 000 x g for 10 minutes. The cells were washed three times in 1 ml of Ultra-pure water by centrifuging at 12,000 rpm for 5 min. DNA extraction and purification was done using ZR Fungal/Bacterial DNA MiniPrep™50 Preps.Model D6005 (Zymo Research, California, USA).50-100 mg of fungal cells was suspended in 200 µl of sterile water. This was transferred into a ZR BashingBead™ Lysis Tube. Exactly 750 µl Lysis solution was added to the tube. The bead containing the solution was secured in a bead beater fitted with a 2 ml tube holder assembly and process at maximum speed for 5 minutes. The ZR BashingBead™ Lysis Tube was centrifuged in a micro-centrifuge at 10,000 x g for 1 minute. 400 µl of the supernatant was pipeted into a Zymo-Spin™ IV Spin Filter in a collection tube and centrifuged at 7,000 x g for 1 minute. This was followed by the addition of 1,200 µl of Fungal/Bacterial DNA binding buffer into the filtrate in the collection tube. After this 800 µl of the mixture was transferred into a Zymo-Spin™ IIC column in a collection tube and centrifuge at 10,000 x g for 1 minute.

The flow through was discarded from the collection tube and the process was repeated to obtain the remaining products. The 200 µl DNA pre-wash buffer was added into the Zymo-Spin™ IIC Column in a new collection tube and centrifuge at 10,000 x g for 1 minute. This was followed by the addition of 500 µl Fungal/Bacterial DNA Wash Buffer into the Zymo-Spin™ IIC column and centrifuged at 10,000 x g for 1 minute. The Zymo-Spin™ IIC column was transferred into a clean 1.5 ml micro-centrifuge tube and 100 µl of DNA Elution Buffer was then added directly to the column matrix. This was centrifuged at 10,000 x g for 30 seconds to elute the DNA. The Ultra-pure resulting filtrate (DNA) obtained was used as a template during the assay. This was transported in ice the laboratory for sequencing.

**2.5.4 PCR amplification**

The PCR reaction was performed on the extracted DNA samples using universal degenerate primers 27F.1 Forward 5'AGRGTTTGATCMTGGCTCAG 3 and 1492R reverse 5'GGTTACCTTGTTACGACTT 3' (De Santis *et al*., 2007) that amplifies the entire 16s Variable region at annealing temperature of 58$^{o}$C. Each PCR reaction contained 5 µl of 10 × Taq buffer, 2 mM MgCl 2,1.5 U Super-Therm DNA Polymerase (Southern Cross), 0.25 mM dNTP's, 0.1 µM of each primer, 1 µl of extracted DNA and Nuclease Free Water (NFW) up to the final reaction volume of 50 µl. The PCR cycle started with an initial denaturation step at 94°C for 10 min. This was followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min and extension at 72°C for 1 min, and a final extension at 72°C for 5 min that was then followed by cooling to 4°C. Few microliters of the samples were run on a 1% agarose gel at 90 V for 30 min in order to verify amplification. The entire PCR reaction was loaded onto a 1% agarose gel and the correct

band size (approximately 1500 bp) was excised. The DNA was recovered from the gel slices by using the GeneJET™ gel extraction kit (Fermentas).

### 2.5.5 DNA Sequencing by Sanger sequencing method

DNA sequencing was performed by Sanger (dideoxy) sequencing technique to determine the nucleotide sequence of the specific microorganism isolated using automated PCR cycle- Sanger Sequencer™ 3730/3730XL DNA Analyzers from Applied Biosystems (Russell, 2002; Metzenberg 2003).

## 2.6 PREPARATION/PRETREATMENT OF PINEAPPLE PEELS

The pineapple fruits were washed with several changes of sterile water and peeled off. The peels were cleaned, cut into small pieces, rinsed in sterile water, oven dried and ground to a mesh size ~ 0.5 mm.

## 2.7 HYDROLYSIS OF GROUND PINEAPPLE PEELS

Fifty gram samples of dried, ground pineapple peels were boiled for 1 hr in;

a) 500ml of 0.5% NaOH
b) 500ml of 0.5% $H_2SO_4$ and
c) 500ml of 0.5% HCl.

The hydrolysates were filtered out and the slurry pH adjusted to 6.5 with sterile lactic acid (a) and 0.2M NaOH (b and c). The hydrolysates were supplemented with 10 ml each of $KH_2PO_4$ (5% w/v), urea (2.7% w/v) and $(NH_4)_2SO_4$ (9% w/v). A 50 g sample of dried, ground pineapple peels boiled in 500ml of distilled water was used as control. The extracts were placed into a sterile container, the reducing sugar, soluble sugar and protein compositions was determined (Sanker *et al*., 2011).

## 2.8 DETERMINATION OF REDUCING SUGAR CONTENT

Sample containing 5 ml of hydrolyzed extracts was added into a beaker, reducing sugar concentrations was determined by a modification of the dinitrosalicylic acid (DNS) method of Miller (1959). DNS (10 g) was dissolved in 200 ml of 0.2M NaOH. Potassium sodium tartrate (300 g) was dissolved in 800 ml of distilled water. The two solutions were mixed and stored in an air tight dark bottle. An aliquot (3 ml) of this reagent was added to tubes containing 1 ml of glucose solution at various concentrations and to distilled water blanks. The tubes were placed in boiling water bath for 10 minutes, cooled to room temperature and diluted by adding 3 ml of distilled water. The solutions were read in a Spectrum spectrophotometer at 540 nm. The readings were used to draw a standard curve for milligrams glucose equivalents per ml against absorbance.

## 2.9 DETERMINATION OF SOLUBLE SUGAR CONTENT

The total soluble sugar was estimated using by Anthrone method according to Mustapha and Berbura (2009). Anthrone reagent (0.2 g) was dissolved in 100 ml of concentrated $H_2SO_4$. Dissolved Anthrone reagent (4 ml) was added to tubes containing 1 ml of glucose solution at various concentrations and to distilled water blanks. The contents were mixed and the test tubes covered. The tubes were placed in boiling water bath for 10 minutes and cooled to room temperature. The solutions were read in a Spectrum spectrophotometer at 620 nm. The readings were used to draw a standard curve for milligrams glucose equivalents per ml against absorbance.

## 2.10 DETERMINATION OF PROTEIN CONTENT

Sample containing 1 ml of extracts (hydrolysates) was mixed with 2 ml of Biuret reagent in a test tube and the mixture was shaken thoroughly and allowed for 15 minutes. The protein

concentration was determined by the Biuret method according to Peterson (1983). Biuret reagent (2 ml) was added to tubes containing 1 ml of Bovine Serum Albumin (BSA) solution at various concentrations and to distilled water blanks. The tubes were shaken thoroughly and allowed for 15 min. The solutions were read in a Spectrum spectrophotometer at 550 nm. The readings were used to draw a standard curve for milligrams protein concentration equivalents per ml against absorbance.

**2.11 INOCULUM DEVELOPMENT**

The selected and identified fungal isolate was cultured on slants of Potato Dextrose Agar. Spores were harvested with 0.1% Tween 80 solution and adjusted with sterile distilled water to final concentration of $10^8$ spores/ml by counting with a haemocytometer under the microscope.

**2.12 FERMENTATION OF HYDROLYZED PINEAPPLE PEELS FOR PRODUCTION OF SINGLE CELL PROTEIN**

From the hydrolyzed samples and control, 90ml each was measured into sterile 250 ml Erlenmeyer flasks and autoclaved at 121$^o$C for 15 minutes. On cooling, 10 ml of the spore suspension ($50 \times 10^8$) was added. Submerged fermentation was carried out in Erlenmeyer flasks with three trial media of the each hydrolysates and control. The experimental set up was aerobically incubated at $30 \pm 2^o$C for a period of 7 days at static condition. Protein concentration of the each fermentation media at the start of experiment were taken and samples were taken at day 1, day 3, day 5 and day 7 to check for protein concentration in each broth media. The culture broth was separated from fungal biomass after incubation period by filtration through Whatman No. 1 filter paper (Sankar *et al.,* 2011).

## 2.13 OPTIMIZATION OF CULTURAL CONDITIONS

### 2.13.1 EFFECT OF NITROGEN SOURCES ON PROTEIN CONCENTRATION OF HYDROLYSATES

From the hydrolyzed pineapple extracts and control, 80ml each were measured into sterile 250 ml Erlenmeyer flasks. To each, 10 ml of different nitrogen sources i.e. sodium nitrate, potassium nitrate, ammonium nitrate, sodium nitrite and ammonium oxalate at 0.2 g/100 ml was added. The Erlenmeyer flasks were autoclaved at 121°C for 15 min. On cooling, the media in the flasks was inoculated with 10ml of the inoculum. Submerged fermentation was carried out in Erlenmeyer flasks with three trial media of the each hydrolysates and control. The flasks were aerobically incubated at 30 ± 2$^{o}$C for a period of 7 days at static condition. Protein concentration of the each fermentation media at the start of experiment were taken and samples were taken at day 1, day 3, day 5 and day 7 to check for protein concentration in each media. The culture broth was separated from fungal biomass after incubation period by filtration through Whatman No. 1 filter paper (Sankar *et al.*, 2011).

### 2.13.2 EFFECTS OF GLUCOSE CONCENTRATION ON PROTEIN CONCENTRATION OF HYROLYSATES

From the hydrolyzed pineapple extracts and control, 80ml each were measured into sterile 250 ml Erlenmeyer flasks. To each, 10 ml of different glucose concentration was added i.e. 2%, 4%, 6%, 8% and 10% (w/v). The Erlenmeyer flasks were autoclaved at 121°C for 15 min. On cooling, the media in the flasks was inoculated with 10ml of the inoculum. Submerged fermentation was carried out in Erlenmeyer flasks with three trial media of the each hydrolysates and control. The flasks were aerobically incubated at 30 ± 2$^{o}$C for a period of 7 days at static condition. Protein concentration of the each fermentation media at the start of experiment were

taken and samples were taken at day 1, day 3, day 5 and day 7 to check for protein concentration in each media by Biuret method according to Peterson (1983). The culture broth was separated from fungal biomass after incubation period by filtration through Whatman No. 1 filter paper (Sankar *et al.*, 2011).

**2.14 DETERMINATION OF FUNGAL BIOMASS**

At the end of incubation, the weight of fungal biomass was recorded after filtering on Whatman No. 1 filter paper and dried at 105°C in a hot air oven until constant weight was attained.

**2.15 DETERMINATION OF PROTEIN IN DRIED FUNGAL BIOMASS**

**Protein Extraction**: Dried biomass (1 g) was suspended in 2 ml of 5% NaOH, 4 ml of 8% urea and 4 ml of 0.05% sodium dodecyl sulphate and incubated at 32$^{o}$C for 16 h. After incubation, the sample was centrifuged and the supernatant tested for protein. Sample (1 ml) was mixed with 2 ml of Biuret reagent in a test tube and the mixture was shaken thoroughly and allowed for 15 minutes. The absorbance was determined at wavelength of 550 nm against a blank (Peterson, 1983).

**2.16 STATISTICAL ANALYSIS**

The results were expressed as mean ± standard deviation (mean ± S.D.) of triplicate experiments. Statistical analysis was performed on data generated from the study using SPSS software. One way analysis of variance (ANOVA) technique was used to compare differences in mean and to establish the extent of significant difference.

# CHAPTER THREE

## 3.0 RESULTS

### 3.1 ISOLATION OF FUNGI FROM PINEAPPLE

Seventy-two fungal isolates were obtained after 3 days of incubation on Potato Dextrose Agar (PDA). Pure cultures were prepared and assigned arbitrary numbers IS 1, IS 2, IS 3, IS 4, IS 5, IS 6, IS 7, IS 8…….to 1S 72.

### 3.2 FUNGAL SELECTION BASED ON CELLULASE ACTIVITY

#### 3.2.1 Fungal selection based on growth on carboxymethylcellulase (CMC agar) plates

All the isolated fungi were screened for their ability to hydrolyse cellulose on CMC agar. The diameter of clear zones were measured and are shown in Table 1. No clear zones were recorded for the fungal isolates; IS 4, IS 9, IS 17, IS 18, IS 19, IS 26, IS 27, IS 32, IS 34, IS 35, IS 40, IS 50, IS 52, IS 53 and IS 68. The highest clear zone was recorded for IS 51 wih 7.4cm, followed by IS 58 with 6.9cm. Fungal isolates that had higher clear zones ranged from 4.0cm – 6.3cm and they include; IS 1, IS 11, IS 16, IS 23, IS 28,IS 42,IS 45 and IS 54.Other fungal isolates had lower clear zones.

#### 3.2.2 Fungal selection based on carboxylmethylcellulase (CMCase) / filter paper / cotton wool activity test

Twenty fungal isolates that gave the highest clear zone diameter on CMCagar were futhur screened for their ability to hydrolyze cellulose substrates namely, carboxyl methyl cellulose (CMC), filter paper and cotton wool. The 20 fungal isolates tested produced different cellulase activities (Table 2). Culture supernatant from the fungal isolates designated IS 25, IS 33 and IS 71 produced the lowest cellulose activities: (0.48, 0.36 and 0.42) mg/ml on CMC (0.16, 0.09 and 0.55) mg/ml on filter paper; and (0.11, 0.12 and 0.16) mg/ml on cotton wool respectively. Fungal

isolates designated IS 28, IS 42 and IS 58 produced high cellulose activities: (3.51, 3.98 and 4.01) mg/ml on CMC (3.19, 2.79 and 3.14) mg/ml on filter paper; and (2.91, 3.04 and 3.27) mg/ml on cotton wool respectively. Culture supernatant from the fungal isolate designated IS 51 produced the best cellulase activities: 4.64 mg/ml on CMC 3.76 mg/ml on filter paper; and 4.12 mg/ml on cotton wool (Table 2), and was therefore selected for further work.

### 3.3 MACROSCOPIC/MICROSCOPIC MORPHOLOGY OF SELECTED ISOLATE

The morphological and cultural characteristics observed for IS 51 includes; fast growing mycelium forming compact cluster, light green conidia over the entire medium and a single concentric ring was found around the point of inoculation. The microscopic examinations were the conidia of the isolate were globose to ellipsoidal and bluish-green colour. Phialides of isolate were short flask shaped arranged in groups. The whole conidiophores systems were not extensively branched. The characteristics observed matched *Trichoderma viride* (Smith 1994; Shaiesta *et al*., 2012).

### 3.4 IDENTIFICATION OF FUNGAL ISOLATE BY DNA SEQUENCING

The result from DNA sequencing of fungal isolate designated IS 51 was obtained as nucleotides. Sequence analysis from resultant nucleotides base pairs was performed by BLAST analysis by direct blasting on American data base http://blast.ncbi.nlm.nih.gov.For the fungal isolate, a read was BLASTED and the resultant top hits with minimum E-score for the BLAST result showing species name was used to name this specific organism. Sequencing result in FASTA format and corresponding identity after BLAST analysis on NCBI website are shown in Table 3. The isolate was identified as *Trichoderma viride* ATCC28038

## 3.5 REDUCING SUGAR, SOLUBLE SUGAR AND PROTEIN CONTENTS OF THE HYDROLYZED PINEAPPLE PEELS

The reducing sugar, soluble sugar and protein contents of the various hydrolyzed pineapple peels extracts are shown in Table 4. NaOH hydrolyzed extract gave the highest carbohydrate and protein content; 138mg/ml total reducing sugar, 298mg/ml total soluble sugar and 9.44mg/ml protein. Extract from HCl hydrolysis had; 131mg/ml total reducing sugar, 279mg/ml total soluble sugar and 7.32mg/ml protein contents, followed by $H_2SO_4$ hydrolyzed extract with 129mg/ml total reducing sugar, 206mg/ml total soluble sugar and 6.28mg/ml protein contents. The unhydrolyzed sample which served as the control gave the lowest carbohydrate and protein content; 83mg/ml total reducing sugar, 107 total soluble sugar and 4.12mg/ml protein.

## 3.6 SINGLE CELL PROTEIN PRODUCTION BY FERMENTATION OF HYDROLYZED PINEAPPLE PEELS

The effects of fermentation on the protein content of hydrolyzed samples are given in Figure 1. The highest protein yield from 9.44 mg/ml to 27.35 mg/ml extract was produced when NaOH was used for hydrolysis. The control produced the lowest protein increase from 4.16 mg/ml to 15.73 mg/ml extracts (Fig. 1). The protein yield produced in 0.5% NaOH hydrolysates was significantly ($p \leq 0.05$) higher than that of $H_2SO_4$ from 6.26 mg/ml to 18.32 mg/ml and HCl hydrolysate from 7.32mg/ml to 16.48 mg/ml. The protein yield increased gradually from the start of the experiment till when it was terminated at day 7. More increase were observed between day 5 and day 7 for HCl, $H_2SO_4$ and control while there was more increase between day 3 and day 5 for the NaOH extract. However there was significant difference ($p \leq 0.05$) between each days of the fermentation.

Table 1: Diameter of clear zone around fungal isolates on CMCagar

| Fungal Isolate | Diameter of clear zone (cm) | Fungal Isolate | Diameter of clear zone (cm) | Fungal Isolate | Diameter of clear zone (cm) |
|---|---|---|---|---|---|
| IS 1 | 4.0 | IS 25 | 1.1 | IS 49 | 0.3 |
| IS 2 | 0.1 | IS 26 | 0 | IS 50 | 0 |
| IS 3 | 3.2 | IS 27 | 0 | IS 51 | 7.4 |
| IS 4 | 0 | IS 28 | 6.3 | IS 52 | 0 |
| IS 5 | 0.6 | IS 29 | 0.4 | IS 53 | 0 |
| IS 6 | 0.8 | IS 30 | 0.8 | IS 54 | 6.1 |
| IS 7 | 0.7 | IS 31 | 0.1 | IS 55 | 0.7 |
| IS 8 | 1.2 | IS 32 | 0 | IS 56 | 0.8 |
| IS 9 | 0 | IS 33 | 1.8 | IS 57 | 0.3 |
| IS 10 | 0.1 | IS 34 | 0 | IS 58 | 6.9 |
| IS 11 | 5.5 | IS 35 | 0 | IS 59 | 0.2 |
| IS 12 | 0.9 | IS 36 | 0.9 | IS 60 | 2.1 |
| IS 13 | 0.7 | IS 37 | 3.6 | IS 61 | 0.1 |
| IS 14 | 0.3 | IS 38 | 0.2 | IS 62 | 0.6 |
| IS 15 | 0.2 | IS 39 | 0.7 | IS 63 | 2.3 |
| IS 16 | 5.5 | IS 40 | 0 | IS 64 | 0.8 |
| IS 17 | 0 | IS 41 | 0.4 | IS 65 | 0.7 |
| IS 18 | 0 | IS 42 | 5.8 | IS 66 | 0.5 |
| IS 19 | 0 | IS 43 | 0.5 | IS 67 | 1.9 |
| IS 20 | 2.7 | IS 44 | 0.9 | IS 68 | 0 |
| IS 21 | 0.3 | IS 45 | 4.1 | IS 69 | 0.5 |
| IS 22 | 0.7 | IS 46 | 0.1 | IS 70 | 0.4 |
| IS 23 | 4.9 | IS 47 | 0.7 | IS 71 | 1.9 |
| IS 24 | 0.4 | IS 48 | 0.5 | IS 72 | 0.7 |

Table 2: Cellulase activities of some selected fungal isolates

| S/N | Fungal Isolate | CMCase activity (mg/ml) | Filter paper activity (mg/ml) | Cotton wool activity (mg/ml) |
|---|---|---|---|---|
| 1 | IS 1 | 2.71 | 2.55 | 1.87 |
| 2 | IS 3 | 1.99 | 1.81 | 1.45 |
| 3 | IS 8 | 0.74 | 0.49 | 0.22 |
| 4 | IS 11 | 2.28 | 2.12 | 2.36 |
| 5 | IS 16 | 2.07 | 1.99 | 2.01 |
| 6 | IS 20 | 1.10 | 1.82 | 0.75 |
| 7 | IS 23 | 1.35 | 1.13 | 1.08 |
| 8 | IS 25 | 0.48 | 0.16 | 0.11 |
| 9 | IS 28 | 3.51 | 3.19 | 2.91 |
| 10 | IS 33 | 0.36 | 0.09 | 0.12 |
| 11 | IS 37 | 2.00 | 1.05 | 1.97 |
| 12 | IS 42 | 3.98 | 2.79 | 3.04 |
| 13 | IS 45 | 1.97 | 1.77 | 1.02 |
| 14 | IS 51 | 4.64 | 3.76 | 4.12 |
| 15 | IS 54 | 3.47 | 3.14 | 3.27 |
| 16 | IS 58 | 4.01 | 2.11 | 3.24 |
| 17 | IS 60 | 1.23 | 1.18 | 1.90 |
| 18 | IS 63 | 1.78 | 2.42 | 2.09 |
| 19 | IS 67 | 0.91 | 0.46 | 0.27 |
| 20 | IS 71 | 0.42 | 0.55 | 0.16 |

Table 3: Sequencing result in FASTA format and corresponding identification of the Fungal Isolate (IS 51)

| Type | Query Length | Sequence nucleotides obtained | Similarity | Gene Bank /Accession Number | Identity Of Isolate Sequenced |
|---|---|---|---|---|---|
| Genomic DNA | 518 | >2_ITS-4-V2_B07_04<br>CCGAGTTTACAACTCCCAAACCCAATGT<br>GAACCATACCAAACTGTGCCTCGGCGGG<br>GTCACGCCCCGGGTGCGTCGCAGCCCCG<br>GAACCAGGCGCCCGCCGGAGGGACCAAC<br>CAAACTCTTTCTGTAGTCCCCTCGCGGAC<br>GTTATTTCTTACAGCTCTGAGCAAAAATT<br>CAAAATGAATCAAAACTTTCAACAACGG<br>ATCTCTTGGTTCTGGCATCGATGAAGAAC<br>GCAGCGAAATGCGATAAGTAATGTGAAT<br>TGCAGAATTCAGTGAATCATCGAATCTTT<br>GAACGCACATTGCGCCCGCCAGTATTCT<br>GGCGGGCATGCCTGTCCGAGCGTCATTT<br>CAACCCTCGAACCCCTCCGGGGGGTCGG<br>CGTTGGGGACTTCGGGAACCCCTAAGAC<br>GGGATCCCGGCCCCTAAATACAGTGGCG<br>GTCTCGCCGCAGCCTCTCCTGCGCAGTAG<br>TTTGCACAACTCGCACCGGGAGCGCGGC<br>GCGTCCACGTCCGTAAAACACCCAACTT<br>CTGAAATG | 99% | AY380909.1 | *Trichoderma viride* ATCC28038 |

Table 4: Reducing sugar, soluble sugar and protein contents of hydrolyzed and control extracts

| Extract | Total reducing sugar (mg/ml) | Total soluble sugar (mg/ml) | Protein (mg/ml) |
|---|---|---|---|
| 0.5% HCl | 131 | 279 | 7.32 |
| 0.5% $H_2SO_4$ | 129 | 206 | 6.28 |
| 0.5% NaOH | 138 | 298 | 9.44 |
| Control | 83 | 107 | 4.12 |

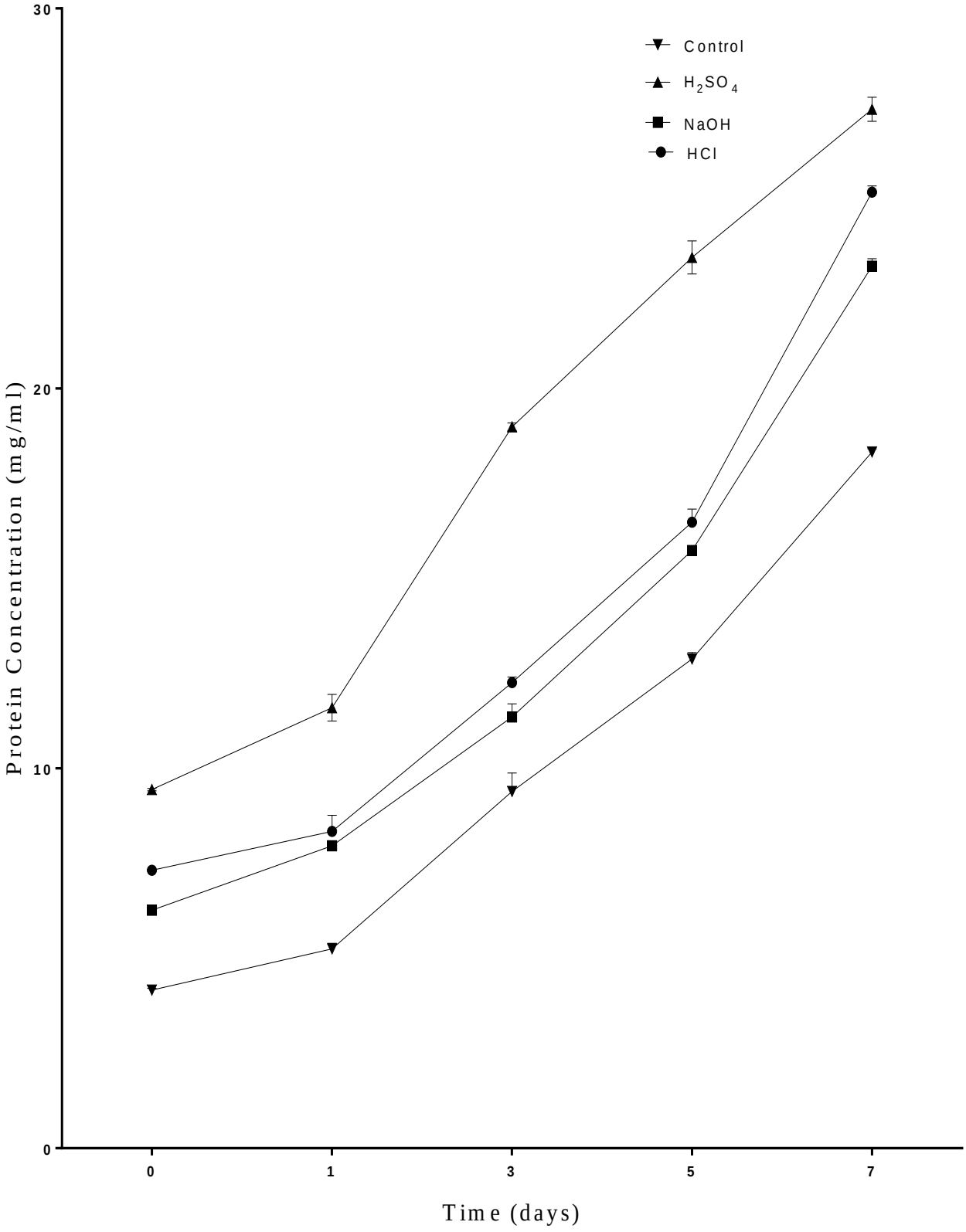


Figure 1: Protein concentration in hydrolyzed samples

## 3.7 EFFECTS OF VARIOUS NITROGEN SOURCES ON THE PROTEIN CONTENT OF THE HYDROLYSATES

Five different nitrogen sources were added to the extracts and their protein yields were recorded

### 3.7.1 Effects of ammonium oxalate on protein content of hydrolyzed samples

When ammonium oxalate was added to the samples, NaOH hydrolysate produced the highest protein from 10.35 mg/ml to 55.44 mg/ml while control has the lowest protein yield from 5.35 mg/ml to 34.16 mg/ml (Fig. 2). At day1, NaOH gave the highest protein yield of 20.68 mg/ml, followed by the control 13.71 mg/ml while $H_2SO_4$ gave the lowest yield of 10.28 mg/ml. However at day 3, $H_2SO_4$ increased to 19.48 mg/ml and control had the lowest value of 17.63 mg/ml. At the end of the fermentation, NaOH extract (55.44 mg/ml) had the highest yield, followed by HCl (48.01 mg/ml), $H_2SO_4$ (34.70 mg/ml) and the control had the lowest value (34.16 mg/ml).

### 3.7.2 Effects of sodium nitrate on protein content of hydrolyzed samples

Protein yield when sodium nitrate was added to the samples were lower than that of ammonium oxalate. However, NaOH hydrolysate also produced the highest protein yield from 9.70 mg/ml to 43.27 mg/ml and control produced the lowest from 4.40 mg/ml to 24.40 mg/ml (Fig. 3).The protein yield produced in 0.5% NaOH hydrolysates was significantly ($p \leq 0.05$) higher than that of $H_2SO_4$ hydrolysate from 6.53 mg/ml to 34.41 mg/ml and HCl hydrolysate from 7.25 mg/ml to 41.66 mg/ml. At day 1, NaOH gave the highest protein yield of 17.46 mg/ml, followed by HCl (14.52 mg/ml), $H_2SO_4$ (10.89 mg/ml) while control gave the lowest yield of 8.62 mg/ml. There was significant increase in the protein yield at day 5, where the protein yield in the NaOH hydrolysate increased to 35.68 mg/ml, $H_2SO_4$ hydrolysate increased to 29.48 mg/ml, HCl hydrolysate increased to 31.88 mg/ml and control had the lowest value of 19.55 mg/ml (Fig. 3).

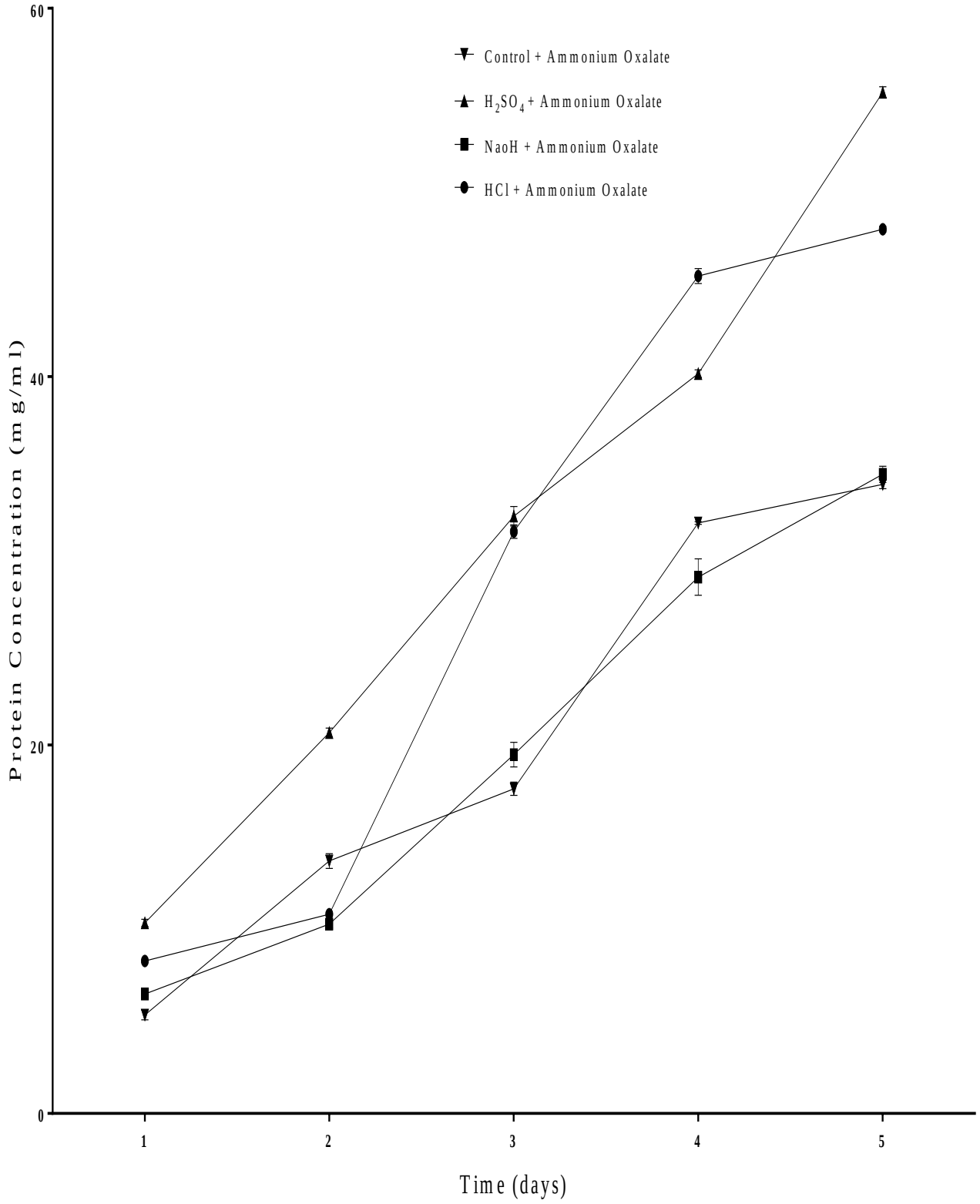


Figure 2: Protein concentration in hydrolyzed samples + ammonium oxalate

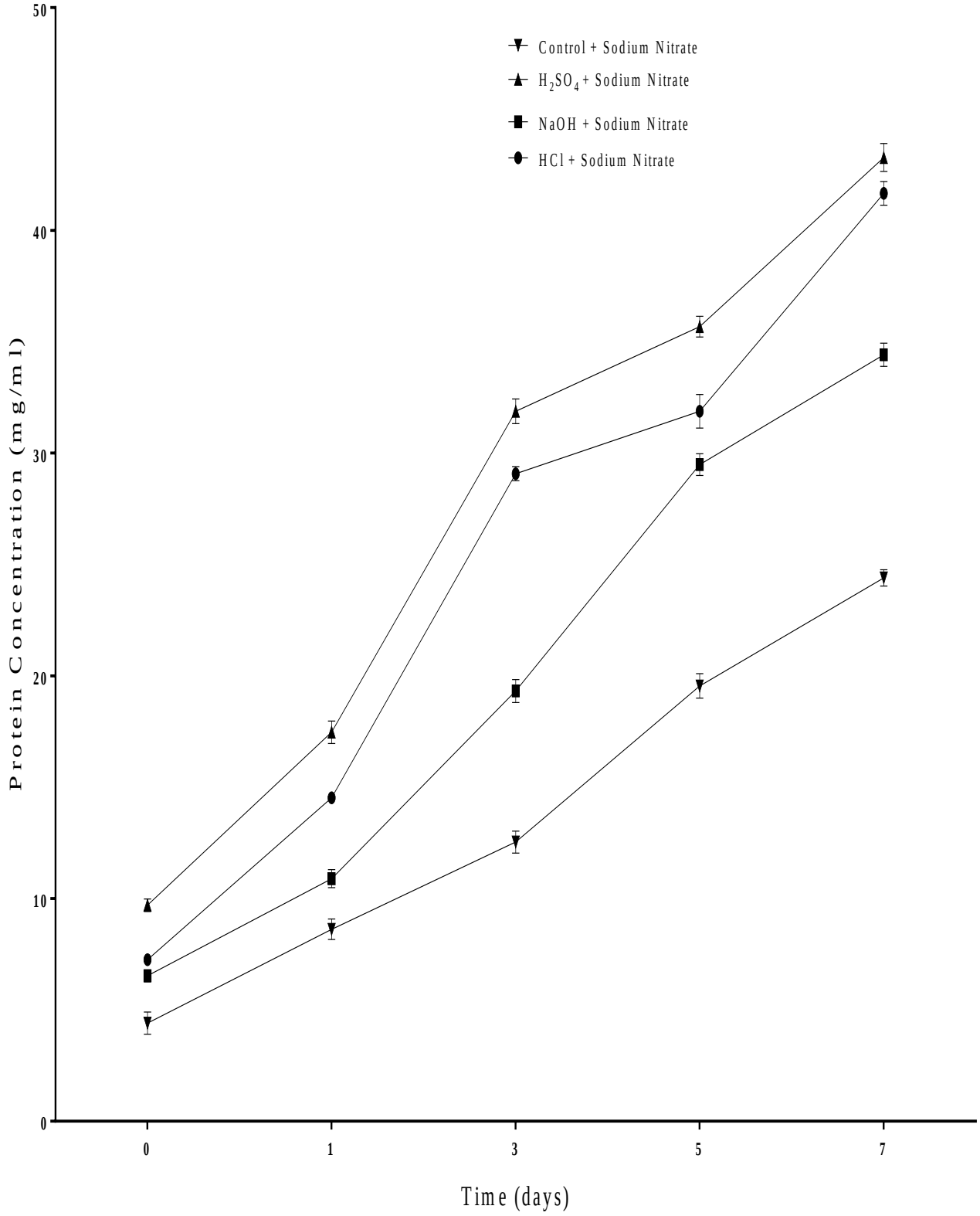


Figure 3: Protein concentration in hydrolyzed samples + sodium nitrate

### 3.7.3 Effects of sodium nitrite on protein content of hydrolyzed samples

Figure 4 shows the addition sodium nitrite to the samples where NaOH hydrolysate also produced the highest protein yield and control produced the lowest. At the start of the fermentation, protein concentrations of the samples were taken and they are; HCl hydrolysate (9.38 mg/ml), $H_2SO_4$ hydrolysate (7.13 mg/ml), NaOH hydrolysate (11.40 mg/ml) and the unhydrolyzed sample which served as the control had 6.35 mg/ml protein content. When the protein concentrations were taken at day 1, there was significant difference ($p \leq 0.05$) when compared with the start of fermentation recorded as day 0. At the end of the fermentation the protein concentrations were; the highest yield was NaOH hydrolysate (50.20 mg/ml), followed by HCl hydrolysate (45.47 mg/ml), $H_2SO_4$ hydrolysate (27.15 mg/ml) and the lowest protein yield recorded was for the control which gave a yield of 26.19 mg/ml (Fig. 4).

### 3.7.4 Effects of ammonium nitrate on protein content of hydrolyzed samples

The highest protein yield when ammonium nitrate was added to the samples was produced by$H_2SO_4$ hydrolysate from 9.66 mg/ml to 46.24 mg/ml and control produced the lowest from 6.25 mg/ml to 27.14 mg/ml (Fig. 5).Protein yield was lower in samples + ammonium nitrate when compared with samples + ammonium oxalate. At day 1 of fermentation there was no significant difference ($p \leq 0.05$) between $H_2SO_4$ hydrolysate and NaOH hydrolysate (17.56 mg/ml and 17.46 mg/ml) respectively. When protein yields were measured at day 5, the values recorded were; HCl hydrolysate (34.40 mg/ml), $H_2SO_4$ hydrolysate (34.61 mg/ml) and NaOH hydrolysate (33.39 mg/ml) where there was no significant difference among the protein content recorded at the end of the fermentation (Fig. 5).

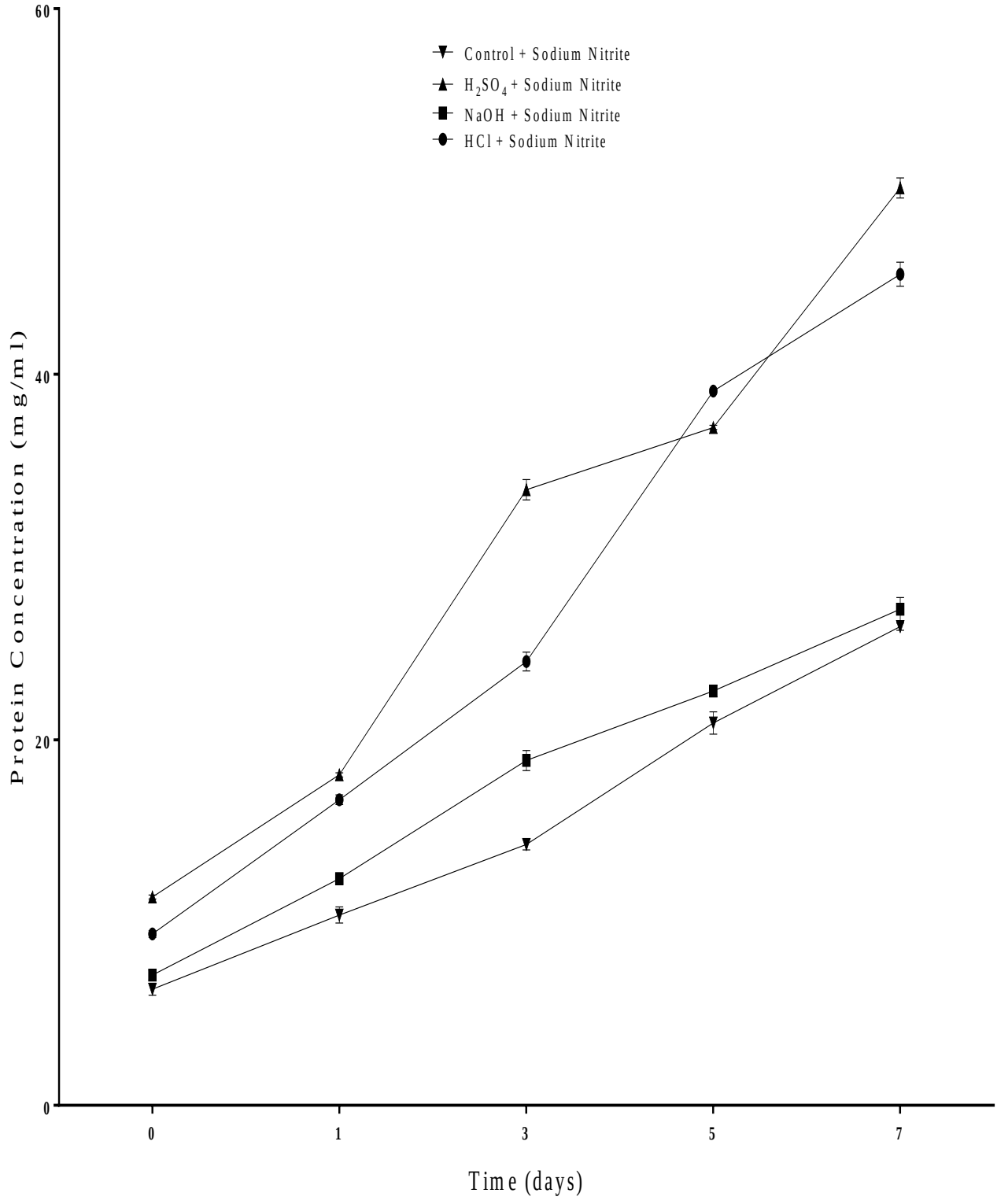


Figure 4: Protein concentration in hydrolyzed samples + sodium nitrite

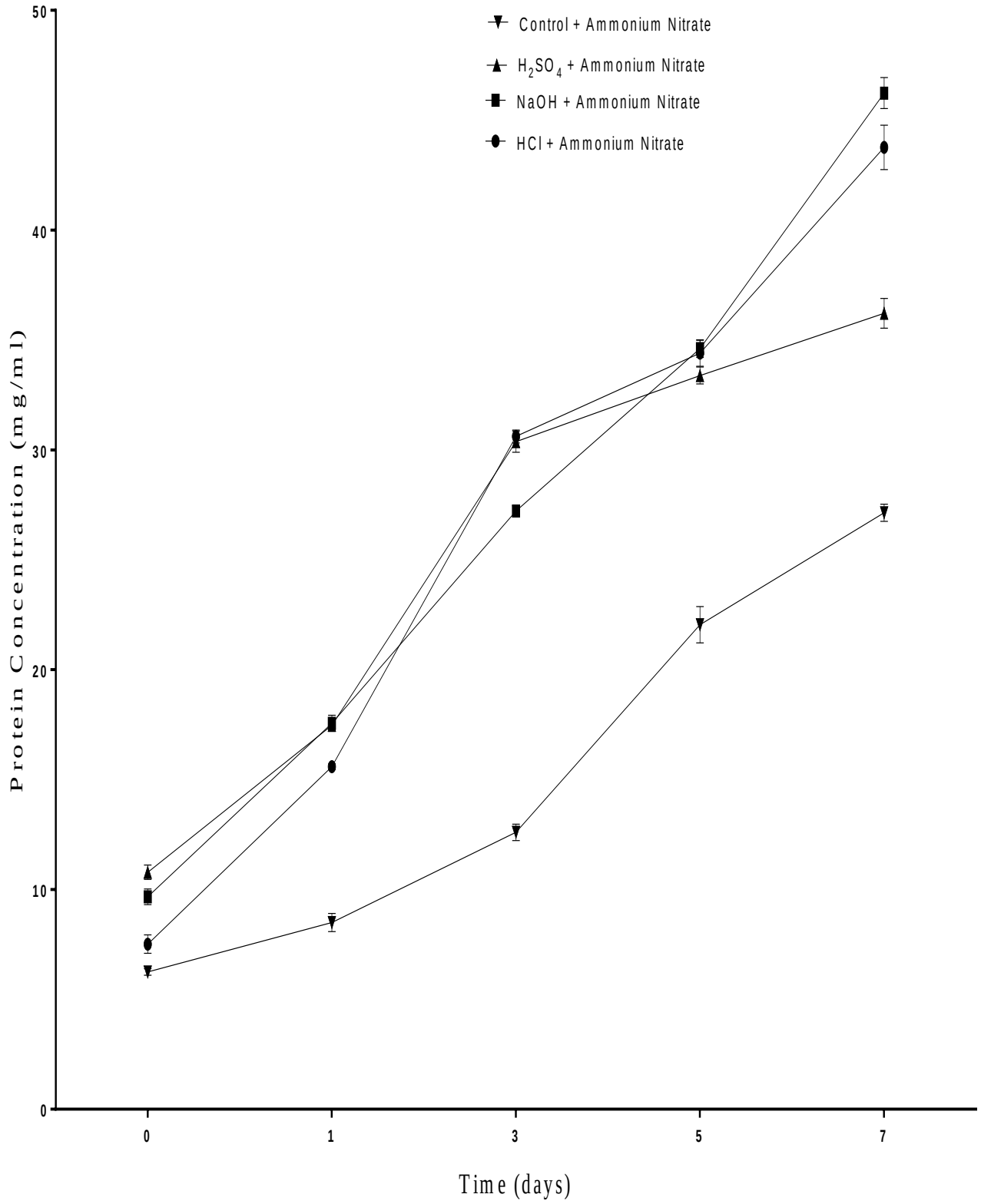


Figure 5: Protein concentration in hydrolyzed samples + ammonium nitrate

### 3.7.5 Effects of potassium nitrate on protein content of hydrolyzed samples

Addition of potassium nitrate to the samples produced the highest protein yield in HCl hydrolysate from 9.22 mg/ml to 40.74 mg/ml and control had the lowest yield from 4.41 mg/ml to 32.15 mg/ml (Fig. 6). Among all the nitrogen sources used, potassium nitrate produced the lowest protein yield for the hydrolyzed samples but it was the best for the unhydrolyzed sample which served as the control in the entire experiment. Figure 6 shows that at the end of the fermentation (day 7), the highest protein yield was HCl hydrolysate (40.74 mg/ml), followed by $H_2SO_4$ hydrolysate (40.44 mg/ml), NaOH hydrolysate (38.45 mg/ml) and the lowest protein yield recorded was for the control which gave a yield of 32.15 mg/ml. Although, HCl hydrolysate produced the highest protein yield, it had no significant difference ($p \leq 0.05$) with NaOH hydrolysate and $H_2SO_4$ hydrolysate.

## 3.8 EFFECT OF DIFFERENT GLUCOSE CONCENTRATIONS ON THE PROTEIN CONTENT OF HYDROLYZED SAMPLES

Glucose at various concentrations was added to the samples. All the hydrolysates and control had the lowest protein yield at 2% (20 g/L) glucose and highest at 10% (100 g/L) glucose.

### 3.8.1 Effect of different glucose concentrations on protein content of HCl hydrolysate

Figure 7 shows the protein yield when glucose was added to HCl hydrolysate. At the start of the experiment there was significant difference ($p \leq 0.05$) in the protein content of the different glucose concentrations with 10% glucose the highest value (12.34 mg/ml) and 2% glucose was lowest (7.32 mg/ml). At day 1, there was no significant difference between the protein yield of 2% glucose and 4% glucose (10.56 mg/ml and 11.28 mg/ml) respectively. Although the protein content for 10% glucose (35.70 mg/ml) was the highest at the end of the fermentation it was not significantly different from that of 8% glucose (34.63 mg/ml).

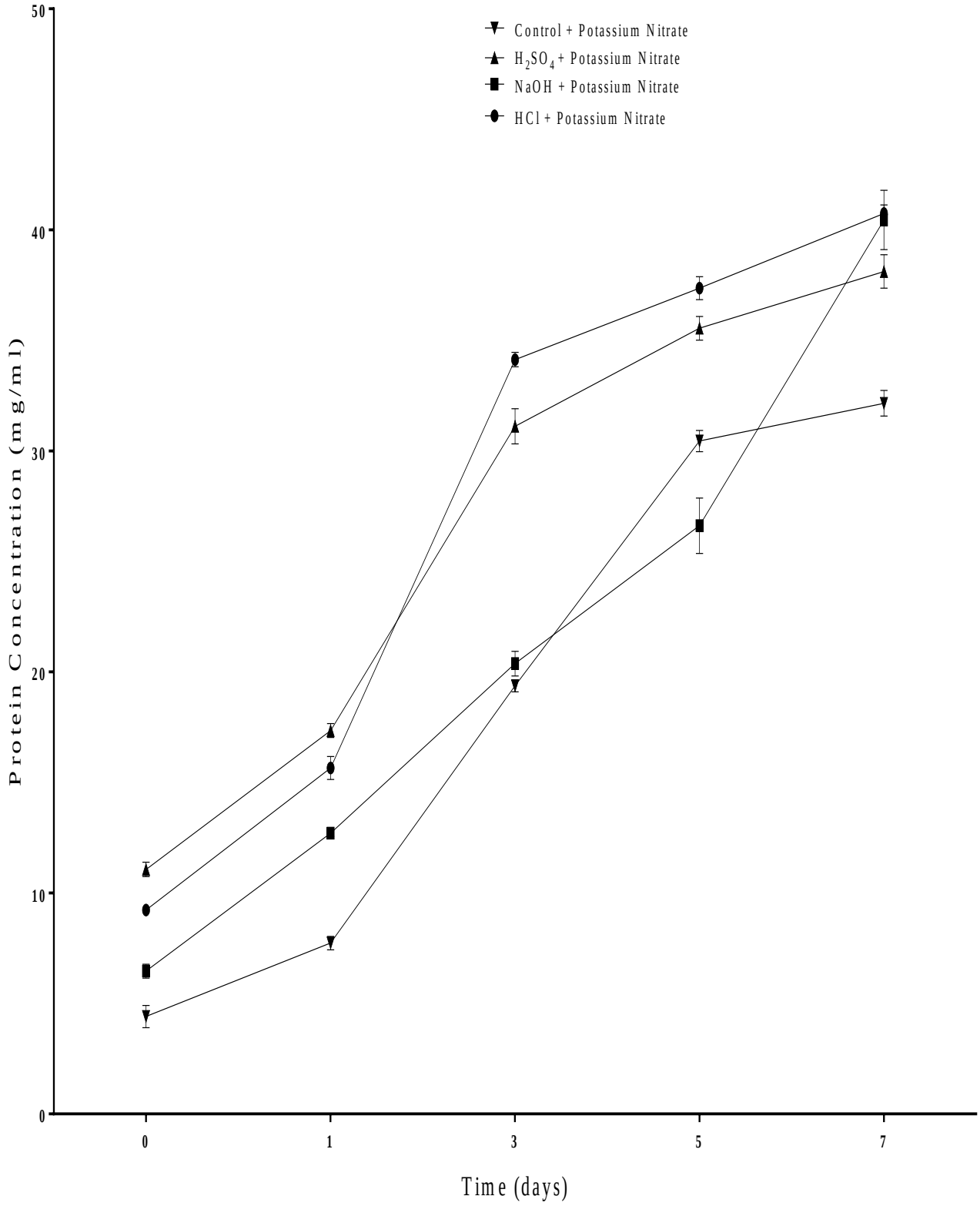


Figure 6: Protein concentration in hydrolyzed samples + potassium nitrate

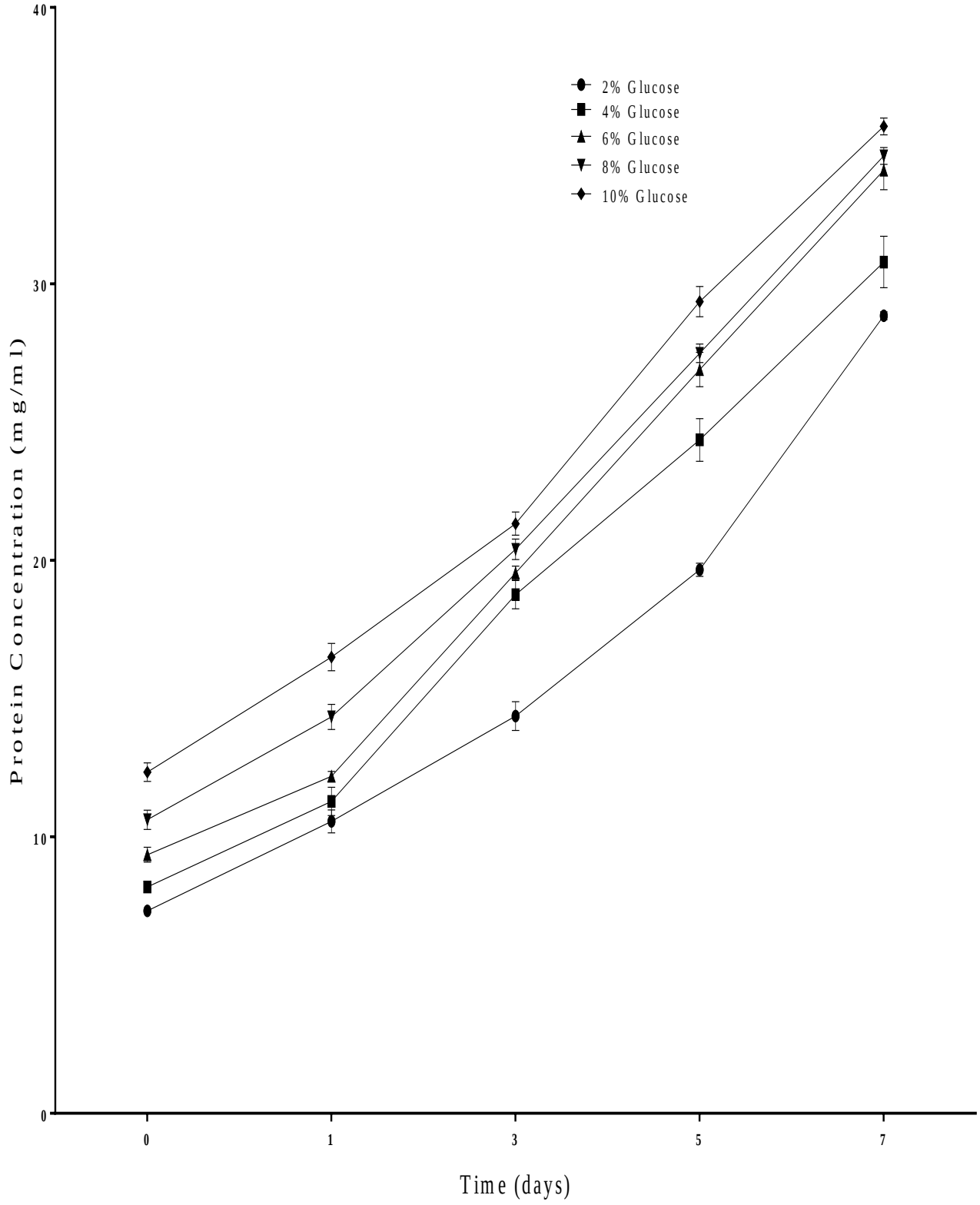


Figure 7: Protein concentration in HCl hydrolysate + different glucose concentrations

### 3.8.2 Effect of different glucose concentrations on protein content of $H_2SO_4$ hydrolysate

At the start of the experiment there was no significant difference ($p \leq 0.05$) in the protein content of the 2% glucose (6.43 mg/ml) and 4% glucose (6.87 mg/ml), also between 6% glucose (8.43 mg/ml) and 8% glucose (9.03 mg/ml). At day 1, day 5 and day 7 there was no significant difference between the value of 8% glucose and 10% glucose (Fig. 8).At day 3, there was significant difference between all the values. The protein content for 10% glucose (37.49 mg/ml) was the highest at the end of the fermentation but it was not significantly different from that of 8% glucose (36.81 mg/ml) and there was significant difference between the protein concentration of 6% glucose (27.91 mg/ml), 4% glucose (29.34 mg/ml) and 2% glucose (27.91 mg/ml) (Fig. 8).

### 3.8.3 Effect of different glucose concentrations on protein content of NaOH hydrolysate

Figure 9 shows the protein concentration in the media when different glucose concentrations were added to NaOH hydrolysate. At the start of the fermentation there was no significant difference between the values of the different glucose concentrations (2%, 4%, 6%, 8% and 10%). There was no significant difference ($p \leq 0.05$) between the protein yield of 6% glucose and 8% glucose at day 1 (11.99 mg/ml and 12.06 mg/ml) and day 5 (32.09mg/ml and 32.37 mg/ml) (Fig. 9).At the end of the fermentation (day 7) the protein content of 10% glucose (42.95 mg/ml) was the highest but it was not significantly different from that of 8% glucose (40.99 mg/ml) and there was no significant difference between 8% glucose (40.99 mg/ml) and 6% glucose (38.79 mg/ml).

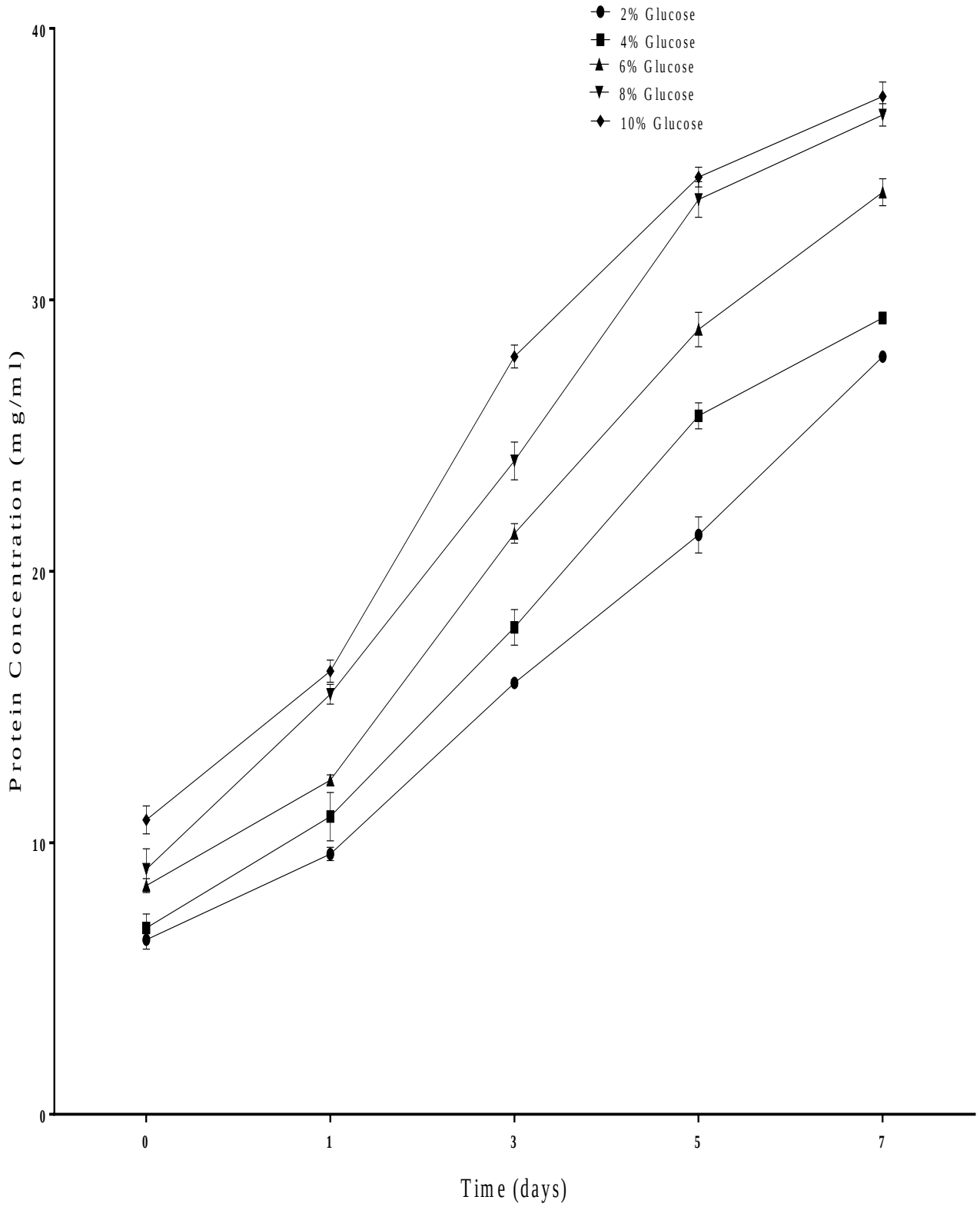


Figure 8: Protein concentration in $H_2SO_4$ hydrolysate + different glucose concentrations

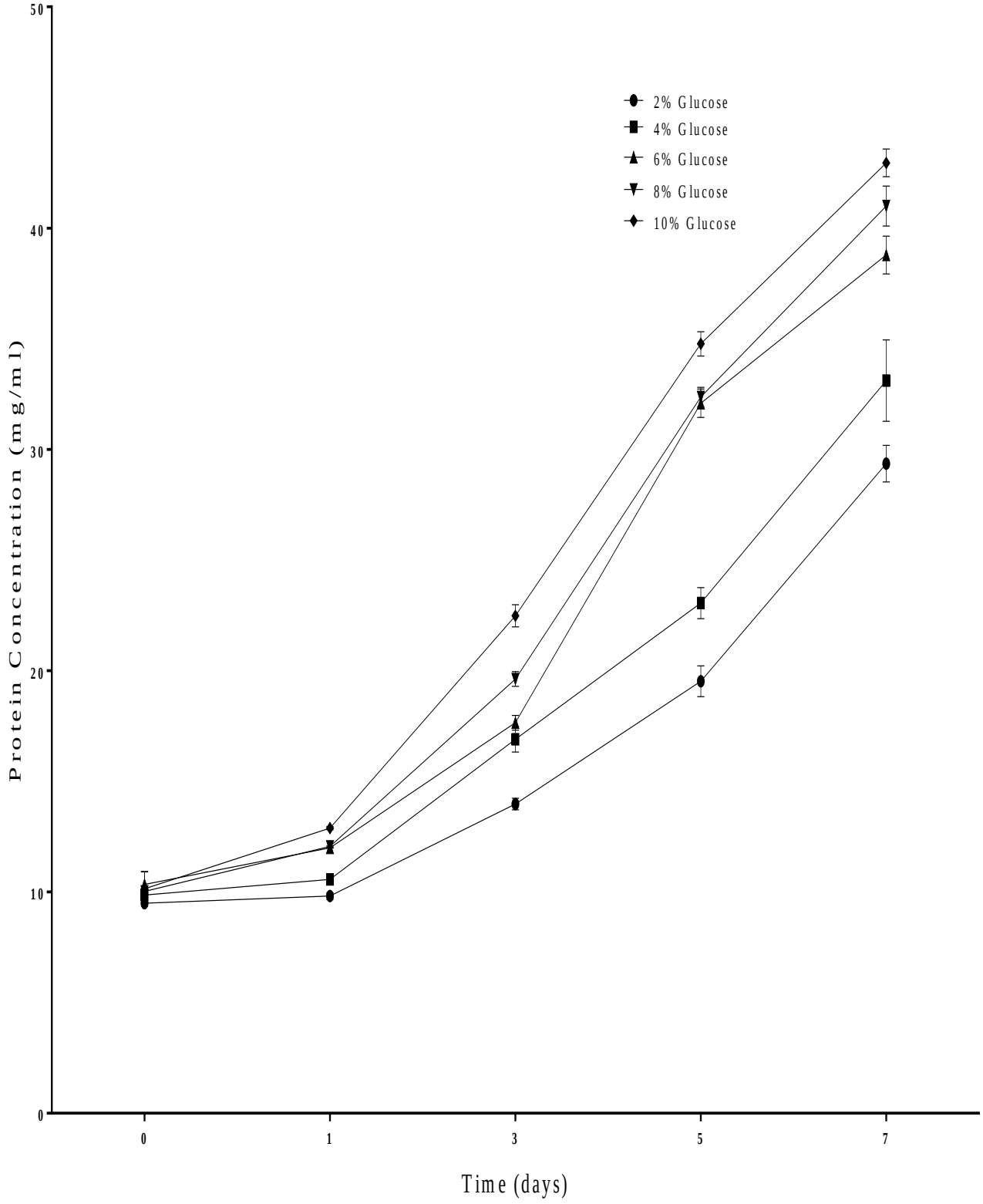


Figure 9: Protein concentration in NaOH hydrolysate + different glucose concentrations

### 3.8.4 Effect of different glucose concentrations on protein content of unhydrolyzed (control) sample

When different glucose concentration was added to the unhydrolyzed sample (control) to know it effect on the protein yield, 10% glucose produced the highest from 7.16 mg/ml to 29.19 mg/ml and 2% glucose produced the lowest from 4.18 mg/ml to 17.05 mg/ml (Fig. 10). At the start of the experiment (day 0), there was no significant difference between the protein yield of 4% glucose (5.32 mg/ml) and 6% glucose (5.52 mg/ml).There was no significant difference ($p \leq 0.05$) between the protein concentration of 2% glucose and 4% glucose at day 1(6.11 mg/ml and 6.47 mg/ml), day 5(13.46 mg/ml and 13.66 mg/ml) and day 7 (17.05 mg/ml and 17.59 mg/ml) respectively. The highest protein yield was from 10% glucose (29.19 mg/ml) (Fig. 10).

## 3.9 WEIGHT OF DRIED FUNGAL BIOMASS

The weight of the dried fungal biomass at the end of each fermentation were recorded and represented in Table 5. The highest weights recorded were from HCl + Sodium nitrate and HCl + Ammonium nitrate (0.66 g) while the lowest weights were from Control, Control + Ammonium oxalate, Control + Sodium nitrate, Control + 2% glucose and Control + 8% glucose where no biomass were harvested (Table 5). A higher weight which ranged between 0.45g – 0.57g for the following fermentation media; $H_2SO_4$ + 6% glucose (0.45 g), $H_2SO_4$ + ammonium oxalate (0.51 g), HCl + Sodium nitrite (0.51 g),$H_2SO_4$+ ammonium nitrate (0.54 g), HCl + ammonium oxalate (0.57 g) and HCl + potassium nitrate (0.57 g). Lower weight which ranged from 0.01 g – 0.15 g were also recorded (Table 5)

## 3.10 PROTEIN CONTENT IN DRIED FUNGAL BIOMASS

The protein content of the dried fungal biomass was measured. The dried fungal biomass contains 109 mg/ml protein concentration.

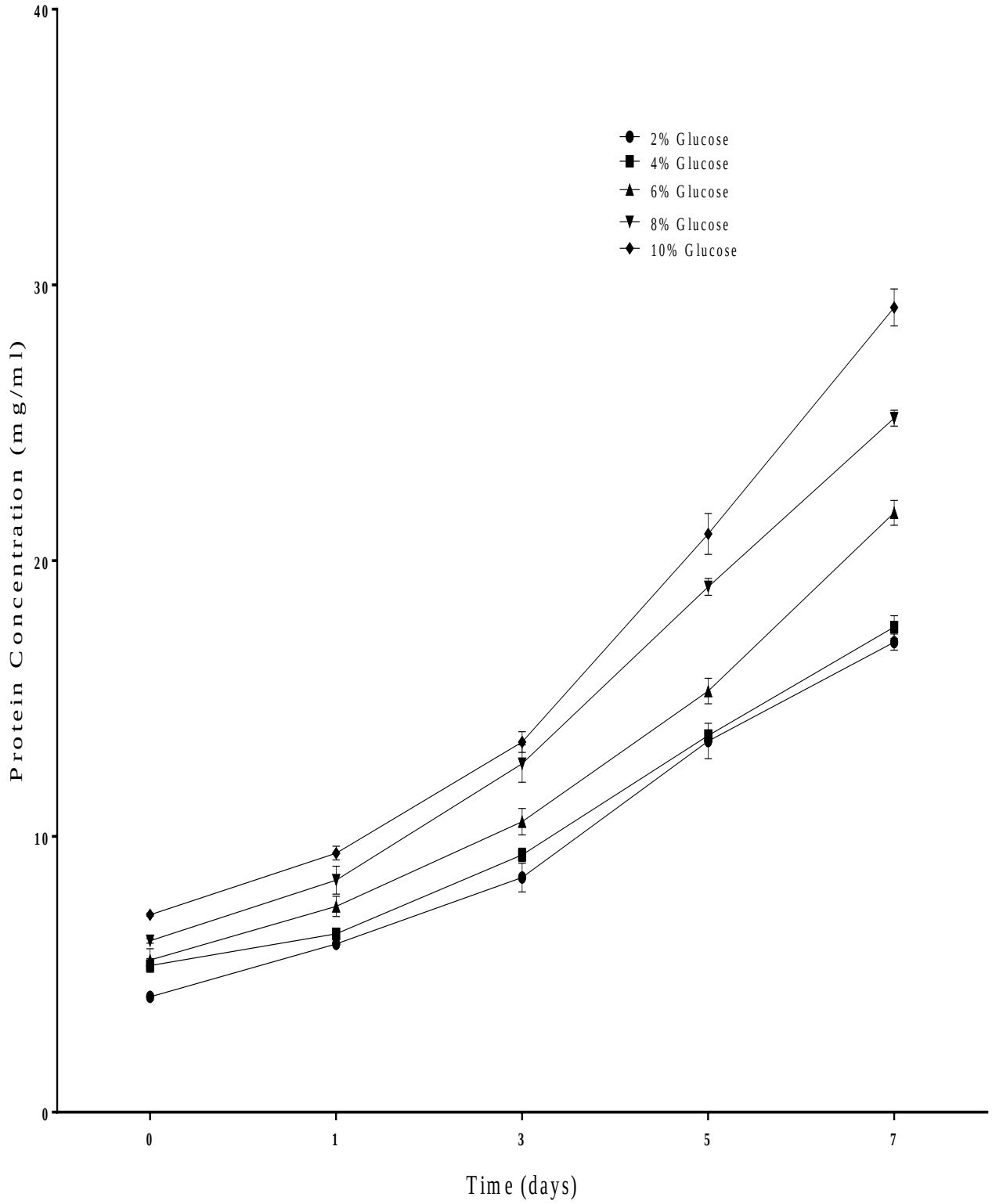


Figure 10: Protein concentration in control + different glucose concentrations

Table 5: Weight of dried fungal biomass at the end of fermentation

| Fermentation Medium | Biomass Weight (g/100ml) | Fermentation Medium | Biomass Weight (g/100ml) |
|---|---|---|---|
| HCl Hydrolysate | 0.09±0.001 | HCl + Potassium nitrate | 0.57±0.003 |
| $H_2SO_4$ Hydrolysate | 0.36±0.002 | $H_2SO_4$ + Potassium nitrate | 0.15±0.001 |
| NaOH Hydrolysate | 0.39±0.002 | NaOH + Potassium nitrate | 0.06±0.001 |
| Control | 0.00±0.000 | Control + Potassium nitrate | 0.03±0.001 |
| HCl + Ammonium oxalate | 0.57±0.003 | HCl + 2% glucose | 0.09±0.000 |
| $H_2SO_4$ + Ammonium oxalate | 0.51±0.002 | HCl + 4% glucose | 0.13±0.001 |
| NaOH + Ammonium oxalate | 0.39±0.002 | HCl + 6% glucose | 0.14±0.002 |
| Control + Ammonium oxalate | 0.00±0.000 | HCl + 8% glucose | 0.15±0.001 |
| HCl + Sodium nitrate | 0.66±0.004 | HCl + 10% glucose | 0.07±0.001 |
| $H_2SO_4$ + Sodium nitrate | 0.39±0.001 | $H_2SO_4$ + 2% glucose | 0.35±0.001 |
| NaOH + Sodium nitrate | 0.21±0.001 | $H_2SO_4$ + 4% glucose | 0.38±0.001 |
| Control + Sodium nitrate | 0.63±0.002 | $H_2SO_4$ + 6% glucose | 0.45±0.001 |
| HCl + Sodium nitrite | 0.51±0.001 | $H_2SO_4$ + 8% glucose | 0.21±0.003 |
| $H_2SO_4$ + Sodium nitrite | 0.21±0.001 | $H_2SO_4$ + 10% glucose | 0.36±0.001 |
| NaOH + Sodium nitrite | 0.09±0.002 | NaOH + 2% glucose | 0.15±0.002 |
| Control + Sodium nitrite | 0.00±0.000 | NaOH + 4% glucose | 0.27±0.001 |
| HCl + Ammonium nitrate | 0.66±0.003 | NaOH + 6% glucose | 0.29±0.001 |
| $H_2SO_4$ + Ammonium nitrate | 0.54±0.001 | NaOH + 8% glucose | 0.31±0.001 |
| NaOH + Ammonium nitrate | 0.12±0.001 | NaOH + 10% glucose | 0.36±0.001 |
| Control + Ammonium nitrate | 0.06±0.000 | Control + 2% glucose | 0.00±0.000 |
| Control + 4% glucose | 0.01±0.000 | Control + 6% glucose | 0.02±0.000 |
| Control + 8% glucose | 0.00±0.000 | Control + 10% glucose | 0.03±0.000 |

# CHAPTER FOUR

## 4.0 DISCUSSION

Several fungal isolates were screened for their ability to hydrolyze cellulose substrates namely, cotton wool, filter paper and carboxyl methyl cellulose (CMC). The 72 fungal strains tested produced different cellulase activities (Table 1 and Table 2). Culture supernatant from the fungal isolate designated IS 51 produced the best cellulase activities, this can be compared to the works of Nwokoro *et al*., (2013) where several fungal isolates were able to produce cellulase activities. The isolate IS 51 was identified as *Trichoderma viride* (Table 3) which has been used by several researchers to produce single cell protein (Durand *et al*., 1993; Bhalla *et al*., 2007; Zhang *et al*., 2008; Mojsov, 2010; Mortta and Santana, 2012; Zeng *et al*., 2016). Fungi have been used as a source of protein in food for centuries, *Aspergillus sp*. and *Trichoderma sp*. have been widely used for SCP production. The choice of fungi for SCP production is governed by many factors including quality and quantity of protein in cells and its lack of toxicity (Spicer, 1971). Accordingly to this specifications, three fungal strains namely- *A. oryzae* MTCC 1846, *A. niger* MTCC1842 and *T. viride* NRRL 1186 were selected for the work of Noomhorm *et al*., (1992). Hydrolyzed pineapple peel extracts contain variable ingredients with major amount of sugars and small amount of protein (Table 4). The result agreed with the observation of Dhanasekaran *et al*., (2011) and Sankar *et al*., (2011) that pineapple extracts contain variable ingredients and may be used as carbon and energy sources for the growth of fungi in the production of single cell protein. The carbohydrate and protein content of pineapple peels are an indication that the waste could serve as a possible alternative substrate for cultivation of fungi (Essien *et al*., 2003). The high moisture reducing and none reducing sugar and crude protein recorded in pineapple wastes makes it a better substrate for single cell protein production (Table 4). The result agreed with the

observation of Nwufo *et al*., (2014) that pineapple waste is a better substrate than other wastes products considered in the research. Also the high yield of biomass observed in pineapple further affirms its position as the best substrates for single cell protein production.

From the results, there were indications that *Trichoderma viride* had variabilities in the consumption of the different carbon sources of the hydrolyzed extracts (Figure 1). The highest and the lowest protein yield of the extracts were observed, highest growth and protein yield of NaOH (27.35 mg/ml) increased significantly at p (<0.05) than control extract (15.73 mg/ml), but the biomass yield were not significant. These differences could be attributed to the variable nutritional values which could serve as sources of nutrients for the growth of the mold in the production of single cell protein. These findings were in agreement with the observation of Bowen and Starper (1989) that there was a higher growth in pineapple peels that has a higher amount of proteins, minerals, vitamins and other soluble carbohydrates which served as source of nutrients. Rogers *et al.*, **(**1972**)** reported 13.3% dry weight crude protein after 4 d growth of *Aspergillus fumigatus* on alkali-treated cellulose. Peitersen (1975) obtained 21–26% dry weight (DW) crude protein by growing *Trichoderma viride* on alkali--treated barley straw for 2 –4 days. Several nitrogen sources were added to the samples, and the protein yields were higher than that of the media that was not supplemented with nitrogen sources. These could be as a result of nitrogenous sources which tend to supplement the nutritional status of the extracts and support the growth of *Trichoderma viride.* These findings were in agreement with the report of Emejuaiwe *et al*. (1998), that the addition of nutrient supplements provided available nitrogen source for the organism thereby enhancing its growth.

The media supplemented with ammonium oxalate [$(NH_4)_2C_2O_4$] gave the highest protein of 55.44 mg/ml for NaOH hydrolysate followed by HCl hydrolysate (48.01 mg/ml) for the samples

(Fig. 2), media supplemented with sodium nitrite ($NaNO_3$) and sodium nitrite ($NaNO_2$) also gave the highest protein of (43.27 mg/ml and 50.20 mg/ml) for NaOH hydrolysate followed by HCl hydrolysate with (41.66 mg/ml and 45.47 mg/ml) respectively for the samples (Fig. 3 and Fig. 4). The media supplemented with ammonium nitrate ($NH_4NO_3$) gave the highest protein of 46.23 mg/ml for $H_2SO_4$ hydrolysate followed by HCl hydrolysate (43.76 mg/ml) for the samples (Fig. 5). Lower protein yield were observed with media supplemented with potassium nitrate ($KNO_3$) which gave the highest protein of 40.72 mg/ml for HCl hydrolysate followed by 40.44 mg/ml for $H_2SO_4$ hydrolysate (Fig. 6). Statistical significant difference at p ($<0.05$) were detected in the highest protein yield of the extracts when supplemented with ammonium oxalate and ammonium nitrate than with potassium nitrate, sodium nitrate and sodium nitrite. This is in agreement with the work of Oshoma and Ikenebomeh (2005) where among all the nitrogen sources investigated, supplementation of the medium with ammonium sulphate [$(NH_4)_2SO_4$] gave the highest biomass yield 1.95±0.03g/l followed by 1.83±0.04g/l when supplemented with ammonium nitrate ($NH_4NO_3$ ). But, the biomass yield was lower when media were supplemented with potassium nitrate ($KNO_3$), sodium nitrate ($NaNO_3$), ammonium chloride ($NH_4Cl$) and control (with no nitrogen supplement).

Different concentrations (2%, 4%, 6%, 8% and 10%) of glucose were also added to the extracts and the protein yields were increased, for all the extracts 2% glucose gave the lowest protein yields while 10% glucose gave the highest protein yields (Fig 7, 8, 9 and10). This is in agreement with the research works of Oshoma and Ikenebomeh (2005); Ojokoh and Uzeh (2005) and Mondal *et al*., (2012) which clearly indicated that higher percentage of protein was found in yeast biomass when *Saccaharomyces cerevisiae* was grown on Glucose Supplemented Fruit Hydrolysates (GSF) indicating that biomass yield can be increased when a carbon source like

glucose is added to the medium. Similar observation had been reported by Yakoub and Umar (2010) with *Penicillium expansum*.

Weight of dried biomass after 7 days of fermentation were taken for each experimental medium (Table 5). Though, increase in the growth and proliferation of fungal biomass in the form of single cell protein (SCP) or microbial protein accounts for part of the increase in the protein content after fermentation (Raimbault, 1998), but there was no significant differences detected in the biomass content of extracts. The maximum amount of biomass produced 0.66g/100ml (6.6 g/l) in this study was higher than the average reported for *Candida utilis* (5.1 g/l), and *Trichoderma viride* WEBL0702 (5.54g/ l) grown on molasses, and winery wastewater treatment, respectively (Nigam, 2000; Zhang *et al*., 2008).

## 4.1 CONCLUSION AND RECOMMENDATION

On the whole, the bioconversion effect of pineapple waste into single cell protein was evaluated using *Trichoderma viride.* The supplementation of their extracts with different glucose and nitrogen sources increased significantly the growth and protein yield of *Trichoderma viride*. The highest protein content of pineapple extract (NaOH hydrolysate) was recorded with 10% glucose as the carbon source and ammonium nitrate as nitrogenous source. The present findings revealed that pineapple waste could be used as effective alternative carbon and energy source for SCP production.

This investigation has demonstrated that fungal biotechnology is an effective tool for the biodegradation of agro industrial wastes thereby reducing pollution. These fungi degraded the polysaccharide content of the peels, converting them to soluble sugars with significant increase

in protein. These biodegradable agro wastes can thus be incorporated into the production of food or animal feed reducing the use of corn in the diet of animals.

The carbohydrate content of the peels is high and when broken down, constitutes a good medium for fungal growth. The single cell protein synthesized by these isolates increased the crude protein level in the hydrolyzed peels. Therefore, the biological treatment using fungi should be employed on a large scale to further increase the nutritive values of these wastes so as to be able to include them in the diet of live stocks. The feed concentrate produced maybe dried to constant weight and stored in air tight condition for subsequent usage. It is recommended that extensive toxicological and acceptability tests should be performed before the product is approved for large scale consumption.

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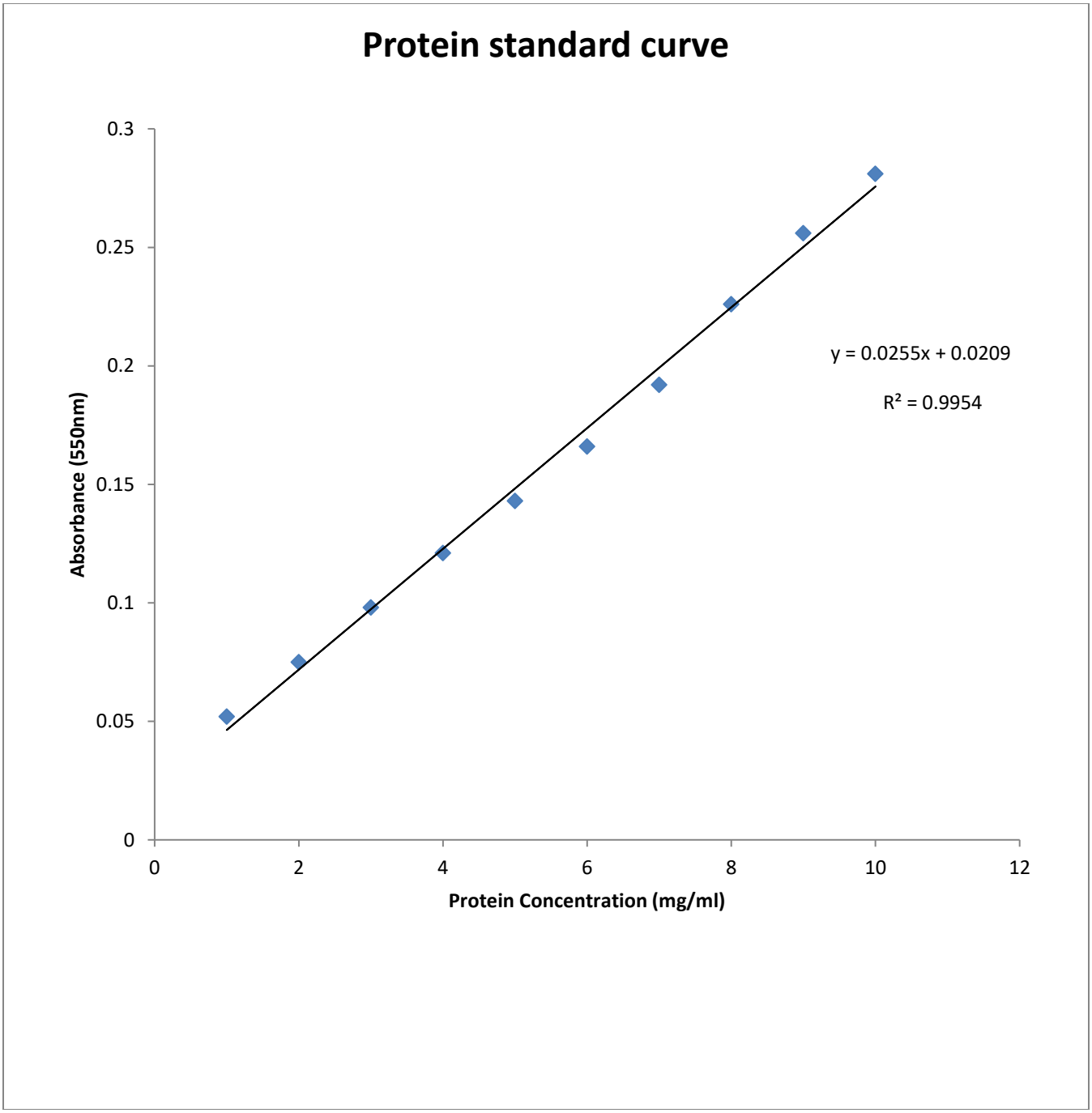
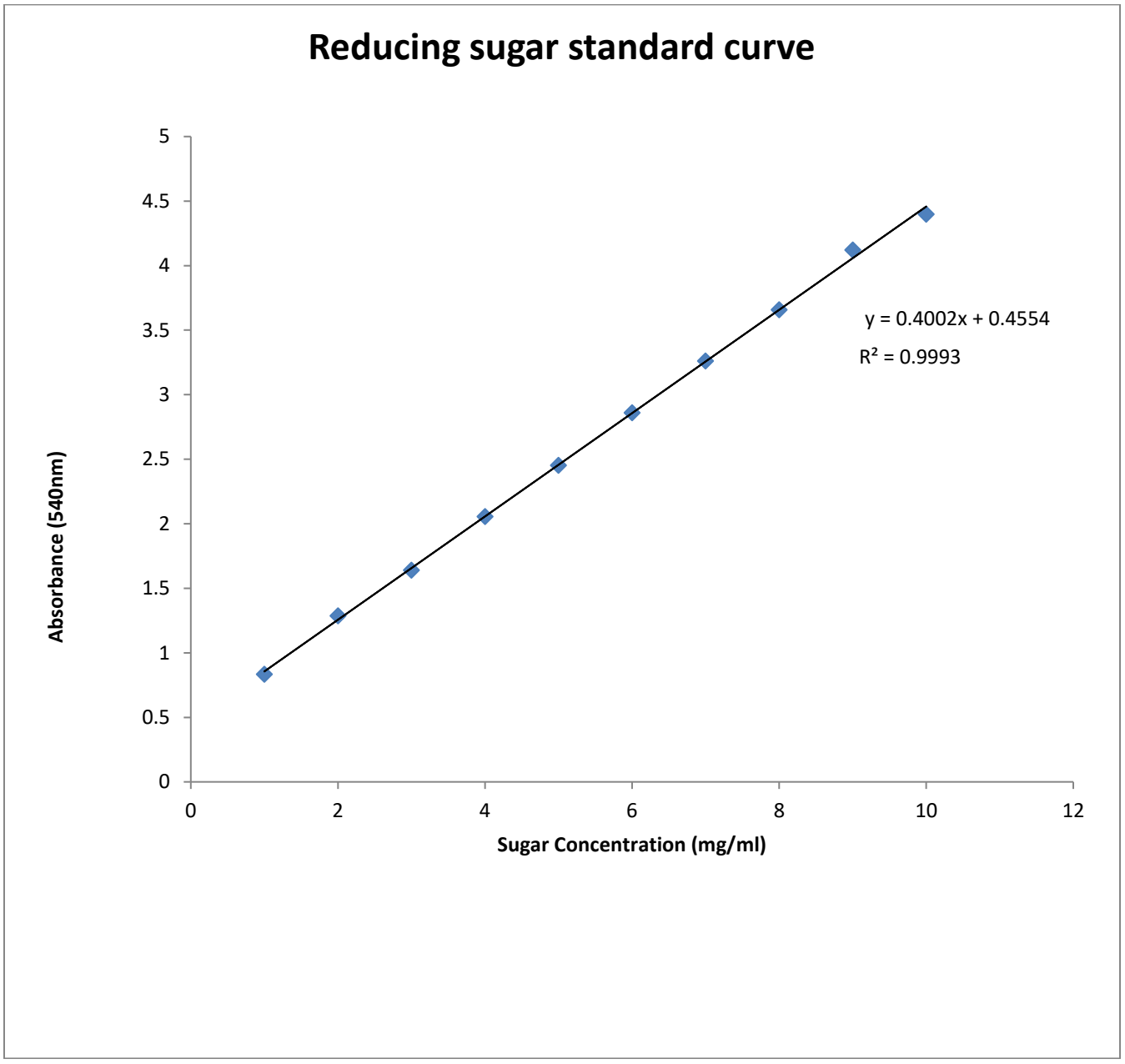
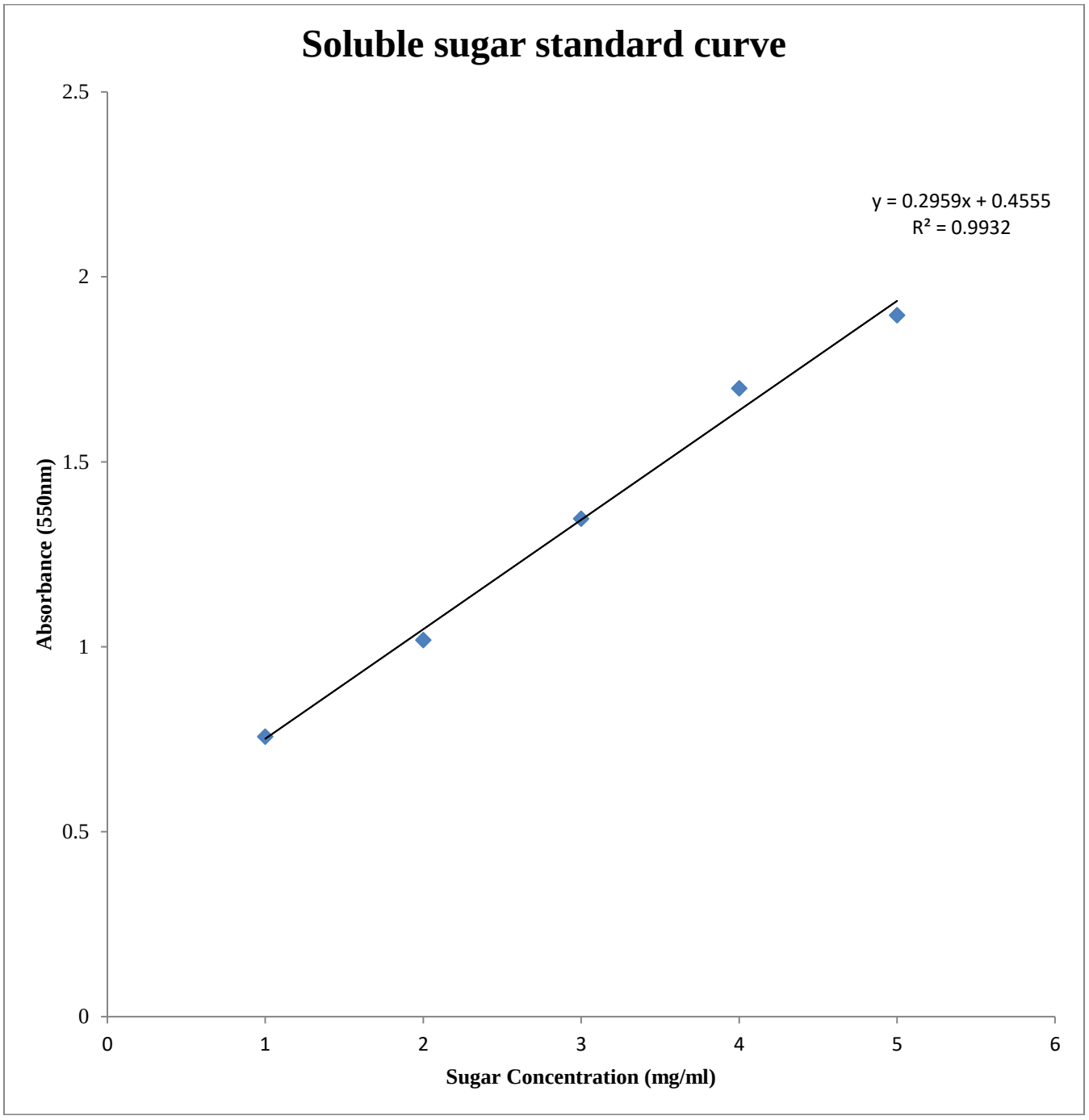
**APPENDIX**


Protein standard curve
Absorbance (550nm)
0.3
0.25
0.2
0.15
0.1
0.05
0
0
2
4
6
8
10
12
Protein Concentration (mg/ml)
y = 0.0255x + 0.0209
R² = 0.9954

**APPENDIX**


Reducing sugar standard curve
Absorbance (540nm)
Sugar Concentration (mg/ml)
y = 0.4002x + 0.4554
R² = 0.9993
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
0
2
4
6
8
10
12

**APPENDIX**


Soluble sugar standard curve
y = 0.2959x + 0.4555
R² = 0.9932
Absorbance (550nm)
Sugar Concentration (mg/ml)
0
0.5
1
1.5
2
2.5
0
1
2
3
4
5
6

**APPENDIX**

Analysis of variance (ANOVA) tables for figures

| | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| Day0 | Between Groups | 43.482 | 3 | 14.494 | 16723.910 | .000 |
| | Within Groups | .007 | 8 | .001 | | |
| | Total | 43.489 | 11 | | | |
| Day1 | Between Groups | 60.854 | 3 | 20.285 | 247.451 | .000 |
| | Within Groups | .656 | 8 | .082 | | |
| | Total | 61.510 | 11 | | | |
| Day3 | Between Groups | 156.529 | 3 | 52.176 | 536.287 | .000 |
| | Within Groups | .778 | 8 | .097 | | |
| | Total | 157.307 | 11 | | | |
| Day5 | Between Groups | 181.021 | 3 | 60.340 | 708.635 | .000 |
| | Within Groups | .681 | 8 | .085 | | |
| | Total | 181.702 | 11 | | | |
| Day7 | Between Groups | 133.356 | 3 | 44.452 | 1028.785 | .000 |
| | Within Groups | .346 | 8 | .043 | | |
| | Total | 133.702 | 11 | | | |

Figure 1: Protein concentration in hydrolysed samples

| | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| Day0 | Between Groups | 42.964 | 3 | 14.321 | 294.780 | .000 |
| | Within Groups | .389 | 8 | .049 | | |
| | Total | 43.353 | 11 | | | |
| Day1 | Between Groups | 205.974 | 3 | 68.658 | 992.168 | .000 |
| | Within Groups | .554 | 8 | .069 | | |
| | Total | 206.528 | 11 | | | |
| Day3 | Between Groups | 549.160 | 3 | 183.053 | 743.641 | .000 |
| | Within Groups | 1.969 | 8 | .246 | | |
| | Total | 551.130 | 11 | | | |
| Day5 | Between Groups | 503.224 | 3 | 167.741 | 566.439 | .000 |
| | Within Groups | 2.369 | 8 | .296 | | |
| | Total | 505.593 | 11 | | | |
| Day7 | Between Groups | 980.181 | 3 | 326.727 | 3617.572 | .000 |
| | Within Groups | .723 | 8 | .090 | | |
| | Total | 980.904 | 11 | | | |

Figure 2: Protein concentration in hydrolyzed samples + ammonium oxalate

| | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| Day0 | Between Groups | 42.889 | 3 | 14.296 | 140.367 | .000 |
| | Within Groups | .815 | 8 | .102 | | |
| | Total | 43.704 | 11 | | | |
| Day1 | Between Groups | 137.537 | 3 | 45.846 | 291.933 | .000 |
| | Within Groups | 1.256 | 8 | .157 | | |
| | Total | 138.793 | 11 | | | |
| Day3 | Between Groups | 715.860 | 3 | 238.620 | 1043.908 | .000 |
| | Within Groups | 1.829 | 8 | .229 | | |
| | Total | 717.689 | 11 | | | |
| Day5 | Between Groups | 427.149 | 3 | 142.383 | 429.717 | .000 |
| | Within Groups | 2.651 | 8 | .331 | | |
| | Total | 429.800 | 11 | | | |
| Day7 | Between Groups | 665.849 | 3 | 221.950 | 824.453 | .000 |
| | Within Groups | 2.154 | 8 | .269 | | |
| | Total | 668.002 | 11 | | | |

Figure 3: Protein concentration in hydrolyzed samples + sodium nitrate

| | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| Day0 | Between Groups | 47.013 | 3 | 15.671 | 286.011 | .000 |
| | Within Groups | .438 | 8 | .055 | | |
| | Total | 47.451 | 11 | | | |
| Day1 | Between Groups | 116.455 | 3 | 38.818 | 410.413 | .000 |
| | Within Groups | .757 | 8 | .095 | | |
| | Total | 117.211 | 11 | | | |
| Day3 | Between Groups | 625.964 | 3 | 208.655 | 862.269 | .000 |
| | Within Groups | 1.936 | 8 | .242 | | |
| | Total | 627.900 | 11 | | | |
| Day5 | Between Groups | 806.300 | 3 | 268.767 | 2199.101 | .000 |
| | Within Groups | .978 | 8 | .122 | | |
| | Total | 807.278 | 11 | | | |
| Day7 | Between Groups | 1379.180 | 3 | 459.727 | 1566.271 | .000 |
| | Within Groups | 2.348 | 8 | .294 | | |
| | Total | 1381.528 | 11 | | | |

Figure 4: Protein concentration in hydrolyzed + sodium nitrite

| | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| Day0 | Between Groups | 37.822 | 3 | 12.607 | 117.734 | .000 |
| | Within Groups | .857 | 8 | .107 | | |
| | Total | 38.679 | 11 | | | |
| Day1 | Between Groups | 165.107 | 3 | 55.036 | 607.849 | .000 |
| | Within Groups | .724 | 8 | .091 | | |
| | Total | 165.831 | 11 | | | |
| Day3 | Between Groups | 656.620 | 3 | 218.873 | 1720.591 | .000 |
| | Within Groups | 1.018 | 8 | .127 | | |
| | Total | 657.638 | 11 | | | |
| Day5 | Between Groups | 331.319 | 3 | 110.440 | 328.935 | .000 |
| | Within Groups | 2.686 | 8 | .336 | | |
| | Total | 334.005 | 11 | | | |
| Day7 | Between Groups | 665.240 | 3 | 221.747 | 416.726 | .000 |
| | Within Groups | 4.257 | 8 | .532 | | |
| | Total | 669.497 | 11 | | | |

Figure 5: Protein concentration in hydrolyzed samples + ammonium nitrate

| | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| Day0 | Between Groups | 77.887 | 3 | 25.962 | 222.297 | .000 |
| | Within Groups | .934 | 8 | .117 | | |
| | Total | 78.822 | 11 | | | |
| Day1 | Between Groups | 159.810 | 3 | 53.270 | 442.136 | .000 |
| | Within Groups | .964 | 8 | .120 | | |
| | Total | 160.774 | 11 | | | |
| Day3 | Between Groups | 502.593 | 3 | 167.531 | 595.348 | .000 |
| | Within Groups | 2.251 | 8 | .281 | | |
| | Total | 504.844 | 11 | | | |
| Day5 | Between Groups | 215.409 | 3 | 71.803 | 121.328 | .000 |
| | Within Groups | 4.734 | 8 | .592 | | |
| | Total | 220.143 | 11 | | | |
| Day7 | Between Groups | 143.556 | 3 | 47.852 | 48.491 | .000 |
| | Within Groups | 7.895 | 8 | .987 | | |
| | Total | 151.451 | 11 | | | |

Figure 6: Protein concentration in hydrolyzed samples + potassium nitrate

| | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| Day0 | Between Groups | 47.423 | 4 | 11.856 | 172.038 | .000 |
| | Within Groups | .689 | 10 | .069 | | |
| | Total | 48.112 | 14 | | | |
| Day1 | Between Groups | 70.868 | 4 | 17.717 | 96.469 | .000 |
| | Within Groups | 1.837 | 10 | .184 | | |
| | Total | 72.704 | 14 | | | |
| Day3 | Between Groups | 87.412 | 4 | 21.853 | 120.500 | .000 |
| | Within Groups | 1.814 | 10 | .181 | | |
| | Total | 89.226 | 14 | | | |
| Day5 | Between Groups | 168.721 | 4 | 42.180 | 145.089 | .000 |
| | Within Groups | 2.907 | 10 | .291 | | |
| | Total | 171.628 | 14 | | | |
| Day7 | Between Groups | 99.414 | 4 | 24.853 | 78.895 | .000 |
| | Within Groups | 3.150 | 10 | .315 | | |
| | Total | 102.564 | 14 | | | |

Figure 7: Protein concentration in HCl hydrolysate + different glucose concentrations

| | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| Day0 | Between Groups | 37.787 | 4 | 9.447 | 37.178 | .000 |
| | Within Groups | 2.541 | 10 | .254 | | |
| | Total | 40.328 | 14 | | | |
| Day1 | Between Groups | 100.189 | 4 | 25.047 | 105.673 | .000 |
| | Within Groups | 2.370 | 10 | .237 | | |
| | Total | 102.559 | 14 | | | |
| Day3 | Between Groups | 275.695 | 4 | 68.924 | 279.912 | .000 |
| | Within Groups | 2.462 | 10 | .246 | | |
| | Total | 278.157 | 14 | | | |
| Day5 | Between Groups | 365.601 | 4 | 91.400 | 281.856 | .000 |
| | Within Groups | 3.243 | 10 | .324 | | |
| | Total | 368.844 | 14 | | | |
| Day7 | Between Groups | 224.790 | 4 | 56.198 | 389.522 | .000 |
| | Within Groups | 1.443 | 10 | .144 | | |
| | Total | 226.233 | 14 | | | |

Figure 8: Protein concentration in $H_2SO_4$ hydrolysate + different glucose concentrations

| | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| Day0 | Between Groups | 1.251 | 4 | .313 | 1.459 | .285 |
| | Within Groups | 2.144 | 10 | .214 | | |
| | Total | 3.395 | 14 | | | |
| Day1 | Between Groups | 18.562 | 4 | 4.640 | 340.045 | .000 |
| | Within Groups | .136 | 10 | .014 | | |
| | Total | 18.698 | 14 | | | |
| Day3 | Between Groups | 120.468 | 4 | 30.117 | 173.692 | .000 |
| | Within Groups | 1.734 | 10 | .173 | | |
| | Total | 122.202 | 14 | | | |
| Day5 | Between Groups | 531.905 | 4 | 132.976 | 352.274 | .000 |
| | Within Groups | 3.775 | 10 | .377 | | |
| | Total | 535.680 | 14 | | | |
| Day7 | Between Groups | 384.413 | 4 | 96.103 | 79.815 | .000 |
| | Within Groups | 12.041 | 10 | 1.204 | | |
| | Total | 396.453 | 14 | | | |

Figure 9: Protein concentration in NaOH hydrolysate + different glucose concentrations

| | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| Day0 | Between Groups | 14.643 | 4 | 3.661 | 72.280 | .000 |
| | Within Groups | .506 | 10 | .051 | | |
| | Total | 15.149 | 14 | | | |
| Day1 | Between Groups | 22.266 | 4 | 5.566 | 58.141 | .000 |
| | Within Groups | .957 | 10 | .096 | | |
| | Total | 23.223 | 14 | | | |
| Day3 | Between Groups | 53.113 | 4 | 13.278 | 57.660 | .000 |
| | Within Groups | 2.303 | 10 | .230 | | |
| | Total | 55.416 | 14 | | | |
| Day5 | Between Groups | 135.881 | 4 | 33.970 | 132.731 | .000 |
| | Within Groups | 2.559 | 10 | .256 | | |
| | Total | 138.441 | 14 | | | |
| Day7 | Between Groups | 316.737 | 4 | 79.184 | 402.278 | .000 |
| | Within Groups | 1.968 | 10 | .197 | | |
| | Total | 318.706 | 14 | | | |

Figure 10: Protein concentration in control + different glucose concentrations