PERFORMANCE CHARACTERISTICS OF FUNGAL DEGRADED RICE BRAN AND PALM KERNEL CAKE SUBSTITUTED IN *Clarias gariepinus* FEEDS

BY

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ABSTRACT

Fish farming enterprise has increased in recent times due to public demand for fish protein. However, rising costs of fish feed ingredients have greatly reduced the profit margin of local fish farmers. Cheap, readily available and locally sourced ingredients may be better substitutes to the costly fish feeds. The aim of this study was to evaluate the performance of fermented Palm Kernel Cake (PKC) and Rice Bran (RB) as substitutes in the diet of *Clarias gariepinus*.

Fungi were isolated from Unfermented PKC (UPKC), RB (URB) and from Naturally-Fermented PKC (NFPKC) and RB (NFRB) ligno-cellulosic substrates using Potato Dextrose Agar (PDA). Isolates were identified and screened for enzyme production (amylase and cellulase) using standard methods. They were then subjected to physico-chemical (pH, temperature, incubation period, carbon and nitrogen sources) analysis to obtain optimal conditions for enzyme production. Proximate analysis of fungi-fermented samples was carried out to select the starters used in degrading the ligno-cellulosic substrates. Starters that gave the highest protein contents were selected. Starter-Degraded PKC (SDPKC) and RB (SDRB) were analysed for proximate and mineral compositions, amino acid profile, anti-nutrition factors, vitamins and heavy metals. The degraded and undegraded ligno-cellulosic bulks were substituted in the diets of fingerling and compared with a standard fish feed as control for three months. Fish performance characteristics [(Mean Weight Gain (MWG), Specific Growth Rate (SGR)] and Feed Conversion Ratio (FCR)) were evaluated. Data were analysed using ANOVA at p=0.05.

Ten fungi isolates obtained were *Aspergillus fumigatus*, *Aspergillus niger*, *Aspergillus clavatus*, *Aspergillus tamarii*, *Aspergillus terreus*, *Aspergillus versicolor*, *Rhizopus stolonifer*, *Rhizopus oryzae*, *Rhizopus* sp. and *Trichoderma* sp. Highest amylase (63.2units/mg) at pH 6.0, 50.0^{o}C, V_{max} 3.9 (UI/mL) and K_m 1.3 (g/mL) was produced by *Aspergillus niger* which grew best on wheat bran (8.0 mg/g) as carbon source and ammonium nitrate (4.6 mg/g) as nitrogen source. Highest cellulase (3.1Units/mg) at pH 8.0, 20.0^{o}C, V_{max} 0.9 (UI/mL) and K_m 0.8(g/mL) was produced by *Rhizopus oryzae* which grew best on RB (7.3 mg/g) as carbon source and potassium nitrate (1.6 mg/g) as nitrogen source. Selected starters, *Aspergillus clavatus* and *Aspergillus tamarii*, gave protein contents of 6.5% and 17.9%. Protein contents of NFPKC, NFRB, UPKC and URB were 5.7, 22.0, 5.2 and 16.0% respectively. Highest zinc composition (520.0µg/g) was recorded in SDPKC and SDRB with reduction in amino acid profile (16.7 – 0.03µg/g). Fermentation decreased oxalate (24.1-18.2 µg/g) among the anti-nutritional factors and had no effect on the vitamin contents, while no heavy metal was detected in the feeds. The MWG of fish fed with URB and UPKC ranged from 18.9g - 35.8g and that of SDRB and SDPKC ranged from 12.8 - 43.3g while that of the control feed was 31.5g. Fish fed SDPKC gave the highest SGR of 1.3g/day and the lowest FCR of 1.4. Significant difference in SGR was observed between fish fed with SDPKC and UPKC.

Fungi degraded palm kernel cake and rice bran supported the growth of *Clarias gariepinus* better than the control feed and could be good sources of fish feed components.

Keywords: Palm kernel cake, Rice bran, Fungal degradation, *Clarias gariepinus* feed.

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Julianah F. Aluko,

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CERTIFICATION

I certify that the research work reported in this thesis for the degree of Doctor of Philosophy (Microbiology) was carried out under my supervision in the Department of Microbiology, University of Ibadan, Ibadan.

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DEDICATION

I dedicate this work to my late husband, Dr. P. O. Aluko, my late father, Pa Benjamen Kolawole, my mother, Beatrice Aduke Kolawole, my sisters, brothers and my children – David Opeyemi, Deborah Oluwafunke and Daniel Oluwaseyi Aluko.

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ABBREVIATION

ADF = Acid Detergent Fibre

ADL = Acid Detergent Lignin

$A.NO_3$ = Ammonium Nitrate

$A.SO_4$ = Ammonium Sulphate

BDH = British Drug House

Ca = Calcium

Cd = Cadmium

CHO = Carbohydrate

Co = Cobalt

CSL = Corn Steep Liquor

Cu = Copper

DOC = Deoiled cake

EFA = Essential Fatty Acid

ES = Enzyme Substrate

F = Fermented

FCR = Feed Conversion Ratio

Fe = Iron

FF = Fish Feed

GRA = Generally Regarded As Safe

IndPKC = Industrial Palm Kernel Cake

K = Potassium

LFMPKC = Palm Kernel Cake from Local feed mill

LPKC = Palm Kernel Cake from local oil mill

Mg = Magnesium

MWG = Mean Weight Gain

NDF = Neutral Detergent Fibre

NFE = Nitrogen Free Extract

PKC = Palm Kernel Cake

PKCSD = Sludge of Palm Kernel Cake (Dry)

$PKCS_W$ = Sludge of Palm Kernel Cake (Wet)

PUFA = Polyunsaturated Fatty Acid

RB = Rice Bran

SBM = Soya Bean Meal

SFPKC = Starter Fermented Palm Kernel Cake

SFRB = Starter Fermented Rice Bran

SGR = Specific Growth Rate

UF = Unfermented

YE = Yeast Extract

CHAPTER ONE

1.0 INTRODUCTION

Feedstuff and animal protein supplies

The increase in human population globally over the last decades has influenced greatly the demand for food products of animal origin. In most developing countries including Nigeria, protein still remains grossly inadequate in the diet of the populace. Ademosun and Kalango (1987) reported that the levels of protein intake in Nigeria represents about one tenth in some advanced countries. Marquis (1985) estimated the animal protein intake of Nigerians to be about 6.5g per capital per day. This is far below the 25g of animal protein recommended (F.A.O. 1997). In consequence there is the need for a considerable development of animal production.

The production of aquatic animals, aquaculture, is currently the fastest growing animal production sector in the world (Ng, 2004). This must be supported by a corresponding increase in the production of formulated diets for the cultured aquatic animals. The world's dependence on its wild aquatic catch for food has become grossly inadequate. According to Food and Agriculture Organization (FAO, 1997), the rate of increase in the total food supply is generally less than the rate of increase in human population. This suggests that food fish is under-produced, thus necessitating an aggressive, intensive aquaculture (Akintomide *et al*., 2005).

The world fish catch for 1900, which was roughly estimated at 4million metric tons (mMT), rose to nearly 100mMT in 1993. About 93,6mMT of food fish was consumed by man from a global aquatic production figure of 118.2mMT in 1998 A.D, while 103mMT of food fish was consumed out of a total production of 132.2mMT in 2003 (F.A.O.,2004). A forecast of about 144mMT of aquatic products is estimated for the year 2010 A.DW by FAO (F.A.O., 1996). In 2025 A.D, when the world's human population is expected to be over 8 billion a total catch of about 165mMT is suggested (Foley, 2013). Nigeria, an African country, is rated by FAO as one of the main producers of fish in the continent. Her annual production figure is given as over 0.5mMT, although an estimate of about 1.2mMT of fish is required for her healthy living, that is less than 50% of the required annual fish intake is caught (F.A.O.,2003). Just about 30 thousand tones of the 0.5mMT are estimated as the country's active produce (fish) for 2003. Going by these data, both the local and international market

opportunities are underutilized (F.A.O., 2003).

One way to improve the supply of animal protein for human consumption is to increase the production of animal feed stuffs. The development of aquaculture is hampered by inadequate supply of feed stuffs which is scarce and expensive (Nwanna, 2003). In recent years, the cost of imported feed ingredients used in commercial aqua feeds in many developing countries has continued to rise due to increased global demand and fluctuation in foreign currency exchange value (Ng, 2004). Even though fish meal continues to be used as a major source of dietary protein in commercial aqua feeds, its escalating cost has stimulated much research into the use of alternative plant protein sources (El-Sayed, 1999).

1.2 Statement of problem

The ultimate goal of any livestock industry is the attainment of sustainable livestock production with minimum costs in the shortest time possible (Eruvbeline *et al*., 2002). This has proved difficult in the developing nations because of the dependency on some conventional ingredients that are either imported and/or expensive where they locally exist (Ojewole and Udom, 2005). For instance, fishmeal, an essential dietary animal protein component of animal feed is usually imported from Denmark, America and some other European countries. Soya bean meal, groundnut cake and some other plant protein sources have also become too expensive (Ojewole and Udom, 2005). This is as a result of the excessive demand for them, thus leading to a disproportionate increase in the costs of animal feed. It is also a known fact that, in the face of teaming population of the developing nations, the conventional protein and energy ingredients being used in feed production or livestock represents products that are better taken advantage of in human nutrition (Ardon *et al*., 1998).

The rising costs of imported ingredients such as fishmeal, soya bean meal; corn flour and wheat flour greatly reduces the profit margins of local fish farmers to such an extent that many local aquaculture enterprises are no longer profitable (Ng, 2004; FAO., 2012). Their products have become too expensive for the majority of the population in Nigeria and elsewhere (Esonu *et al.,* 2003; Tewe, 2003). This is especially true for the culture of lower-value fish species such as catfish, tilapia and carps. Oresegun *et al* (2007) remarked that the lack of reliable catfish feed is still a farmers' nightmare. The increase of popularity of catfish farming has attracted a new breed of investors into aquafeed production. Here is an increasing shift to the production of reliable catfish diets comparable to the imported aquafeed. In 2008, FAO observed that the greatest challenge in aquaclture is in high cost of

feed ingredients which makes the feed industries and farmers to compromise quality for affordability. Agricultural waste and by-products have been extremely employed in ruminant nutrition in many parts of the world as a substitute for concentrate feeds which is usually very expensive (Akinfemi, 2010). There is currently a great interest within the animal feed industry to reduce costs by using locally available feed ingredients.

Aquatic animals are common features in many fresh and marine bodies in the tropics (Fasakin, 2002). Such aquatic materials can constitute a natural resource pool from where dietary animal protein can be sourced (Mercer, 1992). Such aquatic animals include fish, crayfish, shrimps, crabs, squilla and toads. Often-time, by-products such as local fish meal, crayfish waste meal, shrimp waste meal, frog waste meal among others are prepared by the local people and are incorporated into animal feed as protein supplements so as to replace the high cost of conventional protein feed stuffs (Ojewola and Udom,2005). Another unconventional protein source being considered in recent times is the arthropods. Arthropods are insect groups that are known to be rich in crude protein and minerals (Aduku, 1993; Ojewola *et* al., 2003; Ojewola and Annah, 2005).

Fresh animal by-products, for examples blood meal, offal and rumen content, have been in use in the past together with pelleted feed for the rearing of fish. This practice has been forbidden due to health hazards it presents. However, fresh fish is still being used to some extent in feeding of the brood stock of marine fish. Animal materials, for example, ground liver or raw fish, is sometimes fed directly as wet-feed to fish. Unlike terrestrial animals, fish require diets with a high content of protein for good growth (F. A. O., 1987). This creates certain limitations concerning the number of raw materials that can be used as protein supplying fish feed ingredients. Plant material, for example, rice bran or macerated plant leaves, is often provided as a supplementary feed in extensive or semi-intensive fish farming (SFF, 1999).

The high demand for cereals especially maize, for feeding the nation's increasing human population and their use by millers for compounding livestock feed coupled with the need for livestock products have led to the use of unconventional feeds for animal production (lyayi and Tewe, 1994). These unconventional feed materials include sorghum spent grains, wheat offal as well as cassava peels. Food industry by-products and agricultural wastes used in feeding of monogastric animals can be of animal or plant origin. The use of non-conventional feedstuffs for livestock feeding has been demonstrated by Iyayi and Yahaya (1998) on

cassava peels, Abu *et al*. (2003) on sweet potato and Faniyi *et al*. (1997) on sorghum seed hulls. Non-conventional feedstuffs are rich in fibre and this account for their poor utilization in monogastric diets.

Cereals and cereal by products, despite their high carbohydrate contents, form an important component in aquaculture diets. The starch content helps to increase the water stability of the feeds; particularly when wheat is included during processing (New, 1987). Cereals also contribute significantly to the protein and lipid content of the diet. Though deficient in some amino acids (e.g. lysine), they can be used to balance high protein animal and vegetable ingredients. Cereals are often one of the cheapest raw materials that can be included in compound feeds for aquaculture (Gohl, 1981). The increase in human population coupled with high demand for cereal as global human diet require an immediate replacement of this important food in animal feed production.

1.3 Justification

To provide an alternative source of fish feed to avoid the rising cost of conventional feed ingredients. To utilize agriculture (lignocellulosic) by-products, for example rice bran (RB) and Palm kernel cake (PKC) as fish feed to avoid competition of feedstuff with human beings. To reduce the environmental impact from the disposal of these lignocellulosic by-products. To enhance the nutritive value of RB and PKC by subjecting them to fungal degradation (solid state fermentation), to digest the Non Starch Polysaccharides (NSP) so as to improve the bioavailability of their nutrients and increase their protein contents.

Hence the current work is focused on development of alternative ingredient to compound fish feed in replacement of conventional cereal.

1.4. Aims and Objectives of the Project

Based on the above-stated justification, the current work is aimed at achieving the following objectives:

Isolation and identification of fungal species from spontaneously – fermenting rice bran and palm kernel cake.

Physiological characterization including enzymatic studies on the isolated fungi on both rice bran and palm kernel cake.

Nutritional analysis of products of physiological activities of the isolates on the substrates.

Evaluation of the performance and other characteristics of fungal-degraded bulk of these lignocellulosic substrates substituted in the diet of *Clarias gariepinus*.

CHARPTER TWO

2.0 LITERATURE REVIEW

2.1 Fish feeds

Fish, like other animals are fed on adequate quantity diets to improve their growth rate and general production performance. Standard quality feeds found to be expensive have been developed to meet the nutritional needs of some fish species (Rottmann *et al*, 2003). The cost of feeding adequate quantity of good quality diet to fish that are intensively farmed, often forms the larger percentage (often greater than 60%) of the total cost of production. This implies that the cost of feeding will greatly influence the productivity and economics of aquaculture. So, if the cost of feed input is considerably reduced without compromising the final feed quality, the business of farming fish will become more rewarding and less capital intensive, thus being more encouraging to farmers (Akintomide *et al.*, 2008).

There are two possible ways of grouping fish feeds- Natural or artificial feeds and Conventional or Unconventional feeds.

2.2 Natural and Artificial Fish Feeds

2.2.1. Natural Fish Food

Any food material produced by nature as live fish food consists of tiny plants (phytoplankton) and animals (zooplankton) that live in water. Some edible, small water food plants produced by nature for fish are algae, floating duckweeds, leaves, lupin and yeast. Some natural live fish food animals living in water include moina, artemia, daphnia, rotifers, copepods, krill, water insects and worms. Other aquatic food animals are water snails, tadpoles of frogs/toads and small-sized fish (Fermin and Bolivar, 1994).

2.2.2. Artificial/Formulated Fish Feeds

Artificial fish feeds are produced as concentrates (often termed as `*Aqua feeds*') that come in diverse forms and sizes, depending on the size, age group, feeding pattern and environment of the fish in question. *Aqua feeds* are produced from a calculated selection of natural food materials, synthetic products, their by-products and/or wastes. The concentrate ingredients may be a combination of meal/gluten of grains (mainly wheat & corn), fish meal, poultry by-products, oilseed by-products (such as full-fat soy, soybean meal, groundnut cake, cotton

seed cake and sunflower meal), di-calcium phosphate salt and premixes. Standard fish feeds are often packaged and sold as dry or semi-solid granulated, flaked or capsulated feeds, which may float or sink in water (New, 1995).

2.3. Conventional and Unconventional Feeds

2.3.1. Conventional Fish Feeds.

These are fish food materials that are generally accepted for use as fish food or in feed production. Such materials include some natural fish food and feedstuffs used in artificial feed production. These foods include artemia rotifer and daphnia. Some widely accepted feed items for *aqua feeds* production are cornmeal, fish meal and soybean meal (Francis-Floy, 2002).

2.3.2. Unconventional Fish Feeds.

Feedstuffs that may be incorporated as constituents of fish concentrate or directly used as fish food, but are generally unacceptable as standard fish food, and their use is often restricted to few localities. Feed items that are presently being utilised in concentrate formulation were at one time or the other unconventional. The need to source for cheaper (unconventional) feeds of high dietary values arose from the expensive nature of commercial fish diets and conventional feed ingredients that are used in the formulation. Unconventional feedstuffs may either be sourced as edible by-products, wastes derived from human food materials untapped, poorly utilized resources or specially produced as fish feed materials (Akintomide *et al*., 2008).

2.3.3. Sources and Examples of unconventional feedstuffs

Some identified feed items that may be considered for use in feed production include the following:

Poultry: Some poultry by-products that may be gainfully processed and utilised in the production of fish feed include runts, very weak birds of low survivability, carcasses of dead birds. Other wastes of processed birds are intestines and other visceral organs, feathers, heads, legs faecal products and cracked eggs. By-products/wastes obtainable from poultry sources as fish feed materials, are relatively common and generally high in protein content (Sackey, 1989). Hatchery by-products and wastes may also serve as useful feed materials, especially as good protein sources in formulated feed. Some notable examples of hatchery

wastes are infertile or unhatched eggs, dead-in-the embryo, very weak or dead chicks and egg shell (as calcium source) (New *et* al., 1994)

Abattoir: Animal by-products and wastes such as blood meal, offal, rumen content and `fairly' unwholesome meat may be processed and recycled in a hygienic way as fish feed items (Akintomide *et al*., 2008).

Plants and Animals: Plants that may be considered for use as feed ingredients include aquatic plants (duckweed, water velvet and water hyacinth), mushroom, sunflower and breadfruit. Tomatoes, vegetables, melon seed, albizia seed and leaves of potatoes, pawpaw, cassava and banana/plantain may also be considered for use (Francis-Floyd: 2002). In cases of animals including some aquatic animals (water crustaceans, water insects & trash fish), microworms, earthworms, insects (flying reproductive termites and farm flies), tadpoles, snails and rodents (mice, rats and guinea-pigs) are utilized as feed ingredients (Sackey, 1998).

Food Processing Industries: Some useful wastes that may be obtained from food processing industries are broken rice, biscuit dust/waste, baby cereal waste, corn flakes waste, wheat flour dust, bakery waste and cassava flour/fakes waste. A number of these wastes may also serve as good feed binders. Other useful materials include milk dust/waste, fragmented peanut, industrial puree wastes (e.g. tomatoes puree waste), fruit residues obtained from fruit juice industries and some eatery residues. Some of these items may serve as good energy substitutes in place of grains (corn and wheat), some as protein sources, while some others may serve as vitamin/mineral supplements (Bennke, 2002).

2.3.4. Advantages and Disadvantages of using Unconventional Feeds

Unconventional Feeds including cheap source of protein and energy, when carefully selected, bring about least cost of production, thus ensuring maximum profit. Commercialization of such feed items may be money spinning e.g. duckweed, earthworm, tadpole, insect and snail (offal) meals, more investors will be encouraged to farm fish as a result of the reduction in input and an increase in profit margin, encourages waste re-cycling, thus ensuring less environmental pollution and provision of more varieties of feedstuffs to select from (Akintomide *et al*,. 2008).

On the other hand, the use of unconventional feedstuffs may be strenuous and time consuming. Disease may be introduced through the use of unprocessed or improperly processed contaminated wastes e.g. feeding fish cultured in poultry wastes, increase in labor

strength, as more hands may be required for the production or harvesting and processing; extra cost may be incurred in analyzing feed materials that will be used in concentrate production; product acceptability by consumers may at times be a problem e.g. the use of maggot meal in production and more land space may be required for processing (Akintomide *et al*.,2008).

2.3.5. The Economics of using Viable Unconventional Feedstuffs

Cultured fish may be economically raised on a combination of some conventional and unconventional feed materials. Unconventional feedstuffs to be used must be locally available, palatable to cultured fish and easy to produce in large quantities. The overall production cost per kilogram of feed should be cheaper than what was previously obtained before the substitute. Such substitute, should, at least have a similar nutrient value (especially the protein content) to the feed item replaced (Dominy, 2004).

2.4. Agro-industrial by-products

The incorporation of agro-industrial by-products (AIBs) in animal feeds holds tremendous potentials in alleviating the existing critical situation of high cost and inadequate supply of feeds (Longe, 1985; Babatunde, 1989). AIBs are abundantly produced and processed in Nigeria (Fetuga and Tewe, 1985; Omole and Tewe, 1989). In Nigeria the introduction of Structural Adjustment Programme (SAP) in 1980s brought about increasing trend in local food processing and consequently more AIBs. Most of the latter are derived from oil palm, tapioca and sugar cane production. AIBs are used increasingly now in both ruminant and monogastric diets, but they still represent a small proportion of the total diet. Longe (1985), Omole and Tewe (1989) enumerated the available AIBs as oil seed meal, abattoir by-products, poultry by-products, brewery by-products, milling by-products, fruit wastes, starch and sugar processing by-products.

Other agro-industrial residues include rice bran, folder yeast, coconut press cake, sago meal and waste, soy sauce, soybean curd and dairy wastes, plant wastes, cassava, yam and potato peels, biscuit meal and cocoa by-products. Reddish and Scarr (1987) put the supply of AIBs at 738, 217.6 tons per annum in Nigeria.

2.4.1. Rice Bran (RB)

This is the dry, outer cover of rice grain which is always removed during the milling of rice. It accounts for 5-8% of the rough rice weight (FAO, 1964; Houston, 1972). As in other agro-industrial by-products, rice bran is of no direct nutritional value to man. In most mills it is often discarded and allowed to rot away. In some areas, it may be collected and used as litter material or fire making. World rice production is greater than the production of any other single crop (Carter *et al*., 1974).

Since rice bran makes up to 40% of parboiled rice, Nigeria has the potential to produce about 200,000 metric tons of rice bran from 500,000 metric tons of rice produced annually in Nigeria (Wudiri, 1992). As regards its usefulness in animal feed, RB contains 2.9 – 3.6% crude protein, 0.8 – 1.2% ether extract and 39 – 42% crude fibre and 15 – 22% ash (Oyenuga, 1968). RB can best be described as low roughage with a high fibre and lignin content. Such feedstuff when used in animal diets may result in poorer growth than a less fibrous feed (Shqueir *et al*., 1982).

Rice bran is a good source of B group of vitamins. It is the material scoured off before initial rice polishing. It has higher protein content than the original grain and, in the unextracted form, has a high lipid level which is prone to rancidity (Gohl, 1981). Rancid rice bran is much reduced in feed value. The oil is often extracted for human use and the resultant rice bran is favored by feed millers because of its keeping quality. The fibre in extracted rice bran absorbs water and leads to a water unstable pellet (Gohl, 1981).

2.4.1.1. Food use:

Very little data are available on the nutritive value of rice bran done at comparable dietary protein levels. The direct use of rice bran as food has been limited. The addition of 20g of rice bran (0.62g of N) to a white bread diet containing 12.74g of N per day for nine adult women indicated trends towards decreased urinary nitrogen and increased fecal nitrogen excretions (Jank *et al*., 1981). This suggested a slight decrease in protein utilization when bran was incorporated in bread product (Jank *et al*., 1981).

2.4.1.2. Pharmaceuticals:

Historically, the high concentration of the B-group of vitamins in rice bran played a major part in advances in nutrition, medicine and chemistry (Houston, 1972).

Talwalker *et al.* (1965) reviewed the preparation of pharmaceuticals from rice bran. One-step 95% ethanol extraction of RB gave a vitamin B-complex concentrate, and phytin was recovered from extraction of residual bran with 2% hydrochloric acid. Direct saponification of 95% ethanol extract yielded tocopherol concentrate, B-sitosterol, fatty acid, and phytin, with loss of the vitamin B-complex. Two-step extraction with acetone and 95% ethanol followed by saponification of the extracted lipids gave tocopherol concentrate, B-sitosterol, fatty acids, phytin, lecitin, and vitamin B-complex. Only 290-320mg of tocopherol and 500-600mg of sitosterol were recovered per kilogram of bran, together with 5-8g of phytin and 30-37g of fatty acids by direct saponification. The two-step extraction gave 5-8g of phytin, 300-320mg of tocopherol, 120g of free fatty acids, 400-600gmg of B-sitosterol, and 2 - 2.5g of phosphatide (lecithin).

In spite of its abundance, RB has been neglected by the nutritionist because it contains a high level of fibre and is low in protein (Dafwang and Shwaren, 1996) but RB is however rich in oil and is a fairly good source of energy when fed at 5 – 20 level to growers and adult birds (Longe, 1985). Although rice bran is a rich source of vitamin B, oil and good quality protein, it is also rich in anti-nutritional factors such as phytin silica dietary fibre trypsin inhibitor and lectin (haemagglutinin). Bran from parboiled rice is richer in oil (20-25%) but poorer in B vitamins (Kik and Williams, 1945) than bran from raw rice.

2.4.2. Palm Kernel Cake (PKC):

The oil palm (*Eleais guineensis* Jacq) is indigenous to the humid tropical rainforest belt of the West African sub-region where Nigeria is located. It is also adapted to other tropical areas of the world where the soils and climatic regimes of rainfall and sunshine are adequate (Gere, 2004). The cultivation of oil palm has contributed immensely to Nigeria's economy especially before the commencement of petroleum exploration in the 1970s. It is ranked high among the oilseed crops, which provide not only food (fats and oils) and fibre for domestic uses but also raw materials for the food, soaps and detergent, cosmetic and pharmaceutical manufacturing industries (Gere, 2004).

Palm Kernel Cake (PKC), which is a by-product of the African Palm Oil industry, is a possible optimal feed ingredient for animals. The African Palm Oil industry is a stable source of vegetable oil worldwide.

The African oil palm (Elasis *guinensis*) is extensively cultivated in tropical countries and produces more oil per hectare than any other oil – producing plant species (PORLA, 2000). Palm Kernel Cake is the shaft obtained after the extraction of oil from palm kernel. The global production of palm kernel cake (PKC), a by-product of oil extraction from palm kernel, is ever increasing due to the tremendous growth of the oil palm industry in many parts of Asia and Africa (Porla, 2000). The oil palm industry produces about 270,000 tons of palm oil each year (Gere, 2004). The by-products include about 1 million tons of liquid effluents, 270,000 tons of empty bunches, 100,000 tons of pericarp fibre, 160,000 tons of shells and 80,000 tons of palm kernel cake (Porla, 2000). The cake is used in formulated animal feeds. The palm oil sludge may have a potential use in animal feed.

Many plants are grown specifically for the oil which their seeds or fruits produce and which is utilized for human food and other purposes. Vast quantities of by-products from the vegetable oil industry are produced and these form the staple ingredients of animal feedstuffs, being high in protein and low in carbohydrate. All are potential ingredients of aquaculture feeds. Examples of the plants from which products in this category come are the leguminous plants such as soybeans and groundnut, together with mustard, rape, sunflower, coconut, kapok, cotton, oil palm, linseed, poppy, sesame (gingelly) and Para rubber (caoutchouc). The palm kernel cake (African oil palm) is high in protein and saturated fats (Gohl, 1981).PKC which is a by-product of palm oil extraction is an alternative feedstuff that holds some promise if the cultivation of the crop from which it is derived is substantially increased (Tewe, 2003).

Palm kernel meal is abundant in many tropical regions and is a cheap feeding stuff imported by many countries (Ojewole and Ozuo, 2006). PKC is an established feed ingredient for ruminants, supplying valuable dietary sources of protein, energy and fibre. PKC and maize offal are known to have almost similar crude protein levels and are used indiscriminately as feed supplements by local farmers. PKC has also been successfully tested in poultry and swine feeds at low levels of incorporation (Onwudike, 1986; Agunbiade *et al*., 1999). Even though Oyenuga (1968) and Babatunde *et al*. (1975) had earlier reported PKC meal to be unacceptable to pigs and cast doubt on its acceptability by other non-ruminant animals. These

views were based on the high fibre level of PKC (an agriculture by-product) and its gritty nature, which were reported to reduce digestibility, palatability and possibly the availability of nutrients, especially amino acids (Onwudike, 1986; Olomu, 1995).

The low cost and availability of PKC in many tropical countries where aquaculture is practiced have recently generated much interest in its potential use in fish diets. Very little information is currently available on the use of RB and PKC in fish diets.

2.5. Constraints in the use of AIBs.

Longe and Ogedengbe (1989) reported fibrousness of AIBs as the major cause of under-utilization for monogastric feeding. El-Hag and Kundi (1986) identified bulkiness, poor nutritive value for its unsuitability for direct animal use. Further reasons for under-utilization of AIBs include lack of biological screening, contracting responses of animals of different species, physiological state and age. Compositions of the different AIBs along with distance from area of production to area of utilization are reasons limiting their utilization.

As with most plant-based and oilseed meal ingredients, several factors can limit the incorporation of PKC and RB in fish diets. These include relatively low protein content, possible amino acid deficiencies and presence of anti- nutritional factors.

As earlier mentioned above, among the factors which limit the incorporation of PKM into fish diets are its low protein content (Siew, 1989) and the presence of a high level of non-starch polysaccharides (NSPs) in its cell wall materials (Dusterhoft and Voragen, 1991). These NSPs are known to impair the digestibility and utilization of nutrients present in plant feed ingredients such as PKM either by direct encapsulation of the nutrients or by increasing the viscosity of the intestinal content, thereby reducing the rate of hydrolysis and absorption of nutrients (Chort and Annison, 1992).

2.5.1. Anti-nutritional Factors in Rice Bran

Anti-nutritional factors are concentrated in the bran fraction of rice. Most of them are protein in nature and thus heat-labile except for phytin.

2.5.1.1. Phytin:

Phytin is located in globoids of the aleurone protein bodies as potassium and magnesium salt (Bienvenido, 1985). Its phosphate groups can readily complex with cations such as calcium,

zinc, and iron and with protein. Rice bran has a higher phytin content than wheat bran, corn bran, soy barn and oat hulls (Thompson and Weber, 1981). When these cereal fractions were included as fiber at 6% w/w of a chick diet, only rice bran reduced body growth and deposition of zinc, iron and manganese in tibias, which may be explained by the higher phytin acid level in the rice bran diet (1.3%) as compared to that in the other diet (0-0,4%) (Houston: 1972).

A heat-stable factor of pepsin inhibition present in both bran and germ was identified as phytin (Kanaya *et al.*, 1976). A dietary phytate-zinc level is near the minimum requirement of 10-12 mg/g (Morris and Ellis, 1980). Increasing dietary calcium level from 0.75 to 1.75% results in growth depression at phytate-zinc molar ratio >6, indicating that high dietary calcium reduces zinc bioavailability.

2.5.1.2. Trypsin Inhibitor:

Trypsin inhibitor has also been isolated and characterized from rice bran (Tashiro and Maki, 1978, 1979; Maki *et al*, 1980). It is rich in basic amino acids (lysine, arginine and tryptophan), aspartic acid, glutamic acid, praline and cystine (Tashiro and Maki, 1979b) and is an albumin, not a prolamin (Tashiro and Maki, 1979a). Rice bran trypsin inhibitor is a double–headed inhibitor having two different and independent reactive sites based on binding experiments (Maki *et al*., 1980). The partially purified inhibitor was stable at acidic and neutral pH and retained more than 50% of its activity after 30min of incubation at 90^{o}C at pH 2 and 7 (Tashiro and Maki, 1979b). No change in activity was observed during 24 hour of incubation with pepsin approximately 85-95% of the trypsin inhibitor activity was in the embryo, 5-10% in germ-free barn < 1% in polish, and none in milled rice (Barber *et al.*, 1978).

Trypsin inhibitor was inactivated within 6min of steaming of bran at 100^{o}C. By contrast, based on the growth response and pancreatic hypertrophy of chickens fed a rice- bran diet, dry heating the bran for 15 and 30 minutes at 100^{o}C was ineffective in inactivating the trypsin inhibitor, as compared to boiling or autoclaving the bran at 120^{o}C or removing the trysin inhibitor with 1% acetic acid (4: 1, v/ w) for 3 hours (Tsai, 1976). According to Tsai (1976), rice bran trypsin inhibitor differs from soybean trypsin inhibitor in not being water soluble, not being readily destroyed by dry heat, and having a broad spectrum of antiprotease inhibition besides trypsin.

2.5.1.3. Hemagglutinin-lectin:

Hemagglutinins are globulins that agglutinate mammalian red blood cells. Rice bran or germ hemagglutinin agglutinates red blood cells in a way similar to that of wheat germ agglutinin (Ory *et al.*, 1981). Using rabbit and human erythrocyte agglutination, hemagglutinin activity was shown to be only in the germ or primary axes of the rice grain (Benedito de Barber and Barber, 1978; Peumans *et al.*, 1983). Heating bran at 100^{o}C for up to 6min inactivated hemagglutinin activity (Benedito de Barber and Barber, 1978).

Recent interest in rice germ lectin concerns its specific binding to nitrogen –fixing bacteria from the rice rhizosphere (Kortanakul and Boonjawat, 1983; Tabary *et al*., 1987). Lectin is reported to be glucoprotein in nature, for example, containing 27% carbohydrate, predominantly glucose (Takahashi *et al.*, 1973) and being 10% carbohydrate in the molecular weight 14,500 subunit, mainly xylose and arabinose (Indravathamma and Seshadri, 1980), glycine and cystine residues and occurred as the dimmer at neutral and acidic pH (Tsuda, 1979).

2.5.1.4. Dietary fiber:

Dietary fiber may also be considered an autinutitional factor because of its ability to bind mineral cations (Rasper, 1979). Pigmented rice (especially purple to black rice may be high in "tannins," which are concentrated in the bran during milling (Eggum *et al.*, 1981).

2.6. Improvement in AIBs Utilization

Considerable efforts have been devoted to improve utilization of AIBs in practical monogastric nutrition. Onifade (1993) stated that adequate fortification of fibrous diets with micronutrients such as fat and oils, molasses, biotin and niacin could enhance the utilization of dietary fibre as they have extra-caloric effect. Similarly, supplementation of fibrous diet with high quality protein and amino acids improved the efficient utilization of such diets and spared the dilution effect on energy by fibrous feed ingredients (Delorme and Wojeik, 1982).

Other methods employed to improve dietary fibre utilization are moist heating, physical reduction and pelleting, radiation techniques, supplementation of diets with sand of grits, chemical treatment of fibrous diets, nutrient supplementation, use of microbial enzymes and antibiotics (Longe, 1988; Onifade, 1993).

2.6.1. Physical method.

Classified under the physical methods are the following approaches already documented: Soaking in hot water, dry heating, irradiation with high electrons and alteration of particle-sizes with cooking and autoclaving, the antinutritional factors in lima bean were removed (Ologhobo and Fetuga (1986). Cooking removed trypsin-inhibitor activity (Agunbiade, 1992). Sometimes the physical methods do affect the chemical composition of the feed under treatment. Bhatta-Charya and Ali (1985) reported reduction in free fatty acids in rice bran after parboiling and this could be due to volatilization of free fatty acids. During cooking, we have some nutrients loss and while roasting (dry heating) there is evaporation of several volatile substances and inactivation of some enzymes in the foodstuffs.

2.6.2. Chemical methods.

Chemical treatments have been used to improve fibre digestion by both monogastric and ruminant animals. There are a number of acidic and alkaline hydrolytic processes that will solubilise lignin cause disruption of fibre by swelling or improve potential digestibility of fibrous roughages in other ways (Leng, 1991).

According to Guthrie (1974) acid hydrolysis with aqueous ion (Ht) will normally break polysaccharide down to monosaccharide. The improvement of the nutritive value of sodium hydroxide-treated maize cob for West African dwarf sheep (Adeleye, 1980) and sulphur dioxide-treated straws for lambs (Ben Ghedalia and Solomon, 1988) have been reported. The advantage of this approach though numerous, the following disadvantages are worthy of consideration. Chemicals are expensive and are mostly not economically feasible (Keith and Daniels, 1976). Aside the fact that the process is potentially harmful and cannot be handled by in-competent hands, it also results in pollution of the environment (Pearce, 1982). The report of Bersch *et al* (1989) equally showed that autoclave of rice bran was found to produce higher body weight gain in chickens than rice bran, which was raw or chemically stabilized with hydrochloric acid.

2.6.3. Biological Treatment:

Biological treatments include the utilization of microbial proteins, antibiotics, probiotics, enzymes and ensiling. These constitute the most recent methods of enrichment of non-digestible feedstuffs or those imbued with well known antinutrients. Dierick (1989) emphasized that polyphenols such as tannins are not removed by physical or chemical

treatment but by fermentation or germination. The nutritive value of maize in form of lysine and tryptophan contents leading to improvement in biological values and utilizable protein was achieved through germination (Ram *et al*., 1979).

A variety of endogenous enzymes from plants are produced or activated during germination, all having the biological effect of mobilizing the reserved polymers by degrading them to easily available water soluble products (Nout, 1994). Besides ensiling, the most recent additive for improving silage quality is the biological aid. This involves microbial inoculants, metabolic products such as cellulolytic enzymes with easy and safer handling and application. It is neither volatile nor corrosive, aimed at breaking down cell wall to provide wealth of readily available substrates (Dutton, 1987). Enzyme treatment has been reported to have positive effect on alfalfa silage fermentation (Hristov, 1993).

An increase in rumen degradability of the silage *in* vitro and in Sacco was observed with the rapidly degradable fraction of the protein being the most affected. According to Cone (1991), processing feedstuffs with enzymes, rumen enzymes and rumen fluid showed a higher level of degradation than unprocessed ones. Biodegraded feeds contain more free sugars, more protein, less cellulose and lignin with an increased content of ash when compared to the undegraded samples (Yang *et al*., 1993; Zadrazil, 1993; Belewu, 1998).

Other advantages of the biological approach are that it reduces antinutritional substances in the feeds with increase in the availability of dietary nutrients by complementing the activities of the endogenous digestive enzymes of the monogastric (Nout, 1991). Water holding capacity of non-starch polysaccharides is also reduced (Aderolu, 2000) and increase starch, protein and fat digestion.

2.6.3.1. Fermentation

In the strict biochemical sense, the word fermentation refers to anaerobic transformation of carbohydrates into simpler compounds. Through common usage, this term is now applied to any industrial process involving microorganisms. Bacteria, fungi and yeasts are used as enzyme sources and the processes are in two major groups – production by submerged fermentation and production in semi-solid culture.

Majority of bacterial enzymes are produced by submerged fermentation. Semi-solid processes have been employed traditionally for the production of amylases and proteases by *A. oryzae.* Carbohydrates, minerals and buffering substances may be added to the medium

which could be wheat bran. This is moistened with water, sterilized with steam and inoculated with a pure culture of a selected strain of the microorganism. Within 24-48 hours, a suitable enzyme level is achieved with *A. oryzae.* One way to increase the protein content of PKC is by solid-state fermentation with fungus (Ng, 2004). Cheah *et al*. (1998) reported significant increases in the protein content of palm kernel meal (PKM) by solid-state fermentation, with various species of the *Trichoderma* fungus, but the final product has not been tested in animal feed. This process almost doubled the protein content of raw PKC, from about 17% to 32% crude protein (Ng *et al*., 2002). The reducing sugar content of the fermented PKC was also higher compared to that in raw PKC. The nutrient digestibility of PKC was significantly improved.

When the fermented biomass was incorporated into tilapia diets however, a marked reduction in fish growth was observed despite the increased nutritive values (Ng, 2004). It is believed that mycotoxins might have been released during the fermentation (Lim *et al*., 2001). Recently, the use of Solid State Fermentation (SSF) has been shown to be feasible alternative for animal feeding (Deschamps *et al*., 1982). Raimbault and Alazard (1980) obtained a protein level of 20% from the inoculation of cassava root meal with *Aspergillus niger*, a well known lipase producer. The concern for the toxicological characteristic of the fermented product has been addressed by the use of microorganism that has been used traditionally on food and those tested and approved as not being toxic and Generally Regarded As Safe (GRAS).

2.6.3.1.1. Solid State Fermentation (SSF)

This involves the growth of microorganisms on moist substrates in the absence of free flowing water. The necessary moisture (SSF) exists in a complex form which is absorbed within the solid matrix and that was considered advantageous for growth because of the possible efficient oxygen transfer process. In SSF, the water content is quite low and the microorganism is almost in contact with gaseous oxygen in the air, unlike in the case of submerged fermentation (Raghavarao *et al.,* 2002).

Many microorganisms are capable of growing on solid substrates. However, only filamentous fungi are able to grow effectively in the absence of the free water. Bacteria and yeasts grow on solid substrates at 40-70% moisture level, while filamentous fungi were reported to grow under a much lesser water content (Raghavarao *et al.,* 2002). Solid state fermentation has been used in the production of industrial enzymes like amylase and it has great potentials in

the developing countries due to its simplicity of operations, low capital cost and high volume productivity (Mitchel and Lonsane 1990; Akpan *et al*., 1999 b).

2.6.3.1.1.1. Substrates used for the production of enzymes in SSF systems.

Agro-industrial residues are generally considered the best substrates for the SSF processes, and use of SSF for the production of enzymes is no exception to that. A number of such substrates have been employed for the cultivation of microorganisms to produce host of enzymes. Some of the substrates that have been used included, sugar cane bagasse, wheat bran, wheat straw, rice straw, rice husk, soyhull, sago hampas, grape vine trimmings dust, saw dust, corncob, coconut coir pith, banana waste, tea waste, cassava waste, palm oil mill waste, aspen pulp, apple pomace, peanut meal, rape seed cake, coconut oil cake, mustard oil cake, cassava flour, wheat flour, corn flour, steamed rice, steam pretreated willow, starch etc. (Mistra *et al.*, 1994; Tengerdy, 1996).

Solid state cultivation system (SSF) and submerged liquid cultivation system have been used for amylase production, although most research has used liquid culture conditions such as temperature and pH. However, solid state fermentation is gaining interest in recent years due to potential advantages in manufacturing products such as enzyme in high yield at high concentrations and with high specificity (Pandy *et al*., 1999).

However, SSF has some limitations such as a poor pool of microorganisms capable of growth under restricted conditions and the controlling and monitoring of parameters such as Temperature, pH, humidity and air flow (Nahana *et al.*, 1982).

Other attempts at biodegrading agro-industrial by-products (AIBs) include the work of Onilude (1999), Iyayi and Losel (1999), Belewu (1991) and Aderolu (2000). In spite of the factors that inhibit the effective utilization of PKM, supplementation of dietary PKM with synthetic amino acid had been found to enhance its utilization especially in poultry. For instance, Ojewole *et al.* (2006) observed improved weight gain, feed efficiency and decrease cost per Kg weight gain when dietary PKM supplemented with 0.2% of both methionine and lysine was fed to poultry turkey in a tropical environment.

Regarding the utilization of food industry by-products and agricultural wastes some points should be taken into consideration. The increasing utilization of poultry, swine and cattle excrements, solid and liquid, added to feed. They can be used after ensiling or aeration and dehydration in amounts of 5-15% in swine and poultry feed. No negative effect on feed

conversion, weight gain or animal health has been reported, also the utilization of plant waste: - hulls and seed coats, straw and other cellulose-rich sources in the protein biosynthesis. These feed components are used in swine and poultry feeding. These technique can also serve to enrich with protein and vitamins such products as beet pulp, potato, corn and barley pulp (from breweries) by fungi or mixed cultures propagation, and in this way it might play a very important role in the economic utilization of these raw materials as food for monogastric animals.

2.6.3.2. Enzymatic Modification.

Enzymes are able to degrade polysaccharides structure of fibrous feedstuffs, and the growth depression effect of feeding most AIBs is tremendously reduced. According to Cowan *et al*., (1996) addition of enzymes to feed resulted in improved energy availability. Other advantages are reduction in viscosity, reduction in anti-nutritional factors and taste improvement (Cowan *et al*., 1996). The modification of animal feedstuffs using enzyme preparation is an important topic, as increasing the digestibility of the feed ingredients is doubly effective in making better use of the mass of the ingredients and lowering the quantity of polluting wastes produced by the animals (Onilude and Oso, 1999).

2.6.3.2.1. Enzymes.

Enzymes are organic catalysts produced by all living cells which govern the chemical reactions necessary for life processes. Garrett and Grisham (1999) described enzymes as biomolecles that catalyze (increase the rates of) chemical reactions. They are all proteins with molecular weights range from about 10,000 to more than 1,000,000 datoms. Like the other proteins, enzymes consist of chains of amino acids linked together by peptide bonds. An enzyme molecule may contain one or more of these polypeptide chains (Ory and Angelo, 1977).

Like all catalysts, enzymes work by lowering the activation energy for a reaction, thus dramatically increasing the rate of the reaction. As with all catalysts, enzymes are not consumed by the reaction they catalyze, nor do they alter the equilibrium of these reactions. Enzymes can act in several ways, all of which lower the activation energy (Fersht, 1985).

Lowering the activation energy by creating an environment in which the transition state is stabilized (straining the shape of a substrate – by binding the transition state conformation of the substrate/product molecules, the enzyme distorts the bound substrate(s) into their

transition state form, thereby reducing the amount of energy required to complete the transition (Fersht, 1985). Lowering the energy of the transition state, but without distorting the substrate, by creating an environment with the opposite charge distribution to that of the transition state. For example, temporarily reacting with the substrate to form an intermediate Enzyme substrate (ES) complex, this would be impossible in the absence of the enzyme, reducing the reaction entropy change by bringing substrates together in the correct orientation to react. A host of enzymes have been utilized for the degradation of AIBS. These include

2.6.3.2.1.1. Amylase. Alpha amylases (α-amylases) are an important group of enzymes used industrially for conversion of starch into sugars, syrups and dextrins, for liquefaction of various starchy raw materials in brewing and to enhance cleaning power in detergents (Berry and Paterson, 1990; Henson, 1994). A wide variety of microorganisms are known to produce extracellular amylases. Gram positive bacteria and particularly the genus *Bacillus* are prolific producers of amylases and are employed widely in industries. Among the moulds, some species of *Aspergillus Penicillium*, *Cephalosporium*, *Mucor* and *Rhizopus* are known to produce amylases (Crueger and Crueger, 1984).

Extracellular amylolytic enzymes from some yeast have also been characterized (Kelly *et al*., 1985). While fungal-amylases are regarded to be generally more heat-labile than bacterial amylases and therefore not suitable for high temperature processes, there are low temperature industrial processes where fungal α-amylases may have an edge over bacterial amylases such as in detergent production. Moreover, due to the higher saccharifying ability of fungal amylase, it represents a useful agent in the conversion of starch into high maltose syrups and syrups with high dextroses equivalent. In the brewing industries, fungal α-amylase is used to increase the fermentability of wort.

Various works on pre-treatment mechanisms, ranging from delignification, saccharification, irradiation with high electrons, subdivision into micron size particles and steeping in dilute alkali which provide enhanced utilization of food carbohydrate by bacterial enzymes were reviewed (Millet *et al.*,1980; Beuvink and Mulder, 1989). Graham and Aman (1991) in their own conclusion stated that treatments that disrupt the endosperm cell walls in barley can increase the proportion of the diet digestibility prior to the large intestine.

2.6.3.2.1.2. Cellulase. Cellulase is a complex of enzymes containing chiefly endo and exo β glucanases plus cellobiase. It occurs in abundance in nature and constitutes one half of the approximately 150 billion tons of organic materials that are photosynthesized annually (Hall

and Slessor, 1976). There are three different kinds of enzymes believed to be involved in crystalline cellulose decomposition; endo- β-1, 4-glucanase, exo-β-1, 4-glucanase and β-glucosidase (Emert *et al*., 1974). Cellulases are a group of hydrolytic enzymes capable of degrading cellulose to smaller sugar components like glucose units (Onsori *et al*., 2005). Native cellulose is very resistant to microbial attack (Dunlap *et al*., 1971). Its high crystalline structure and the presence of lignin reduce its susceptibility to attack by microorganism. Pretreatment of the cellulolytic substrate with 40% alkali (NaOH) is required to alter the structure of cellulose and to remove lignin (Han and Anderson, 1974; Despande *et al*, 1978). Of the cellulolitic microorganisms, fungi have been the subject of most of the intensive study because of their high cellulolytic activity, simple culturing and higher biomass production. The cellulase system in fungi is considered to comprise three hydrolytic enzymes (i) the endo-(1, 4)-β-D-glucanase (synonyms: endoglucanase, endocellulase, carboxymethyl cellulase [EC 3,2,1,4], which cleaves β-linkages at random, commonly in the amorphous parts of cellulose: (ii) the exo-(1,4)-β-D-glucanase (synonyms: cellobiohydrolase, exocellulase, microcrystalline cellulase, Avicelase [EC 3.2.1.9]),which releases cellobiose from either the crystalline parts of cellulose; and (iii) the β-glucosidase (synonym: cellobiase [EC 3.2.1.21]), which releases glucose from cellobiose and short-chain cello oligosaccharides (Bhat and Bhat,1997)

2.6.3.2.2. Enzyme Inhibitors:

Protease and amylase inhibitors are widely distributed in seed tissues including cereal grains. Inhibitors tend to be heat stable. There are numerous reports that trypsin inhibitor, chymotypsin inhibitor and amylase inhibitor levels are reduced during fermentation (Chaven and Kadam, 1989; Reddy and Pierson, 1994).

2.7. Anti-Nutritive Factors in Animal Feedstuffs.

Anti-nutritive substances are defined as “those generated in natural feedstuffs by the normal metabolism of the species from which the material originates and by different mechanisms exerting effect contrary to optimum nutrition.”

These anti-nutritive substances are often referred to as “toxic factors”, because of the deleterious effects they produce when eaten by animals. The term “toxic factor” however is misleading, because there is an implication that the substances are lethal beyond a certain level of intake. In fact, for most animals, they are not and produce less effect such as reduced

growth, poor food conversion, hormonal changes and occasional organ damage.

2.7.1. Endogenous Anti-nutritional Factors Present In Plant Feedstuffs.

The presence of endogenous anti-nutritional factors within plant feedstuffs is believed to be the largest single factor limiting their use within compounded animal and fish feeds at high dietary levels. Although these factors vary in their individual toxicity to fish, a large proportion of them can be destroyed or inactivated by heat treatment processes (Tacon and Jackson, 1985).

2.7.1.1 Food Toxicant.

Toxicant has been defined as any substance causing signs of toxicity in animals or symptoms of toxicity in humans. These substances may be added deliberately, introduced unintentionally as a consequence of human activities or an intrinsic part of the plant or animal food material as it was formed in nature (Strong, 1974). Many chemical components of natural food products have been identified as toxicants and some of these include - Cyanogenic glycoside hamagglutinin, Saponin, Gossypol, Goistrogen, Trypsin inhibitor, Oxalates, Phytates and antivitamins (Onwuka, 2005).

2.7.1.2. Mycotoxins.

Cereals constitute the most important food and feed sources which are affected by various mycotoxic fungi. The problem of natural occurrence of mycotoxins in cereals aggravated to some extent due to rapidly changing agricultural technology (Hesseltine, 1974). Mycotoxins are a group of highly toxic secondary metabolites of fungi produced under certain favorable environmental conditions (Singh *et al*, 1983). Because of their potent toxic nature and fairly common occurrence under natural conditions, mycotoxins have attracted word-wide attention in recent years. The diseases or physiological abnormalities resulting due to ingestion of mycotoxins are known as **mycotoxicosis (**Bilgramis and Sinha, 1984).

In general, mycotoxins and particularly aflatoxins seem to pose great problem in the tropics than in the temperate regions but no part of the world can be considered to be mycotoxin-free zone due to the movement of various foodstuffs from one part of the globe to the other (Bilgrami and Sinfa, 1984). Association of mould produced toxins with food commodities has been known since Biblical times but their roles in inciting disease syndrome was realized

only when it was discovered that ergotism was caused due to consumption of barley and rye infected with *Claviceps purpurea* (Barger, 1931).

The severity of mycotoxin problem was realized during World War ll. Some of the important commodities which have been found to be naturally contaminated with one or the other mycotoxins are: maize usually contaminated with aflatoxin B1 and Zearalenone, Rice has been reported to be contaminated with aflatoxin B and some others like citrinin, Sterigmatocystin and Ochratoxin has been detected as natural contaminants of rice, wheat has also been found to be contaminated with aflatoxin B and Sterigmtocystin, sorgum was found to be naturally contaminated with all the four aflatoxins B1,B2,G1 and G2, Pulses and oilseeds also contain aflatoxin B, dry fruits and spices have orchratoxin, citrin and zearalinone.

2.8. Biodegradation of Materials.

Biodegradation is defined as the decomposition of a substance through the action of biological agents especially microorganisms (Lederberg, 1992).

2.8.1. Mechanism of Biodegradation. Biodegradation is a process of decay initiated by microorganisms resulting in complete breakdown or mineralization of complex materials into simple constituents (Aidoo *et al*., 1982). The vital role of biodegradation includes, recycling of biological building blocks, preventing the accumulation of debris and reduction of toxic materials in the environment.

2.8.1.1. In Vitro Biodegradation

Solid state fermentation is an alternative because sophisticated equipment is not usually required and the product can easily be harvested (Aidoo *et al.,* 1982). Willey et al (2008) reported that most bios33ynthetically formed compounds (including plant materials which make up majority of the feedstuffs) are degradable under aerobic and anaerobic conditions. This means that for every compound however complicated, there exist some microorganisms that have the ability to degrade it either partly or completely.

The fragments that are produced can be utilized by other organisms Biodegradation in livestock feeding is fast becoming a tool to increase feed utilization and digestion of nutrients within the feed. Some anti-nutritional factors like polyphenols are also removed along side with synthesis of some amino acids needed by the animal.

2.8.1.2. In Vivo Biodegradation.

The fact that a symbiotic relationship exists between the gut micro-flora and the host has been long established (McNeil, 1984). This is attested to by the findings of Salyer *et al* (1979) in their review of the breakdown of polysaccharides in colon. The concentrations of high molecular weight carbohydrate were much lower in all parts of the colon than in the ileum, indicating appreciable breakdown of polysaccharide had occurred. The microflora are able to obtain a fraction of their energy from food by fermentation and provide the end products, principally short chain fatty acids (SCFA) or Volatile Fatty Acids (VFA) that are absorbed by the host animal, who is then able to metabolize them fully. The energy contained in the structural carbohydrate of plants is made available by bacterial breakdown (McNeil, 1984) since the complex carbohydrate cannot be digested by the gut enzymes of monogastric animals.

Soluble polysaccharides are broken down by enzymes associated with the cell while the complex carbohydrate like guar gum needed the action of extracellular enzyme secretion. Starch, cellulose and hemicellulose are among those degraded by extracellular bacterial enzymes to hexoses and pentoses, which are later metabolized inside the bacteria. Subsequent metabolism via glycosis or pentose phosphate pathway produces pyruvate which is rapidly metabolized to the end products viz short chain fatty acid (SCFA), hydrogen, carbon dioxide, methane and sometimes lactic acid (McNeil, 1984).

The above described process is at the expense of so many factors when it comes to the pace and ease of operation. According to Salyer *et al*, (1979) the following among others are the factors affecting the rate of fibre breakdown, accessibility of the area of the polysaccharides in the plant cell wall, competition for substrate, low metabolic rates or the presence of inhibitory substances like lignin could affect the amount of polysaccharide that is utilized by bacteria *in-vivo*.

The ability of the intestinal microflora to degrade fibre components is highly variable and dependent on various dietary nutrients and source of plant fibre. Fermentation of dietary fibre depends on solubility nature, chemical structure, particle size, flora and the transit time in the digestive tract (Cummings, 1982). Other factors like the condition of the biotype (slow transit time, pH, humidity and temperature) are highlighted by Nyman and Asp (1982). Kenelly *et al.* (1981) stressed the importance of chemical factors as they influence fermentation of carbohydrate. He stated that bacterial fermentation might be readily fermentable carbohydrate

sources, which appears to be required for optimum bacterial growth.

Dietary fibre with more heterogenous monosaccharide composition have a more variable fermentation rate and individual short chain fatty acid production than starch which consist of polymers of glucose molecules and in this sense represent a uniform class of complex carbohydrate (McBurney *et al*.,1990). With prolonged feeding of dietary fibre, the growth of greater cellulolytic bacteria is ensured (Varel and Pond, 1985) and more short chain fatty acid was discovered in animals fed on high fibre diet than those on low fibre diet.

2.8.2. Organisms Involved in Biodegradation.

In recent years, the new potential of using microorganisms as biotechnological sources of industrially relevant enzymes has stimulated renewed interest in the exploration of extracellular enzymatic activity in several microorganisms (Billinski and Stewart, 1990; Akpan *et al*., 1999b; Buzzini and Martini, 2002). A large number of microorganisms, including bacteria, actinomycetes, yeast and fungi produce different groups of enzymes.

2.8.2.1. Fungi:

Fungi are very heterogeneous, with the typical fungi having a range of features that separate them from other organisms. Many of them are filamentous, which means that they consist of hyphae, grow at their tips, and branch periodically to create a network of hyphae called mycelium. The filamentous fungi of the class Phycomycetes such as the Genera *Mucor* and *Rhizopus*, have been found to be suitable for SSF because of their ability to grow significantly in the absence of free water (Moo-Young *et al.,* 1983). The spectrum of microbial cultures employed for production of various enzymes in solid state fermentation systems include: *Trichoderma species* like *T. viride,* T. *reesei* and *Aspergillus* species like *A. niger*, *A. flavus, A. fumigatus* and *Rhizopus* species like *Rhizopus stolonifer* and *Penicillium* species. *Candida* species like *C. rogosa* (Moo-Young *et al*., 1983).

2.8.2.2. Bacteria

The list of bacteria involved in SSF fermentation includes *Pseudomonas* species, *Streptomyces* sp., *Staphylococcus* sp., *Bacillus* sp.(Moo-Young *et al.,* 1983).

2.8.3. Biodegradation of Polysaccharides:

2.8.3.1. Starch

Starch is a major reserve carbohydrate of all higher plants. In some cases, it accounts for as high as 70% of the un-dried plant material (Prasanna, 2005). It occurs in the form of water insoluble granules. The size and shape of the granules are often characteristic of the plant species from which they are extracted (Prasanna, 2005). When heated in water, the hydrogen bonds holding the granules together begin to weaken and this permits them to swell and gelatinize. Ultimately, they form paste or dispersion, depending on the concentration of polysaccharide. Starches are produced commercially from the seeds of plants, such as corn, wheat, sorghum or rice, from the tubers and roots of plants such as cassava, potato, arrowroot and the pit of sago palm. The major commercial source of starch is corn from which it is extracted by a wet milling process.

Starch is a heterogenous polysaccharide composed of two high molecular weight entities called amylose and amylopectin. These two polymers have different structures and physical properties. Starch may be separated into its two components by addition of a polar solvent, for example, n-butanol, to a dispersion of starch (Banks and Greenwood, 1975). The insoluble amylose complex can then be separated from soluble amylopectin fraction. Amylose is composed of linear chains of -1, 4 linked D-glucose residues. Hence it is extensively degraded by amylase. Some amylose is not totally degraded to maltose by this enzyme. Amylose has a degree of polymerization of several thousands of glucose units (Banks and Greenwood, 1975). Because of the molecular shape and structure of amylose, it is not stable in aqueous solution and retrogrades (precipitate spontaneously). This is because linear chains align themselves by hydrogen bonding and thus forms aggregates. This process is irreversible.

Retrograded amylose will only dissolve in alkaline solution. Amylose has considerable viscosity in alkaline solutions due to its molecular shape. Amylose forms complex with iodine to form intense blue colour and this forms the basis of a method for quantitative determination of amylase.

Amylopectin may account for 75 to 85% of most starches (Fogarty, 1983). It has molecular weight in excess of 10^7-10^8 and has a branched structure composed of chains of about 20-22 -1, 4 linked D-glucose residues. Amylopectin which is branched by β -1, 6 linkages may

contain 4 to 5% β -1, 6-D- glucosidic bonds. In aqueous solutions, amylopectins are relatively stable due to branched molecules and are not able to form compact aggregates. There is no apparent relationship between the limiting viscosity number and the degree of polymerization. Due to the nature of branched structure, the iodine binding power is reduced (Fogarty, 1983). The branched components of starch are amylopectin which has different types of chain referred to as A, B, and C chains.

The hydrolysis of starch may be carried out using either acid or enzyme as catalyst. Enzyme hydrolysis has several advantages: it is more specific, therefore fewer by-products are formed and hence yields are higher. Conditions for enzyme hydrolysis are milder, therefore, refining stages to remove ash and color is minimized. The enzymatic hydrolysis of starch has been practiced on an industrial scale for many years and is gradually replacing the traditional acid hydrolysis process (UnderKofler *et al*., 1965).

Cellulose, Hemicellulose and Lignocellulose

2.8.3.2. Cellulose

Cellulose is the most important of the polysaccharides in plants and is found in all species. It is the major structural component of cell wall in wood. Cellulose fibres have long been an important raw material in industry as well as fibres from wood (paper), seed hair (cotton), bast fibres (flax, jute), and leaf fibres (hemp). Cotton is the purest form of natural cellulose (Stewart *et al.*, 1974).

Cellulose is the only one of the proximate constituents that can be isolated as a relatively pure product of known chemical structure. The other proximates are all determined as mixtures, often of uncertain chemical composition (Stewart *et al.*, 1974). The first stage in the isolation of cellulose is a delignification process yielding a product consisting of cellulose plus varying amounts of other polysaccharides. The latter collectively known as hemicellulose.

2.8.3.3. Hemicelluloses

Hemicelluloses are heteropolysaccharides consisting of short branched chains of hexoses (mostly mannose) (Kuhad *et al*., 1997).

2.8.3.4. Lignocellulose:

Lignocellulosic biomasses from plants are the most abundant agricultural residues in the world. It accounts for more than 60% of the total biomass production (Kuhad *et al*., 1997). They are constantly being replenished by photosynthesis.

The lignocellulosic materials of plants consist of three main components, namely: - cellulose, hemicellulose and lignin (Betts, *et al.*, 1988). The chemical properties of the compounds of lignocelluloses make them a substrate of enormous biotechnological value (Malherbe and Cloete, 2003). Large amounts of lignocellulosic waste materials generated through forestry and agricultural practices, paper-pulp industries and many agro-industries pose significant environmental problem.

A large percentage of the lignocellulose wastes are often disposed off by biomass burning, which is not restricted to developing countries alone, but is considered a global phenomenon (Levine, 1996). However, the huge amounts of residual plant biomass considered as waste can potentially be converted into various different value-added products including biofuels, chemicals and cheap energy sources for fermentation, improved animal feed and human nutrients.

The three types of polymers it contains (cellulose, hemicelluloses and lignin) are strongly intermeshed and chemically bonded by non-covalent forces and bycovalent cross-linkages. A great variety of fungi and bacteria can fragment these macromolecules by using a battery of hydrolytic or oxidative enzymes. In native substrates, binding of the polymers hinders their biodegradation. The diversity of cellulosic and lignocelluosic substrates has contributed to the difficulties found in enzymatic studies. Fungi are the best-known microorganisms capable of degrading these three polymers; because the substrates are insoluble, both bacterial and fungal degradation have to occur exocellularly, either in association with the outer cell envelope layer or extracellularly. Microorganisms have two types of extracellular enzymatic systems: the hydrolytic system, which produces hydrolases and is responsible for cellulose and hemicelluloses degradation; and a unique oxidative and extracellular ligninolytic system, which depolymerizes lignin (Perez *et al.*, 2002).

The more important and difficult problem of waste fibre utilization requires microorganisms that can utilize lignocelluloses. Unfortunately at present there are no known microorganisms that can utilize natural lignocelluloses at rates of commercial interest. It is necessary,

therefore, to pretreat the waists. The purpose of the pretreatment is twofold: to expose the cellulose by removal or modification of the lignin, and to reduce the crystalline fraction of the cellulose. Alkali treatment based on recent modifications of the Beckman process has been proposed s a direct method for converting straw, maize cobs, etc., into a more digestible feed (Rexen, 1975). This method does not increase the protein content of the feed because there is no microbial growth. It does, however, increase the availability of cellulose to the rumen bacteria.

2.8.4. Influence of Microbial Biodegradation / Nutritional Improvement of Materials

2.8.4.1. Protein

One way to increase the protein content of PKC is by solid-state fermentation (SSF) with fungus (Ng, 2004). Cheah *et al.*, (1989) reported significant increases in the protein content of PKM by SSF with various species of the *Trichoderma* fungi. This process almost doubled the protein content of raw PKC, from about 17% to 32% crude protein (Ng *et al.*, 2002). Recently, the use of SSF has been shown to be feasible alternative for animal feeding (Deschamps *et al.*, 1982). Raimbault and Alazard (1980) obtained a protein level of 20% from the inoculation of cassava root meal with *Aspergillus niger*, a well known lipase producer.

2.8.4.2. Ash

Biodegraded feeds contain more free sugars, more protein, less cellulose and lignin with an increase content of ash when compared with the undegraded samples (Yang *et al.*, 1993; Zandrazil, 1993; Belewu, 1998). The reducing sugar content of the fermented PKC was also higher compared to that of raw PKC.

2.8.4.3. Energy: - The energy in feed is not available until the complex molecules are broken down to simpler molecules by digestion. The products of digestion are then absorbed into the body of the animal where oxidation processes occur which release the energy. According to Cowan *et al.* (1996), addition of enzymes to feed resulted in improved energy availability.

2.9. Performance characteristics.

Biodegradation increase starch, protein and fat digestion. The nutrient digestibility of PKC was significantly improved. Enzymes are able to degrade polysaccharides structure of fibrous feedstuffs and the growth depression effect of feeding most AIBs is tremendously reduced.

Other advantages are reduction in viscosity, anti-nutritional factors and taste improvement. The modification of animal feedstuffs using enzyme preparation is an important factor in increasing the digestibility of the feed ingredients (Onilude and Oso, 1999).

2.10. Fish.

Fish are cold blooded animals that live in water. Their metabolism and activities are directly influenced by the water qualities. Fish is very important to man. It is a vital source of man's food. It is the man's most important single source of high quality protein, providing 16% of animal protein consumed by world population (FAO, 1997). Apart from food value, fish have been noted to possess medicinal values. It is one of the sources of essential amino acids and other much needed nutrients for body activities. Fish oil consumption has recorded positive effect on cardiovascular problems by reducing the serum cholesterol (Conigilo, 1992). Dietary responses to short-term diets of fish and the positive effects on protein-calorie malnutrition, asthma, arthritis, auto-immunity, coronary heart diseases and arteriosclerosis have been independently and unanimously reported (Gerhard *et al.*, 1991, Cobiac *et al.*, 1991). It is an important international object of trade generating foreign exchange with per annum internationally traded value estimated to US$51 billion (FAO, 2000).

It is also a source of employment, with over 36 million people employed (worldwide) directly through fishing and aquaculture (FAO, 2000). As many as 200 million people derive direct and indirect income from fish (Garcia and Newton, 1997). Most of the by-catches are converted into fishmeal in the feed industry for livestock and aquacultural purposes. Fish oil, a by-product used in the manufacturing industries for soap making, production of cod liver oil. Fish serves recreational and aesthetic purpose, with increased awareness about ecotourism, sport fishing is practiced globally. For scientific purpose, a lot of skilled personnel in fisheries related fields are engaged in various forms of research, coming up with new innovations in fisheries management (Akintomide *et al.*, 2005).

2.10.1. Nutrient Requirements of Fish

Nutrient requirement refer to the defined needs of the body in terms of protein, energy, minerals and vitamins to enable it to survive and reproduce. The extent to which animals and fish can maximize production is directly related to the level and quality of dietary nutrients supplied above the maintenance requirements. Halver (1972) observed that fish nutrient requirement vary from species to species, age, sex, reproductive state and the environment.

Healthy state of animal like fish is a manifestation brought about by the combination of a set of organic elements known as **nutrients** brought together in different proportions at feeding (Falayi, 2009a). The currently recognized feed nutrients are Carbohydrates, Proteins, Lipids, Vitamins, Minerals and Water.

2.10.1.1. Carbohydrate requirement:

Carbohydrates (CHO) form about 75 percent of all the dry matter in plants and the chief source of energy and heat in the feed of animals. Carbohydrate feedstuffs include: the sugars, the starches, the cellulose, gums and other related substances (Falayi, 2009). The role of carbohydrate in fish nutrition is vital. Carbohydrate requirements have been established for warm water fish. Digestible CHO requirements for optimum growth in some fishes have been indicated.

Robert (1977) indicated that the diets which produced optimum growth parameters in catfish contained digestible CHO levels ranging from 5.6% to 22.5% of the diet. Singh and Nose (1967) reported that CHO can spare protein in fish rations. In a study with channel catfish 0.23g of CHO per 100g of feed was found to spare 0.05g protein (Dupree and Huner, 1984). The ability of catfish to use starch as an energy source give rise to protein sparing effect while the presence of adequate starch in the diet for energy allows catfish to meet amino acid requirement. Ayinla and Faturoti (1990) also successfully included corn starch in the diets of *C. gariepinus* fingerling to spare protein for growth.

2.10.1.2. Protein and amino acid requirement

Protein requirement is one of the most important factors in fish nutrition. It is required by fish for both body building and maintenance and its effective utilization depends on its amino acid composition (Stickney and Lovell, 1977). About ten of the amino acids have been identified as been indispensable for fish growth such that it has to be provided in the diet because they cannot be synthesized by the fish body (Mertz, 1972 and Halver, 1976). These indispensable amino acids are leucine, lysine, methionine, phenylalanine, threonine, tryptophan, valine, arginine histidine and isoleucine. These amino acids are the building blocks for flesh, enzymes, eggs, milt, antibodies and some hormones. The balance of these amino acids in proper proportion is determinant for success or failure of any fish husbandry programme. Dietary protein requirements are said to be size dependent, that is, smaller fish

require higher levels of protein for maximal growth than larger fish. For optimum growth, a protein content of at least 35% has been recommended for fish (Jauncey, 1982).

Weight gain of fish is essentially linear with protein content in the feed and is directly proportional to protein of the diet at a range of 20 – 40%, but 50% protein does not improve growth (Dupree and Huner, 1984). It is therefore important not to feed more protein than can be used by the fish because no benefit is realized in growth, the excess may not only cost more but can be damaging to the fish by inducing deleterious side-effects such as growth depression and increased susceptibility to induced biotic stress and diseases.

2.10.1.3. Lipids and Essential Fatty Acids Requirement:

Dietary lipids which include free fatty acids, triglycerides, phospholipids, oil, waxes and sterols, according to Stickney (1984) are important in the nutrition of catfish as they provide energy and are involved in the biological membranes and also act as carriers of fat-soluble vitamins. News (1987) explained that dietary lipid serve both as sources of energy and its component fatty acids, of which are essential dietary components for the growth and survival of the recipient animal. The essential fatty acid (EFA) cannot be synthesized in the body of the animal hence have to be produced in the diets. They are involved in the structural building of the biomembranes. The polyunsaturated fatty acids (PUFA) are precursors for prostaglandins (five carbon cyclic components required in trace quantities which exhibit activities similar to hormones).

Igbinosum and Talabi (1982) reported that in the catfish, growth was best in the fish diets containing 10.89% vegetable oil and least in those containing 9.09% vegetable oil. They also observed a positive correlation between growth rate and hepatic phospholipids, cholestrerol, triglycerol and free fatty acids. Whole body lipids was also found by Watanabe (1977) to increase as dietary lipid level increased and as the percentage of fat in a diet increases the percentage of the body protein decreased.

2.10.1.4. Vitamins

Vitamins are complex organic substances, usually of comparatively small molecular size (molecular weight usually less than 1000). They are distributed in feedstuff in small quantities and form a distinct entity from other major and minor food components (Cho *et al.*, 1985). Vitamins are needed for normal growth, maintenance, and reproduction of animals. Deficiency have been found to cause depressed appetite and reduced growth rate in channel

catfish. Other symptoms include discoloration, lack of coordination, nervousness, hemorrhages, lesions, fatty liver and increased susceptibility to bacterial infection (Dupree and Huner, 1984). *Clarias* fingerling requires vitamin B_1 (thiamin), B_2 (riboflavin), B_6 (pyridoxine), pathothenic acid, folic acid, niacin and vitamin C (ascorbic acid) for proper growth (Omitoyin, 1997).

2.10.1.5. Minerals

Minerals are required for maintenance of salt and water tissue balance for the metabolism of other nutrients and for major structural elements on the tissue of fish. Most studies of mineral requirement by Nose and Arai (1979); Dupree and Huner (1984) have involved calcium and phosphorous. Calcium can be absorbed in sufficient quantity from the water and dietary phosphorous has been reported for fish.

2.10.1.6. Water and its Quality

Water is fish habitat without which it will not survive or do anything. The continuous supply of regular and clean water is essential for maximum performance of fishes (Falayi, 2009 a). Water is available in large quantity in rivers, lakes, pools, ponds, dug outs or reservoirs. Water quality variable is many characteristic (physico-chemical and biological) of water that affects the survival, reproduction or management of fish in many ways (Falayi, 2009a). If water quality is excellent, the survival, growth and reproduction will be enhanced.

The quality of nutrients in solution in the water varies considerably and determines the different natural productivities which in turn depend on the physico-chemical characteristics. The importance of chemical factors is due to their lethal and sublethal effects on culturable organisms and also their effect on biological productivity (Omitoyin, 1995). Among the important water quality parameters include temperature, dissolved oxygen and pH (hydrogen ion concentration).

2.10.2. Other Needs of Fish

2.10.2.1. Nutritional Bioenergetics in Fish

2.10.2.1.1. Energy metabolism in Fish.

Energy metabolism in fish is similar to that in mammals and birds with two notable exceptions: Fish do not expend energy to maintain a body temperature different from that of their environment and secondly the excretion of waste nitrogen requires less energy in fish than it does in homoeothermic land animals (Falayi, 2009b). Energy is lost from the body of a fish in the faeces, urine, gill excretion and heat. Small amounts are also lost from external body surface. The energy needs of fish are supplied by fats, carbohydrates and proteins. The amount of energy required by fish is affected by species, age, sex, water temperature activity and water quality. Using a protein feed of 40%, Omitoyin (1989) recommended the use of energy level between 3,100-3,200 kcal/kg in *C. gariepinus* broodstock diet.

2.10.2.1.2. Temperature

The temperature of the water affects the activity, behavior, feeding, growth and reproduction of all fishes. Next to oxygen, water temperature may be the single most important factor that affects the welfare of a fish during its life span (Dupree and Huner, 1984). Increase in water temperature is inversely related to dissolve oxygen and water pH.The higher the temperature the lower the level of dissolved oxygen (DO) and pH of the water.

Each fish species have their natural thermal tolerance outside which they cannot live. The optimum temperature for maximum growth rate and feed conversion as observed by Varreth and Bieman (1987) is 27.5%. The rate of metabolism in fish is also temperature dependent. As temperature increases basal metabolism increases. Nutrient requirements of fish are temperature dependent and that near certain upper thresholds, feed digestibility, metabolism and nutrient utilization are enhanced. Temperature also has effect on the reproduction of *C. gariepinus* (Delince *et al*, 1985).

2.10.2.1.3. Dissolved Oxygen (DO)

Dissolved oxygen is probably the most critical water quality variable in fish culture. Maintaining adequate DO concentrations in catfish ponds therefore is critical to successful fish farming. The atmosphere is 21% oxygen by volume, but this oxygen is not directly available to fish. For fish to utilize oxygen it must be dissolved in water. The exact

concentration of DO required maintaining fish in healthy condition is variable; however, a DO of 4mg/l or more will prevent the detrimental effects of low oxygen (Schwedler, 1983). Adequate DO is necessary not only to prevent massive fish kill but also to keep fish healthy and make them grow well. Low DO concentrations adversely affect fish even at levels which do not cause mortality, making them more susceptible to parasites and diseases (Plumb *et al.*, 1976). DO concentration as low as 1mg/l can be tolerated by healthy catfish for a short period, however prolonged exposure to 1.5mg/l DO can cause histological damage to catfish (Scott and Rogers 1980). In addition fish do not feed or grow when concentration remain continuously below 4 or 5mg/l. More oxygen is needed at feeding because much of biological and chemical activities are taking place, and there is the need to supplement normal oxygen composition of ponds by external or additional aeration (Falayi, 2009a).There is a direct relationship between feeding rates, dissolved oxygen and other water quality characteristics (Dupree and Huner, 1984).

2.10.2.1.4. Hydrogen Ion Concentration (pH)

pH is a measure of the hydrogen ion (H^+) concentration and indicates the level of acidity or alkalinity of the water. Fish grow best in water at pH 6.5 - 9.0 and prefer slightly alkaline water close to neutral pH. The pH of water is greatly influenced by the concentration of carbon dioxide; however the optimal acceptable pH level varies with species. The acid and alkaline death points for pond fish are approximately pH 4 and pH11 respectively.

2.10.3. Catfish

Catfish of the genus *Clarias* are wildly distributed in Africa and have been the focus of long term aquaculture interest. Other catfishes that are cultured include *Clarias anguillaris, C, ischeriensis, C, submarginatus Heterobranchus bidosalis, H. longifillis, Chrysichthys nigrodigitatus, Bagrus sp., Synodontis sp., but C. gariepinus* is undoubtedly the fish choice of farmers. Nigeria is the highest producer of this Clariid catfish in the world (Williams *et al*., 2007). They are sometimes referred to as mudfish. They belong to the Siluriformes, and are similar in shape. They are scaleless fish with smooth skin and soft-rayed fins. They possess the characteristic elongated, paired barbells that resemble cat whiskers (so they are referred to as `cat' fish), and a pair of spines (each attached to the base of the pectoral fins) in most cases.

They have accessory respiratory organ (`lung') with which they breathe in atmospheric air to compliment the function of their gills. African catfish (*Clarias* and *Heterobranchus*) are tropical fishes with dorso-ventrally flattened, bony head and elongated body being omnivores; they feed on planktons, some plants insects' larvae and worms, animal wastes and a host of other items. They are hardy and tolerant to most strenuous conditions to which other fishes are sensitive, so they are easier to farm.

The Catfish is indigenous to Nigeria. It was first officially discovered in a brook in Igbo ora, Oyo State in 1923 and was derogatively called the Nigeria Mud Catfish (Taiwo, 2008). The Nigeria mud catfish also known as the African sharp tooth catfish (*Clarias gariepinus*) is undoubtedly remarkable and fascinating fish. It became Nigeria`s contribution and a special gift to Fisheries and the science world after it was taken to Europe as well as other countries in Africa like Jonkershock Fish Hatchery in the Western Cape Province of South Africa in 1941 by Douglas Hey. It was from there the Nigeria catfish became known as the "African mud catfish" (Taiwo, 2008).

2.10.3.1. *Clarias gariepinus* (Burchell, 1822) as a culture species in Nigeria

In Africa aquaculture, *C. gariepinus* and *C.anguillaris* are the most important *Clarias* species used, although other species may be used. Apart from *Tilapia*, *C. gariepinus* is the most abundant culturable species in Nigeria (Tugels, 1984; Adekoya et al., 2001). The importance attached to *C. gariepinus* in aquaculture in Nigeria is because it is a highly esteemed fish and commands high commercial value in our market. It is very hardy and has arborescent air-breathing organs which enable it to tolerate low dissolved oxygen level and other adverse aquatic conditions where most other cultivable species cannot survive. It is highly fecund but does not easily spawn under captive conditions (F.A.O., 2006). African catfish in Nigeria, routine spawning has been common (Oresegun *et al*., 2007). These qualities, coupled with its high economic value, low bone content, fine flavor, high growth rate and its ability to feed on virtually anything make the fish highly recommendable for farming in Nigeria (Omitoyin.1995). It also has a high yield of tastful flesh (Ajana *et al*., 2006).

2.10.3.2. Feeding Requirement of *C. gariepinus:* The *Clarias* is described as omnivorous fish by Welman (1948); Lewis (1974); Bard *et al.* (1976) Olatunde (1983) and with a propensity for being carnivorous Bard *et al*. (1976). They live mostly in river bottoms, swamps and muddy places (hence the nomenclature- mud fish) where they feed on a variety of food ranging from mud, planktons weeds to insects (and their larvae), crustaceans, worms,

snails, frogs and small fish. The species of fish preyed upon include *Labeo senegalensis*, *Ctenopoma kingsleyae* and Tilapia (Bakare, 1968; Ita, 1971; Lewis, 1974). *Clarias gariepinus* can thus be described as an opportunistic feeder, feeding *on* virtually any*thing* that comes its way.

It was in Europe that it's feeding, growth and reproductive requirements of 35-45% crude protein were discovered. The catfish was later translocated back to Nigeria its original home in the early 80s from the Netherlands as Dutch *Clarias* to grow and flourish as the number one source of animal protein in the world with the most balanced amino acid profile (Taiwo, 2008). This species of *C gariepinus* is mostly cultivated in ponds by fish fermers and it is refered to as Dutch *C. gariepinus* while the species that are generally found in the wild is refered to as Local *C. gariepinus*.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Sampling and Culture Methods

3.1.1 Collection of Samples:-

1. Samples of lignocellulosic Materials.

Rice bran (RB) was collected from a rice mill at New-Bussa in Niger State and at Bodija market in Ibadan, Oyo State. Palm Kernel cake and its different varieties of Palm Kernel meal (PKM), Palm Kernel Sludge (PKS) and De-oiled Cake (DOC) were collected from feed mills, local and industrial oil mills at various locations within Ibadan City. All the samples were placed inside sterile plastic containers and transported to the Postgraduate Laboratory, Microbiology Department, University of Ibadan for immediate analysis. Each sample was coded before analysis.

2. *Clarias gariepinus* fingerling samples.

Fingerling samples of *Clarias gariepinus* (Burtchell, 1822) were obtained at the Hatchery Unit of the National Institute of Freshwater Fisheries Research (N.I.F.F.R.), New – Bussa. Niger State. They were transported inside a clean bucket with water to the Green House in the Limnology Programme of the same Institute where the feeding trial was conducted. They were weighed on a weighing balance, distributed into aquaria (60cm x 30cm x 30cm) containing 40 litres of water in 10 numbers, acclamatized for 3days to empty their stomach contents before feeding them with the different feed samples.

3. Feed Components.

The different feed samples used for the feeding trial experiment include- Unfermented Rice Bran (URB), Unfermented Palm Kernel Cake (UPKC), the mixture of URB and UPKC, Fermented Rice bran (FRB), Fermented Palm Kernel Cake (FPKC). the mixture of RRB and FPKC, Fish feed obtained from the Nutrition Programme of N.I.F.F.R., as commercial feed, Fish feed with one of it's components substituted with URB, UPKC, FRB, FPKC, RB fermented with starter culture, PKC fermented with starter culture separately.

3.1.2. Spontaneous fermentation

Samples of Rice Bran (RB) and those of Palm Kernel Cake (PKC) and the different varieties were subjected to spontaneous fermentation. Fifty grams of each of the samples was weighed into clean, sterile Erlenmeyer flasks plugged with Aluminium foil. Fifty millilitres (50ml) sterile distilled water was added. The bottles were left at Laboratory temperature (30 ^{0}C) for four days after which isolation was made from each.

3.1.3. Isolation of fungi

The dry samples as well as the wet samples (spontaneously fermented) were cultured by Pour plate method (Clark *et al*., 1958) using Potato Dextrose Agar (PDA) (Difco). The PDA powder (39g/l) was weighed and dissolved in 1 litre of distilled water according to the manufacturer's instructions, autoclaved at 121^{o}C and 15psi (1.05kgcm^{-2}) for 15minutes; allowed to cool to about 50^{o}C. Two ml of streptomycin was added aseptically to the PDA. One gram of RB and PKC samples (fermented and non-fermented) was weighed into sterile Petri dish. The prepared PDA was then poured aseptically on the samples and allowed to solidify. All the plates were incubated at (28^{o}C) $\pm$ 2^{o}C for up to ten days in Gallemkamp Incubator (Genlab, 250L). Fungal growth was monitored on a daily basis from day two up to the tenth day. Temperature and pH values of the wet samples were measured on daily basis as well. The fungal growths observed were identified based on their colonial morphology and microscopic features compared with pictures of already identified fungi (Kulovant *et al*, 1991).

Table 3.1: Nature and Source of experimental lignocellulosic samples.

Type	Form	Code	Source
Rice Bran	Unextracted	RB_1	New-Bussa, Niger State.
	Unextracted	RB_2	Bodija Market, Ibadan, Oyo State.
Palm Kernel Cake	(A)Expeller Residue	PKC	
		LPKC	**Local Oil Mill**
		$LPKC_1$	Glorious Palm Kernel Mill, Sasha, Ibadan.
		$LPKC_2$	Iyana Church, Olodo, Ibadan.
		$LPKC_3$	New Garage, Ibadan.
		LFMPKC	**Feed Mill.**
		$LFMPKC_1$	Adom Feed Mill, Orogun, Ibadan.
		$LFMPKC_2$	Goodness and Mercy Feed Mill, Orogun, Ibadan.
		IndPKC	**Industrial Palm Kernel Cake.**
		$IndPKC_1$	Sudit Oil Mill, Ijebu-Ode Road, Ibadan.
		$IndPKC_2$	Sudit Oil Mill, Ibadan-Lagos express road, Ibadan.
	(B)Sludge	**PKCS**	**Palm Kernel Cake Sludge.**
	(a) Dried	$PKCS_D$	New-Garage, Ibadan.
	(b) Wet	$PKCS_W$	New-Garage, Ibadan.
	(C)Solvent Extracted Deoiled Cake	DOC	Sudit Oil Mill, Ibadan-Lagos Express road, Ibadan.

3.1.4. Collection of Pure Cultures

Pure cultures were obtained by sub-culturing different fungal isolates on separate PDA plates. Sterile needle was used to pick a particular type of fungal isolate onto a separate PDA plate. This was done for all the different isolates on any PDA plate with mixed fungal growth. The plates were incubated at (28^{o}C) ± 2^{o}C for 5 days in an Incubator (Genlab, 250L).

3.1.5 Maintenance of Stock Cultures

Each of the pure cultures was sub-cultured onto freshly prepared PDA slants every two weeks in sterile McCartney bottles. They were kept in the refrigerator at 4^{O}C for use.

3.1.6. Identification Procedures

The different fungal growths observed were identified. Their colonial morphologies and microscopic features were compared with contents of different Compedia (Domisch *et al*, 1980; Kulovaut *et al.*, 1991).

3.1.6.1. Morphological identification of colonies

The color and size of hyphae were observed on the plate. The color produced at the reverse side of the plate was also observed

3.1.6.2. Microscopic identification

A few hyphae were placed and teased out on a drop of Lactophenol blue on a clean slide with a needle. A clean cover slip was placed on the preparation and observed under the microscope using the x10 and x40 objective lens. These observed features were matched with features of already identified fungi in Literature.

3.1.7. Physiological characterization and enzymatic studies of fungal isolates

3.1.7.1.1. Amylase.

Preparation and sterilization of medium

Starch Yeast Extract Medium (SYEM) of Bernett and Fergus (1971) with the following composition- Soluble starch - 5g, Yeast Extract - 2g, Dipotassium hydrogen phosphate (K_2HPO_4) - 1g and Distilled Water - 1 litre was prepared. Aliquots of 30ml were put into 150ml clean conical flasks, sterilized at 121^oC, 15psi (1.05kg/cm^2) for 15minutes.

Preparation of Inoculums

Seed Medium of Cavazzoni and Adami (1992) of the following composition- D-glucose - 39g, Magnesium Sulphate ($MgSO_4.7H_2O$) - 2g, Dipotassium hydrogen Phosphate (K_2HPO_4) - 1g, Ammonium Sulphate $(NH_4)_2SO_4$ - 5g, Yeast Extract - 2g, Peptone - 1g and Distilled Water - 1 litre at pH 6.0.

The above components sequentially dissolved inside clean conical flask was sterilized under pressure of 15lb/in^2 (psi), 1.05 kg/cm^2 at 121^oC for 15minutes, cooled down and inoculated with fungal isolate. The inoculated seed medium was incubated for 3days at 45^oC to provide inoculums.

Determination of inoculums size (Olutiola *et al*, 1991)

The inoculums size was ddetermined using haemocytometer (Improved Neuber Counting Chamber). The spore suspention in 1ml of the seed was stained with lactophenol blue (2%). A Pasteur pipette was used to deliver 0.1ml of the stained spore suspention into the haemocytometer by capillary action. The haemocytometer was mounted on an Olympus microscope and the spores counted with X40 objective. The spores in the 4 squares of 0.04mm^2 (at the corners of the central square) with one middle square was counted to obtain the numbers of spore. The value obtained was converted to the number of cells per millimeter of suspension as follows

Area of one small square = $\frac{1}{400}$ mm^2

Depth of one small squrare = $\frac{1}{10}$ mm

Volume of one small square = $\frac{1}{400} \times \frac{1}{10}$ mm^3

Numbers of cell counted in 100 small square = X

Since the volume of one small square = $\frac{1}{400} \times \frac{1}{10}$ mm^3 =1:4000

The number of cells per mm^3 = $\frac{X}{100} \times \frac{400}{1} \times \frac{10}{1} = \frac{4000X}{100}$

If the dilution of the cells 1 in 35 for example

The number of cells per cm^3 of undiluted sample = X x 4000 x $\frac{35}{100}$

The number of cells counted was 3 x 10^5 x 4000 = 12 x 10^8.

Inoculation and Incubation of Basal Medium

The sterilized 30ml of the SYE medium above was inoculated with 1 ml of each of the seed media inoculated with different fungi isolate. They were incubated for 4days at 45^oC.

Harvesting for Fungal growth and Enzyme studies:-

The content of each flask was filtered through a preweighed Whatman No.1filter paper after incubation. The biomass produced was harvested and the dry weight of the mycelia was determined. The filtrate was was used for enzyme assay.

Biomass determination:

The biomass determination in all stages of the work was done according to Kashmin *et al.* (2006).The mycelium produced in each of the flask; after incubation was filtered with a pre-weighed filter paper. The filter paper was dried in an oven at 85^oC for 24 hours (Sri Lakshm and Varasimba, 2012). It was cooled in a dessicator and weighed after filtration; the wet filter paper with the trapped mycelia was weighed. It was dried at 80^oC for 30min and weighed. It was returned to the hot air oven and weighing was done until the weight was constant. The mycelia dry weight was obtained as shown bellow

The mycelia weight = $(w_2-w_1) - (w_3-w_1)$

Where w_1 = weight of filter paper before filtration

w_2 = wet weight of filter paper after filtration

w_3 = dry weight of filter paper after filtration.

The fungus culture filtrate collected for enzyme studies.

Amylase Assay:-

One ml of each of the fungus culture filtrate and the control filtrate was incubated with 1ml of the assay medium containing 1% soluble starch in 0.02 M Phosphate buffer (pH 6.9) for 1 hour at 45^{o}C. 5 M NaOH was added to bring the reaction to a halt. One ml of 3.5 – Dinitrosalicylic acid (DNSA) reagent was added to each of the filtrate – Starch-reaction mixture for each of the identified fungi isolated as well as the control. They were boiled in water at 100^{o}C for 10min to estimate the amount of reducing sugar released according to Bernfeld (1955). The transmittance was determined at 540nm using Jenway 6051 colorimeter. The treated control filtrate was used to set the transmittance at 100% (Bernfeld, 1955: Giraud et *al*., 1991).

Standard Curve: - The transmittance of 1ml maltose solutions of 0.125 mg, 0.25 mg, 0.5 mg, 1.0 mg and 2.0 mg per ml was determined and used to construct a curve of percent transmittance as related to mg of maltose per ml.

The amount of reducing sugar released by 1 ml of each of the fungus filtrate from the 1% starch assay medium was extrapolated and calculated from the Standard Curve. The results were reported as units of amylase. One amylase unit is the amount of enzyme in 1 ml of filtrate which releases 1mg of reducing sugar from 1 % starch solution in 1 hour at 45 ^{0}C at pH 6.9.

3.1.7.1.2. Cellulase

Preparation and Sterilization of Medium:-

Oatmeal Chaff (OMC) Medium of the following medium - Oatmeal Chaff - 20g, Yeast Extract - 5g and 1litre of distilled water, adjusted to a pH of 6.8 with 0.2N NaOH. It was sterilized at 121^{o}C, 15psi (1.05kg/cm^{2}) for 15minutes.

Oatmeal chaff was obtained by washing Quaker oats in a muslin cloth with water. Most of the starch content was washed off leaving only the chaff. The chaff was dried in an oven at 85^{o}C for 48 hours (Oso, 1978).

Preparation of Inoculum:-

The seed medium prepared and used for amylase studies above was also used as inoculums for this cellulase studies.

Inoculation:-

Sterilized OMC medium dispensed in 30ml into clean 150ml conical flasks was inoculated with 1ml of the seed medium. Incubation of the inoculated OMC Medium and harvesting for growth and enzyme studies were done as described for amylase above.

Cellulase Assay

Cultures were filtered through a preweighed Whatman filter paper after incubation at 45^{o}C for 5days. The cellulolytic activity of the filtrate was determined using the method of Reese and Mandels (1963). One ml of the fungus filtrate was incubated with 9ml of assay medium containing 0.55% carboxymethyl cellulose (CMC) in 0.55 M acetate buffer (pH 5.5) for 1hour at 45 ^{0}C. The filtrate of uninoculated control was obtained and similarly assayed. The amount of reducing sugar produced was estimated by adding 1 ml of filtrate- CMC reaction mixture with 1ml of DNSA reagent (see appendix for preparation) boiled in water at 100 ^{0}C for 10 mins and cooled in water. Transmittance was determined at 540 nm using Jenway 6051 colorimeter. The filtrate – CMC reaction mixture of the uninococulated control was used to set the transmittance at 100% (Bernfeld *et al*, 1955).

3.1.7.2. Optimization of Environmental Conditions for Fungal Growth and Enzyme Production

3.1.7.2.1. Effect of Temperature on Fungi Growth and Enzyme Production:

Amylase

Sterilized 30ml of SYE Medium was inoculated with a drop of seed medium inoculated with *Aspergillus niger* which is the fungi isolate that produced amylase best of the total of 10 isolates tested for amylase production. This was incubated at the following different temperatures - 10^{o}C, 25^{o}C, 30^{o}C, 35^{o}C, 40^{o}C, 45^{o}C, 50^{o}C, 55^{o}C, and 60^{o}C. Cultures at different temperature were incubated for 3, 5 and 7days. Each culture was filtered, mycelia weight was determined and amylase was assayed from the filtrate

Cellulase

Sterilized 30ml of OMC Medium was inoculated with one ml of seed medium inoculated with *Rhyzopus oryzae* which is the fungi isolate that produced cellulase best out of the 10 fungi isolated for cellulase production. This was also incubated at the same temperature, filtered mycelia weight determined as for amylase above and cellulase from the filtrate.

3.1.7.2.2. Effect of Time (day) on Fungi Growth and Enzyme Production:

Amylase

Sterilized 30ml of SYE Medium inoculated with inoculums of *A.niger* was incubated for 2, 3, 5, 7, 9, 11 and 12days at 45^{o}C. Mycelial weight was determined and the filtrate was used for amylase assay.

Cellulase

Sterilized 30ml of OMC Medium inoculated with the inoculums of *R.oryzae* was incubated for periods of 2, 3, 5, 7, 9, 11 and 12days. The cellulolytic activity of the filtrate was determined using the method of Reese and Mandels (1963).

3.1.7.2.3. Effect of different pH on Fungal Growth and Enzyme Production:

Amylase

The 30ml of SYE Medium was adjusted to different pH of – 4, 5, 6, 7, 8, 9 and 10 with acid and base, sterilized, cooled down and inoculated with the inoculums of *A.niger*. Cultures of each of the fungi grown at different pH were incubated at 45^{o}C for 3, 5 and 7days.

Cellulase

The 30ml of OMC Medium was adjusted to pH of 4, 5, 6, 7, 8, 9 and 10 with acid and base, sterilized, cooled down and inoculated with inoculum of *R. oryzae.* The cultures of each of the fungi grown at different pH were incubated at 45^{o}C for 3, 5 and 7days.

3.1.7.2.4. Effect of Inoculum size on Fungal Growth and Enzyme Production

Amylase

Sterilized 30ml of SYE Medium in different 150ml conical flask was inoculated with 1, 2, 3 and 4 ml of the spores of inoculums of *A, niger*. Incubation was at 45^{o}C for 3, 5 and 7days

Cellulase

Sterilized 30ml of OMC Medium in different 150ml conical flask was inoculated with 1, 2, 3, and 4 ml of the spores of the inoculum of *R.oryzae*. Incubation was at 45^{o}C for 3, 5 and 7days.

3.1.7.2.5. Effect of Carbon Source on Fungal Growth and Enzyme Production

The effects of the following carbon sources were determined on fungal growth, Amylase and Cellulase production-

Simple Carbon sources – D-glucose, Sucrose, Fructose, Maltose, Xylose, Manitol, Galactose, Sorbitol, Lactose, Starch, Saccharose, Raffinose, Mellibiose and Trehalose.

Complex carbon sources – Rice bran, Wheat bran, Palm Kernel Cake, Oatmeal Chaff and Sugar cane baggase.

Amylase

Five gram each of the different carbon sources were added to the following components

Yeast Extract - 2g, Sodium nitrate - 3g, Dipotassium hydrogen phosphate - 1g, Magnesium sulphate - 0.5g and dissolved in 1 litre of distilled water.

The resultant medium in 30ml aliquots were sterilized and inoculated with 1ml of inoculum of *A niger* and incubated for 3, 5 and 7days at 45^{o}C.

Cellulase

Five grams of different carbon sources were added to 5g of Yeast Extract, 3g of Sodium nitrate and dissolved in 1 litre of distilled water. The aliquots of each medium of carbon source in 30ml were sterilized and inoculated with the inoculum of *R. oryzae*. Incubation was at 45^{o}C for 3, 5 and 7days.

3.1.7.2.6. Effect of Nitrogen Sources on Fungal Growth and Enzyme Production

The effects of the following Nitrogen Sources were tested on mycelia growth and amylase and cellulase production.

Inorganic Nitrogen sources – Ammonium nitrate, Ammonium sulphate and Potassium nitrate. Organic Nitrogen sources – Casein, Urea, Yeast Extract and Peptone and Corn Steep Liquor (CSL).

Amylase

Medium for determining the effect of Nitrogen Sources on Fungal growth and amylase production is composed of 5g of Soluble Starch, 2g of Glucose, 1g of Dipotassium hydrogen phosphate (K_2HPO_4), 0.5g of Magnesium sulphate ($MgSO_4.7H_2O$), 2g of the Nitrogen source and 1litre of distilled water. Sterilized media (30ml) containing each of the nitrogen sources were inoculated with 1 ml of inoculums of *A. niger* and incubated at 45^{o}C for 3, 5 and 7days.

Cellulase

The above nitrogen sources were incorporated in 2g to the following- Oatmeal Chaff - 20g, Glucose - 5g and 1litre of distilled water. Aliquot of 30ml medium was sterilized, inoculated with 1 ml of the inoculums of *R. oryzae*, incubated at 45^{o}C for 3, 5, and 7days.

3.1.7.3. Optimization of all the above conditions on the production of Enzyme

The points at which enzyme was produced best in each of the above cultural conditions at which enzyme will be produced maximally were determined.

3.1.7.3.1. Temperature (^{o}C)

Amylase

At temperature of 20^{o}C, amylase was able to liberate the highest reducing sugar. To get the temperature at which the enzyme will be produced optimally. SYE medium in 30ml aliquots were inoculated with inoculums of *A.niger* and incubated at 10^{o}C, 20^{O}C, 25^{0}C, 30^{O}C, 35^{O} C, 40^{o}C, 45^{O}C, 50^{O}C, 55^{O}C and 60^{O}C for 3, 5 and 7days. The culture medium was filtered with Whatman No. 1 filter paper and the mycelia weight was determined. The filtrate was later used for amylase assay.

Cellulase

At 20^{o}C, the highest cellulase was produced. The temperature at which the enzyme will be produced maximally was determined by incubating the OM chaff medium inoculated with *R. oryzae* at 10^{o}C, 20^{O}C, 25^{O}C, 30^{O}C, 35^{O}C, 40^{O}C, 4.5^{O}C, 50^{O}C, 55^{O}C and 60^{o}C for 3, 5 and 7days. Whatman filter paper No. 1 was used to filter the cultured medium. The mycelia weight was determined and the filtrate used for cellulase assay.

3.1.7.3.2. Time (days)

Amylase

At day 7, amylase production was the highest. The day the enzyme will be produced maximally was determined by inoculating SYE medium with *A. niger* and incubated at 45^{o}C for days 2, 3, 5, 7, 9, 11 and12.

Cellulase

At day 3, cellulase yielded librated the highest reducing sugar. The culture medium for cellulase inoculated with *R. oryzae* was incubated for 2, 3, 5, 7, 9, 11 and 12days. The weight of the mycelia obtained was determined and the filtrate used for cellulase assay.

3.1.7.3.3. Inoculum size

Amylase

Highest amylase value was produced with 1 ml of inoculums of *A. nger*. To determine the inoculums size that will produce amylase optimally, 30ml of SYE medium was inoculated with 1, 2, 3, 4, 5, 6 and 7 ml of inoculums size, incubated at 45^{o}C for 3, 5 and 7 days. The medium was filtered. The mycelia were harvested, washed with distilled water, dried to a constant weight at 80^{o}C. The filtrate was used for amylase assay.

Cellulase

The highest cellulase production was with 4 ml of inoculums of *R.oryzae* in 30ml of OM chaff medium. To determine the inoculums size of *R. oryzae* that will produce cellulase optimally, 1, 2, 3, 4, 5, 6 and 7 ml of the inoculum size of *R. oryzae* were inoculated into OM chaff medium, incubated at 45^{o}C for 3, 5 and 7days. The cultured medium was filtered to obtain the mycelia weight and filtrate for cellulase assay.

3.1.7.3.4. pH

Amylase

Of all the pH values tested, amylase production was highest at pH 7. The optimum pH was determined adjusting the pH of the SYE medium to 5.5, 6.0, 6.5, 7.0, 7.5, 8.0 and 8.5. Incubation was carried out at 45^{o}C for 3, 5 and 7days. The culture medium was filtered and the filtrate was used for amylase assay.

Cellulase

At pH 4, the cellulase production was highest. The optimum pH was determined by adjusting the OM chaff medium to different pH of 3.0, 3.5, 4.0, 4.5, 5.0, 5.5 and 6, sterilized, cooled down and inoculated with *R. oryzae*. Incubation was done at 45^{o}C for 3, 5 and 7days. The filtrate of the culture medium was used for cellulase assay. The mycelia dry weight was obtained by rinsing the mycelia with distilled water and dried in an oven at 50^{o}C for 8 hours.

3.1.7.3.5. Carbon sources

a. Simple carbon

Amylase

Out of all the simple carbon sources used at 5% levels, xylose performed best. To get the optimum level of xylose, concentrations of 2, 3, 4, 5, 6, 7, and 8g per litre were prepared and inoculated with *A. niger* inoculums and incubated at 45^{o}C for 3, 5 and 7days. The filtrate obtained from the culture was used for amylase assay.

Cellulase

Xylose was also the simple carbon source that performed best at 5% inclusion. The level of its optimum performance was determined by preparing different concentrations of 2, 3, 4, 5, 6, 7 and 8 gram per litre into the cellulase medium for carbon sources. Incubation was done for 3, 5 and 7days at 45^{o}C. The filtrate was used for cellulase assay and the mycelia weight was determined.

b. Complex carbon

Amylase

Oatmeal chaff performed best out of all the complex carbon sources used at 5% concentration. To get its optimum concentration, different concentrations of 2, 3, 4, 5, 6, 7 and 8 gram per litre were prepared and inoculated with inoculum of *A niger*. Incubation was at 45^{o}C for 3, 5 and 7days. Filtrate from the culture medium was used to assay for amylase.

Cellulase

Corn Steep Liquor (CSL) at 10% concentration was the complex carbon source which produced cellulase that librated the highest sugar content. To determine the concentration at which the optimum cellulase will be produced, 7%, 8%, 9%, 10%, 11%12% and13% was included in the cellulase culture medium as carbon sources. At 45^{o}C, incubation was carried out for 3, 5 and 7days. The filtrate from the culture medium was used for cellulase assay and the mycelia weight was also determined.

3.1.7.3.6. Nitrogen sources

Inorganic Nitrogen sources

Amylase

Ammonium sulphate of all the inorganic nitrogen sources, at 0.5g/l produced the the highest amylase. The concentration at which it will produce amylase optimally was determined by inoculating amylase culture medium with different concentrations of ammonium sulphate (between 0.5 – 3.5g/l) with *A.niger,* incubated at 45^{o}C for 3, 5 and 7days. After which the culture was filtered. The filtrate obtained was used for amylase assay.

Cellulase

Potassium nitrate (KNO_3) in 5% concentration produced cellulase that yielded the highest reducing sugar. To get the concentration at which it will perform optimally, concentrations of 2, 3, 4, 5, 6, 7 and 8 gram per litre of KNO_3 were prepared in the cellulase medium as nitrogen sources. One ml of inoculums of *R. oryzae* was inoculated into the medium and incubated at 45^{o}C for 3, 5 and 7days. The filtrate of the culture medium was used for cellulase assay while the mycelia weight was determined.

Organic Nitrogen sources

Amylase:

The best organic nitrogen source was Yeast Extract at 2% concentration. To get its optimum concentration, different concentrations of 0.5%, 1%, 1.5%, 2%, 2.5%, 3% and 3.5% were prepared in the amylase culture medium as nitrogen source, sterilized and inoculated with the inoculum of *A. niger*. The filtrate obtained when the culture medium was filtered was used for amylase assay.

Cellulase

Urea was the organic nitrogen source that produced cellulase that yielded the highest reducing sugar. Different concentrations of 2, 3, 4, 5, 6, 7 and 8 grams per litre were prepared in the cellulase medium for nitrogen sources, sterilized and inoculated with 1 ml of *R oryzae* inoculum. Incubation was done at 45^{o}C for 3, 5 and 7days. The filtrate from the culture medium was used for cellulase assay and the mycelia weight was determined.

3.1.7.4. Characterization of the Amylase and Cellulase Enzymes

3.1.7.4.1. Effect of temperature on the activities of the enzymes

Amylase

One ml of the fungi culture filtrate was added to 1ml of 1% soluble starch in 0.02M phosphate pH 6.8 and incubated for 10minutes at 10^{o}C, 20^{O}C, 20^{O}C, 30^{O}C, 40^{O}C, 50^{O}C, 60^{O}C, 70^{O}C, 80^{O}C, 90^{O}C and 100^{o}Crespectively. The activity of the enzyme was determined according to Bernfeld *et al.* (1955). The mixture of 1ml of the enzyme reaction with 1ml of Dinitrosalycilic acid (DNSA) was boiled in water for 10minutes, cooled in water and the absorbance read at 540nm with SP 600 spectrophotometer.

Cellulase

One ml of 1% Carboxylmethyl Cellulose (CMC) in 0.55M acetate buffer at pH 5.0 was added to 1ml of fungal culture filtrate was incubated for 45^{o}C at 10^{o}C, 20^{O}C, 30^{O}C, 40^{O}C, 50^{O}C, 60^{O}C, 70^{O}C, 80^{O}C, 90^{O}C and 100^{o}C. The activity of cellulase was determined according to Bernfeld *et al.* (1955).

3.1.7.4.2. Temperature Stability

The crude enzyme alone in 1ml was kept at different temperatures of 10^{o}C, 20^{O}C, 30^{O}C, 40^{O}C, 50^{O}C, 60^{O}C, 70^{0}C, 80^{O}C, 90^{O}C and 100^{o}C for 10minutes, after which the activity of the enzyme was determined.

Amylase

The steps of amylase assay were followed. One ml of amylase assay medium (1g of soluble starch in 0.02M phosphate buffer at pH 6.8) was added to 1ml of enzyme kept at the different temperatures above and incubated at 45^{o}C for 1hour. The reducing sugar was determined according to Bernfeld *et al* (1955).

Cellulase

The steps of cellulase assay were followed by adding 9ml of cellulase assay medium (1% CMC in 0.55M Sodium acetate buffer at pH 5.0) to 1 ml of enzyme and kept at the temperatures above and incubated at 45^{o}C for 1hour. The reducing sugar produced was determined according to Bernfeld *et al* (1955).

3.1.7.4.3. Effect of Time on the activities of the enzymes

Amylase

The mixture of 1ml of enzyme (amylase) filtrate and 1ml of SYE mediun was incubated at 45^{o}C for 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100mins. The reducing sugar was determined according to Bernfeld *et al* (1955).

Cellulase

The mixture of 1ml of enzyme (cellulase) filtrate and 9ml of CMC was incubated at 45^{o}C for 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 minutes. The reducing sugar was determined according to Bernfeld *et al* (1955).

3.1.7.4.4. Effect of pH on the activities of the enzymes

Amylase

One ml of enzyme filtrate was mixed with 1ml of SYE medium adjusted to different pHs 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12 was incubated at 45^{o}C for 10mins.

Cellulase

One ml of enzyme filtrate was mixed with 9ml of cellulase substrate adjusted to different pHs of 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12 and incubated at 45^{o}C for 10mins (Bernfeld *et al*, 1955).

3.1.7.4.5. pH Stability

Amylase

One ml of phosphate buffer of different pHs of 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12 was added to 1ml of enzyme filtrate and was incubated at 45^{o}C for 10mins. One ml of this was mixed with 1ml of amylase enzyme substrate, incubated for 1hour at 45^{o}C.

Cellulase

One ml of acetate buffer of different pHs of 3, 4, 5, 6, 7, 8, 9, 10, 11 and12 was mixed with 1ml of enzyme filtrate and was incubated for 10mins at 45^{o}C. One ml of this was added to 9ml of cellulase enzyme substrate and incubated for 1hour at 45^{o}C.

The reducing sugar librated for amylase and cellulase was determined according to Bernfeld *et al* (1955).

3.2.7.4.6. Effect of Substrate concentration on the activities of the enzymes

Amylase

Starch, the substrate for amylase was prepared in phosphate buffer of pH 6.0 at different concentrations of 0.5%, 1%, 1.5%, 2%, 2.5%, 3%, 3.5%, 4%, 4.5% and 5% respectively. One ml of enzyme filtrate was added to 1ml of each of these substrate concentrations, incubated for 1hour at 45^{o}C

Cellulase

CMC, the substrate for cellulase was dissolved in acetate buffer of pH 5.0 at different concentrations of 0.5%, 1%, 1.5%, 2%, 2.5%, 3%, 3.5%, 4%, 4.5% and 5% respectively. One ml of of the enzyme filtrate was added to 9ml of each of the cellulase enzyme concentrations, incubated at 45^{o}C for 1hour. The reducing sugar librated for amylase and cellulase was determined according to Bernfeld *et al* (1955).

3.1.7.4.7. Effect of the enzyme concentrations on their activities

Amylase

The amount of enzyme filtrate added to 1ml of SYE medium to give different enzyme concentrations of 0.5%, 1%, 1.5%, 2%, 2.5%, 3%, 3.5%, 4%, 4.5% and 5% respectively. These were incubated at 45^{o}C for 1 hour.

Cellulase

The enzyme filtrate was added to 9ml of CMC in volumes that gave different enzyme concentrations of 0.5%, 1%, 1.5%, 2%, 2.5%, 3%, 3.5%, 4%, 4.5% and 5% respectively. Incubation was carried out at 45^{o}C for 1 hour. The librated reducing sugar for amylase and cellulase was determined according to Bernfeld *et al* (1955).

3.1.7.4.8. Effect of different Inhibitors on the activities of the enzymes

The effect of the following Inhibitors was determined on the activities of the enzymes - Ethylene diamine tetra acetic acid (EDTA), Mercuric chloride ($HgCl_2$), Potassium cyanide (KCN), Benzoic acid ($C_7H_6O_2$) and Urea (NH_2CONH_2).

The stock solutions of .1M, 0.2M, 0.3M, 0.4M, 0.5M, 0.6M, 0.7M, 0.8M, 0.9M and1M respectively was prepared for each of the inhibitors above .

Amylase

A mixture 1ml of each the stock solutions of the inhibitors with 1ml of 1g of soluble starch in 100ml of phosphate buffer pH 6.8. One ml of amylase enzyme filtrate was added to the inhibitor – starch mixture and incubated at 45^{o}Cfor 10minutes. The enzyme activity was measured by the DNSA method.

Cellulase

A mixture of 0.1ml of each of the stock solutions of the inhibitors with 1ml of 1% CMC in sodium acetate buffer of pH 5.0. Zero point five (0.5) ml of the enzyme was added to the inhibitor–CMC mixture, incubated for 10min at 45^{o}C. The enzyme activity was measured by the DNSA method.

3.1.7.4.9. Effect of different Anions on the activities of the enzymes

The effects of the following Anions were determined on the activities of the enzymes - Sodium nitrate ($NaNO_3$), Iron sulphate ($FeSO_4$), Iron chloride ($FeCl_2$), Ammonium nitrate (NH_4NO_3) and Ammonium chloride (NH_4Cl).

The stock solutions of each of the above anions were prepared by varying their concentrations of 0.1M, 0.2M, 0.3M, 0.4M, 0.5M, 0.6M, 0.7M, 0.8M, 0.9M and 1M respectively. The molecular weight of each of the anions dissolved in 1litre of distilled water gives 1M solution of the anions.

Amylase and Cellulase

The activities of amylase and cellulase under influence of the different molar concentrations of anions were determined as for the inhibitors above.

3.1.7.4.10. Effects of different Cations on the activities of the enzymes

The effects of the following Cations were determined on the activities of the enzymes - Calcium chloride ($CaCl_2$), Magnesium sulphate ($MgSO_4$), Manganese sulphate ($MnSO_4$), Potassium nitrate (KNO_3) and Sodium chloride (NaCl).

The stock solutions of the cations in different molar concentrations of 0.1M, 0.2M, 0.3M, 0.4M, 0.5M, 0.6M, 0.7M, 0.8M, 0.9M and1M was prepared. The molecular weight of the each of the cations in 1litre of distilled water makes 1M solution of the cation.

Amylase and Cellulase

The activities of amylase and cellulase with the effect of the different molar concentrations of these cations were determined as for the inhibitors above.

3.1.8. *In-vitro* biodegradation of lignocellulosic substrate samples.

Procedure

Each of the 10 fungal isolates was washed as starter culture with 1litre of sterile distilled water which was used to wet 100g of the RB and PKC samples separately and mixed thoroughly to ensure evenly distribution. The fungi were allowed to degrade the samples for two weeks (personal communication with a lecturer in the Department of Microbiology) after which the Proximate composition was determined. The sample with the highest protein

content was substituted in the fish feed.

3.1.8.1. Proximate Analysis of Biodegraded Sample:

Procedure

Two grams of the samples (dried and fermented Rice Bran (RB) and Palm Kernel cake (PKC) was weighed into pre-weighed porcelain crucibles. The crucibles were placed inside an oven at 105 ^{0}C for 18 hours and later placed in a desiccator to cool down for about 30 minutes and weighed. They were put back into the oven for 2 hours and weighed. This was repeated until the decrease in weight did not exceed 0.05 mg per gram of the sample. The weight loss was reported as Moisture content.

Calculation:-

M0 = weight in g of porcelain crucible

M1 = weight in g of crucible and sample before drying

M2 = weight in g of crucible and sample after drying

M1 – M0 = weight of sample prepared for drying

3.1.8.1.1. $$\% \text{ Moisture content} = \frac{M_1 - M_2}{M_1 - M_o} \times 100$$

3.1.8.1.2. % Dry matter content = 100 - % moisture content

3.1.8.1.3. Total Ash Determination (AOAC, 1980)

Procedure

Porcelain crucible was washed and dried in an oven and weighed. Two gram of dried material of each of the samples above was weighed inside the pre-weighed porcelain crucibles. They were placed in a muffle furnace at 55^{o}C for 6 hours. The furnace was allowed to cool down for 4 hrs. The crucible was placed in a dessicator with stopper top, cooled down and weighed.

$$\% \text{ Ash} = (\text{Weight of crucible+ ash}) - \frac{\text{Weight of empty crucible}}{\text{Sample weight}} \times 100$$

3.1.8.1.4. Crude Protein Determination

KjeIdahl Nitrogen method (AOAC, 1980)

Boric acid Preparation

About 400 g boric acid crystals was dissolved in 6 litres of boiled deionized water. It was mixed and was made up to a volume of about 9 litres with more boiled deionized water and cooled. One hundred millilitres of bromocresol green solution (100 mg of the bromocresol green in 100 ml alcohol) and 70 ml methyl red solution (100 mg of methyl red in 100 ml alcohol) were added at room temperature. Deionized water was used to dilute it up to 10 litres. It was carefully mixed.

Procedure

Zero point five (0.5) g of the sample was weighed into the digestion flask, kjeldahl catalyst tablets were added. Ten ml of concentrated H_2S0_4 was added and 20 ml of distilled water, the flask was fixed into the digestor and was put on for about 8 hours until a clear solution was obtained. It was cooled and the digest was transferred into 100 ml volumetric flask, distilled water was added to make it up to mark. The distillation apparatus was set up and rinsed for 10 mins after boiling. Ten ml of boric acid was put into a conical flask. Two drops of indicator in order to trap the ammonia gas involved was added. Five millilitres of the digest was added into the kjeldahl distillation flasks. The conical flasks and distillation flasks were fixed in place and 20 ml of 40% NaOH was added through the glass funnel into the digest. The steam exit was closed. Distillation was done for 15 mins as soon as the solution reached the mark of 25 ml and the boric acid indicator turns green. The distillate was titrated with 0.05 N HCl.

Calculation

$$\% \text{ Total Nitrogen} + 14.01 \frac{\text{Sample titre} - \text{blank titre}}{10 \times \text{Sample weight}} \text{ N}$$

N = Normality of the acid

% Protein (crude) = % Total Nitrogen X Conversion factor (6.25)

3.1.8.1.5. Crude Fat Determination (Soxhlet Method) (AOAC, 1980)

Procedure

250ml extraction flasks were washed and dried.

They were dried in an oven at 105^{o}C – 110^{o}C for 30 mins, allowed to cool down in a dessicator and weighed. The soxhlet extractor was set up with reflux condenser and the water flow through the condenser. Two gram of dried sample was folded inside filter papers and transferred into a fat-free extraction thimble and was plugged lightly with cotton wool.

The thimble was placed in the extraction barrel and petroleum ether (boiling poit 40 – 60^{o}C) was added until it was siphoned once in the flask directly below it. Flasks were heated and samples were refluxed for about 8 hours.

The thimbles were removed from the extraction barrel after extraction and dried.

The barrels were replaced and the solvent was distilled off until the extraction flasks were almost dried

The flasks containing the solvent were detached and dried in the oven at 105^{o}C – 110^{o}C for 1 hour for the solvent to evaporate completely.

The flask was weighed and the fat content was calculated as follow

$$\% \text{ Fat} = \frac{(\text{Weight of flask + fat}) - (\text{weight of empty flask})}{\text{Sample weight}} \times 100$$

3.1.8.1.6. Crude Fibre Determination (AOAC, 1980).

Procedure

One gram of defaulted samples were weight into 600 ml beaker and 100 ml of 1.25% H_2SO_4 was added and boiled under refluxed for 30 minutes beginning from the time boiling started and was filtered after the flasks were removed. The residues were washed with hot distilled water. The sample was rinsed back into the beaker and 100 ml of NaOH was added and boiled under reflux for 30 minutes. The digested sample was rinsed into a pre-weighed crucible with acetone and dried inside oven to a constant weight and weighed (WA). They were ashed in a muffle furnace at 500^{0}C for 6 hours, cooled down and weighed (weight WB).

The loss in weight during incineration is equivalent to the amount of crude fibre.

$$\% \text{ Crude fibre} = \frac{\text{Weight A} - \text{Weight B}}{\text{Sample weight}} \times 100$$

3.1.8.1.7. Nitrogen Free Extract (NFE) Crampton and Harris, 1969

NFE value of a food substance is obtained by adding up the percentage values of its ash, crude fibre, ether extract and crude protein values or contents analyzed and subtracting them from 100 (Crampton and Harris, 1969)

Calculations

NFE (%) on dry basis = 100% - (ash (%) on dry basis + crude fibre (%) on dry basis + ether extract (%) on dry basis + protein (%) on dry basis).

3.1.8.1.8. Cellulose

This was determined according to the method of Van Soest and Wine (1968). The cellulose content was calculated thus

ADF value – ADL value.

3.1.8.1.9. Acid – Detergent Fibre Determination: - (ADF) AOAC, 1980; Van Soest, 1963.

Procedure

Two grams of each sample was weighed into beakers for refluxing. One hundred ml of acid Detergent solution cooled to room temperature was added along with 2ml decahydronaphthalene. They were filtered on a previously tarred crucible, which was set on the filter manifold using light solution. The filtered mats were broken up with a rod and washed twice with hot water

They were washed with acetone until no more color was removed; lumps were broken up so that the solvent came into contact with all particles fiber.

Acetone was sucked out totally from the acid detergent fiber, dried at 105 ^{0}C for 8 hours, cooled down to room temperature in a desiccator and weighed

Calculations

$$\% \text{ ADF} = \frac{A-B}{S}$$

A = Weight of paper or crucible with fiber
B = tarred weight of paper or crucible
S = Sample weight

3.1.8.1.10. Neutral Detergent Fibre (Cell wall constituents) NDF: - (Goering and Van Soest, 1970; Van Soest *et al.*, (1991).

Procedure

Samples were grinded to pass through a 1mm Screen. One gram of each sample was placed in Berzelius beaker and refluxed through 100ml cold (room temperature) neutral detergent solution was added, followed by 2 ml decahydronaphthalene and then 0.5 g sodium sulphite. They were heated up to boil for 5 to 10 minutes and the heat was reduced as the boiling started to avoid foaming. The boiling was adjusted to an even level and refluxed for 60 minutes timed from onset of boiling.

Previously tarred crucibles were placed on filtering apparatus. Beakers were swirled to suspend solids and fill crucibles. After the crucibles were filled, vacuum was created; low vacuum was used at first and increased as more force is needed. Samples were rinsed into crucibles with hot water. Vacuum was removed, mats were broken up, and crucibles were filled with hot water. Liquid was filtered and washed.

Residues were washed twice with acetone and sucked dry. The crucibles were dried at 105^0C overnight (12 hours) and weighed.

The yield of recovered neutral-detergent fiber was reported as cell walls.

Calculations

% NDF (Cwc) = Dry weight of crucible and residue - Dry tarred weight of crucible

Weight of initial sample =100

3.1.8.1.11. Lignin Detergent (Acid Detergent) (ADL) :- (AOAC, 1980)

Procedure

Acid-detergent fiber (ADF) was prepared for each sample.

Crucibles containing the ADF of each sample were placed in a glass tray with one end of the tray raised higher to drain away acid from the crucibles. The contents of the crucibles were covered with cooled (5^oC) 72% H_2S0_4 and stirred with a glass rod to a smooth paste with all the limps broken. Crucibles were filled up about half way with acid and stirred. They were refilled with 72% H_2S0_4 and stirred at hourly intervals as acid drained away.

The crucibles were kept at 23^oC (room temperature) and acid filtered off as much as possible with vacuum after 3 hours. The contents were washed with hot water until they were free from acid, rinsed and contents washed with acetone. Crucibles were dried at 100^oC overnight and weighed. Crucibles were ignited in a muffle furnace at 500^oC for 3 hours, cooled to 250^oC and transferred to a desiccator, cooled to room temperature and weighed.

Calculations

$$\% \text{ ADL} = \frac{\text{(Dry weight of crucible + contents) – (Dry weight after ashing)}}{\text{Original weight of ADF Sample}} \text{ X } 100$$

3.1.8.1.12. Determination of Amino Acid Profile

The amino acid profile in the sample was determined using methods described by Spackman *et al*. (1958). The sample was dried to constant weight, defatted, hydrolyzed, evaporated in a rotary evaporator and loaded into the Technicon sequential Multi-sample Amino Acid Analyzer (TSM).

Defatting Sample

A known weight of the dried sample was weighed into extraction thimble and the fat was extracted with chloroform/methanol (2:1 mixture) using soxhlet extraction apparatus as described by AOAC (1980). The extraction lasted 15 hours.

Nitrogen Determination (Smyth *et al*, 1963)

A small amount (200 mg) of ground sample was weighed, wrapped in Whatman filter paper (No. 1) and put in a Kjeldhal digestion flask. Concentrated sulphuric acid (10 ml) was added.

Catalyst mixture (0.5 g) containing sodium sulphate (Na_2SO_4), copper sulphate ($CuSO_4$) and selenium oxide (SeO_2) in the ratio of 10:5:1 was added into the flask to facilitate digestion. Four pieces of anti-buping granules were added.

The flask was then put on Kjedhal digestion apparatus for 3 hours until the liquid turned light green. The digested sample was cooled and diluted with distilled water to 100 ml in standard volumetric flask. Aliquot (10 ml) of the diluted solution with 10 ml of 45% sodium hydroxide were put into the Markhlam distillation apparatus and distilled into 10 ml of 2% boric acid containing 4 drops of bromocresol green indicator until about 70 ml of distillate was collected. The distillate was titrated with hydrochloric acid (standardized 0.01 N) to grey colored end point (Smyth *et al*., 1963). The percentage nitrogen in the original sample was calculated using the formula:

$$\text{Percentage Nitrogen} = \frac{(a - b) \times 0.01 \times 14}{W \times C} \times 100$$

Where:

a = Titre value of the digested sample

b = Titre value of blank sample

v = Volume after dilution (100ml)

w = Weight of dried sample (mg)

C = Aliquot of the sample used (10ml)

14 mg =Nitrogen constant in mg

100 = Conversion factor to percentage

Hydrolysis of the Sample (Ijarotimi and Keshinro, 2011)

A known weight of the defatted sample was weighed into glass ampoule. Seven ml of 6NHCL was added and oxygen was expelled by passing nitrogen into the ampoule (this is to avoid possible oxidation of some amino acids during hydrolysis e g methionine and cystine). The glass ampoule was then sealed with Burnsen burner flame and put in an oven preset at 105 ^{o}C $\pm$ 5 ^{o}C for 22 hours. The ampoule was allowed to cool down before broken open at

the tip and content was filtered. The filtrate was then evaporated to dryness at 40^{o}C under vacuum in a rotary evaporator. The residue was dissolved with 5 ml to acetate buffer (pH 2.0) and stored in plastic specimen bottles, which were kept in the freezer.

Loading of the Hydrolysate into the Technicon Sequential Multi – Sample Amino acid (TSM) Analyzer

The amount loaded was between 5 to 10 microlitres. The TSM analyzer is designed to separate and analyze free acidic, neutral and basic amino acids of the hydrolysate. The period of analysis lasted 76 minutes.

Method of Calculating Amino Acid Values from the Chromatogram Peaks (Speckman *et al*, 1958)

The net height of each peak produced by the chart recorder of TSM (each representing an amino acid) was measured. The half-height of the peak was accurately measured and recorded. Approximate area of each peak was then obtained by multiplying the height with the width at half-height.

The norleucine equivalent (NE) for each amino acid in the standard mixture was calculated using the formula:

$$NE = \frac{\text{Area of Norleucine Peak}}{\text{Area of each amino acid}}$$

A constant S was calculated for each amino acid in the standard mixture:

Sstd = NE std x mol. Weight x µMAAstd

Finally, the amount of each amino acid present in sample was calculated in g/16 N or g/100 g protein using the following formula:

$$\text{Concentration (g/10 g protein)} = NH \times W(a) \frac{NH}{2} \times Sstd \times C$$

$$\text{Where} \quad C = \frac{\text{Dilution} \times 16}{\text{Sample wt (g)} \times N\% \times 10 \times \text{vol. Loaded}} \quad NH \times W(nleu)$$

Where: NH =Net height

W = Widh @ half height

Nleu = Norleucine.

Sample wt (g) x N% 10 x vol. Loaded

3.1.8.1.13. Test for Presence of Toxic Substances in Fungal Mycelia and in Degraded Products

The presence of toxic substances like Alkaloids, Oxalate Phytate, Tannin, Phytic acid, Trypsin inhibitor, Cyanogeni glycoside, Hemagglutinin was tested for in (a) Feed Samples (fermented and unfermented samples). The toxic substances tested for in the mycelia of the fungi include Mycotoxin, Aflatoxin, Fumosin, Alkaloid and Oxalate (Davies, 1989; AOAC, 1984 and Henry, 1993).

3.1.8.1.13.1. Test for Aflatoxin-producing ability: -

Mycelia from the isolated mould was picked with a sterile loop and placed on the surface of coconut extract agar (CEA) aseptically. The plates were incubated at room temperature for 7 days; observation was made after the 3rd day of incubation. The plates that produced orange-yellow pigmentation in CEA were observed under UV lamp. The orange–yellow pigmentation is a rapid presumptive test for aflatoxin production. Aflatoxin producing colonies were detected under long UV light (365 nm) by blue fluorescence on the reverse side after 5 days of growth (Davies *et al*., 1987). Isolates that produced a blue fluorescence under UV light (365 nm) were considered aflatoxin producing

3.1.8.1.14. Test for Anti-nutritional Factors (ANF)

3.1.8.1.14.1. Quantitative Test for Alkaloids

Two grams of finely ground sample was weighed into a 100 ml beaker; 20 ml of 80% alcohol was added to give a smooth paste. The mixture was transferred to a 250 ml flask and more alcohol was added to make up to 100 ml and 1g magnesium oxide added. The mixture was digested in a boiling water bath for 1.5 hrs under a reflux air condenser with occasional shaking. The mixture was filtered while hot through a small Buchner funnel. The residue was returned to the flask and redigested for 30 min with 50 ml alcohol after which the alcohol will be evaporated. Hot water was added to replace the alcohol lost. When all the alcohol has been removed, 3 drops of 10% HCL was added. The whole solution was later transferred into a 250 ml volumetric flask. Ten ml of zinc acetate solution and 5 ml of potassium ferrocyanide solution was added, thoroughly mixed to give a homogenous solution.

The flask was allowed to stand for a few minutes, the solution was filtered through a dry filter paper and 10 ml of the filtrate was transferred into a separatory funnel and the alkaloids present were extracted vigorously by shaking with five successive portions of chloroform. The residue obtained was dissolved in 10 ml hot distilled water and transferred into a kjeldahl tube with the addition of 0.20 g sucrose and 10 ml conc. H_2SO_4 and 0.02 g selenium for digestion to a colorless solution to determine %N by Kjeldahl distillation method. %Nitrogen got is converted to % total alkaloid by multiplying by a factor of 3.26 that is, % alkaloids = %N × 3.26 (Henry 1993, ACO Analysis, 1992).

3.1.8.1.14.2. Qualitative Test for Oxalate (Sigrid Pel ds zus *et al*, 1998).

Ten millilitres of distilled water was added to 0.2g of inoculum. Two ml of 5% potassium permanganate ($KMnO_4$) was added. The production of a gas with an odor of acetaldehyde as a result of reduction of $KMnO_4$ by the inoculums indicated the presence of oxalate.

3.1.8.1.14.3. Quantitative Determination of Oxalate (Maga, 1983; AOAC, 1984 and Sigrid Pel ds zus *et al*, 1998).

One gram of inoculum was boiled with 40 ml water for 30 mins under reflux. Ten ml of 20% $NaCO_3$ was added and boiled for another 30 mins. The mixture was filtered and washed with hot water till it does not show any alkaline reaction. The content was washed with water and filtered. The filtrate was concentrated by heating to a small volume and cooled. With constant stirring, HCl was added drop wise until the final acid concentration after neutralization was about 4% at which stage a heavy precipitate appeared (which was allowed to flocculate). The extract was filtered carefully into a 250 ml flask and made up to mark. It was kept overnight and filtered.

Aliquot of this filtrate was put into a 400 ml beaker, diluted with water to 200 ml, made just ammoniacal and reacidified with Lactic Acid. Ten ml of a 10% calcium chloride solution was added in the cold and stirred well to allow calcium oxalate precipitate to appear. It was allowed to settle overnight. The clean supernatant was carefully decanted off through Whatman No. 42 filter paper, without disturbing the precipitate. The precipitate was dissolved in HCl, oxalic acid was precipitated by adjusting the pH with ammonium hydroxide solution. The content was boiled and allowed to settle overnight. Oxalic acid was determined by titrating against 0.05 N $KMnO_4$ solution.

1ml of 0.05N $KMnO_4$ = 0.00225 anhydrous oxalic Acid

=% Oxalic Acid.

$$= \frac{\text{Titre Value}}{2} \times 0.00225 \times \frac{100}{1}$$

= T.V X 0.1125. (Maga, 1983).

3.1.8.1.14.4. Qualitative Test for Fumosin (Mansson *et al*, 2010)

Inoculum (0.1g) was dissolved in 2ml of water. One ml of 10% $FeCl_3$ (Ferric Chloride) solution was added. The development of a purple to violet color which turned red after 2 drops of 10% H_2SO_4 was added indicated the presence of fumosin.

3.1.8.1.14.4.1. Quantitative Determination of Fumosin (A.O.A.C., 1984; Mansson *et al*, 2010)

Sixty five ml of 85% acetonitrile was added to 0.1 g of inoculum and warmed on the steam bath to 50^{o}C with occasional swirling. It was allowed to cool at room temperature for 30 mins. The mixture was filtered through a double layer hardened Whatman No. 1 filter paper into a 100ml volumetric flask and made up to the mark with 85% acetonitrile solution.

Standard solution of Fumosin of range 0-10 µg/ml was prepared from 1000ppm Stock Fumosin by taking 0, 2 ml, 4 ml, 6 ml, 8 ml and 10 ml and made up to 50 ml with 95% acetone. Two milliters of 95% Dimethyl formanide (DMF) was added to each of the solutions of standards and sample aliquot.

The absorbance of standard solutions and sample aliquots were read after color development with 95% DMF at a wavelength of 560 nm.

$$\% \text{ Fumosin} = \frac{\text{Absorbance of sample x Average gradient x Dilution Factor}}{10{,}000}$$

3.1.9. Evaluation of the performance and other characteristics of fungi-degraded bulk of these lignocellulosic substrates (RB and PKC) substituted in the diets of *Clarias gariepinus*.

3.1.9.1: Cultivation of *Clarias gariepinus* – The aim of fish culture principally is to produce fish food for human consumption. The cultivation of fish (*Clarias gariepinus*) can be done in either earthen or concrete tanks, reinforced plastic, wooden troughs or cages (Omitoyin,

2007). This research work was carried out in experimental tanks called aquaria (glass)

3.1.9.1.1: Stocking of fish: The glass aquaria were washed and filled three quarter with water. Ten number *Clarias gariepinus* fingerlings were weighed (initial weight), after acclimatization and stocked into each of the aquaria.

3.1.9.1.2: Fish feeding trial: Feeding trial was conducted 4 times on *Clarias* gariepinus fingerlings as follows-

(1) Fingerlings of local species of *Clarias gariepinus* were fed directly with (a), unfermented rice bran, (b), unfermented palm kernel cake, (c), mixture of a and b, (d) spontaneously fermented rice bran, (e) spontaneously fermented palm kernel cake and (f), the mixture of d and e (were not substituted in the standard fish feed) and fish feed (7 different treatments) for 90 days (Omitoyin, 1995).

(2) Fingerling of Dutch species of *C. gariepinus* were fed with the same 7 different treatments as with the local species in (1) above for 90 days

3) Fingerling of Dutch of *C. gariepinus* species were fed with – a) unfermented rice bran, b) unfermented palm kernel cake, c) spontaneously fermented rice bran and) spontaneously fermented palm kernel cake (on Table 2 above) substituted in the standard fish feed for 90 days. **Techniques in ration formulation:** Generally, fish rations are balanced first on the basis of crude protein requirements because of its importance in and on growth, egg composition and production, meat production and other protein related physiological implications or factors (Falayi, 2009d). Other nutrient values to be considered are energy, fat/oil, fibre and mineral contents (Akintomide, 2008).Formulating the rations can be accomplished manually or by the use of computer (Linear programming).Manual computation can be achieved by using one or a combination of two or more of the following methods: (a) .Pearson's square, (b). Algebraic or quadratic or simultaneous equation, (c). Trial by error, (d) Least cost feed, (e). A combination of a – c. The algebraic method was used in formulating the feed in this fish feeding trials (Ovie and Ovie, 2010). See appendix

The metabolizable energy (M.E.) of the different treatments used for the feeding trials on Table 3.2 below was obtained from their proximate contents according to Jenssen, 1989.

M. E. = 38.79 x % Crude Protein (C.P) + 87.24 x % Fat + 18.22 x Nitrogen Free Extract (NFE).

Table 3.2: The seven (7) treatments used for feeding trials I (fingerling of local *C. gariepinus* Burtchel, 1822) and 2 (fingerling of Dutch *C. gariepinus* Burchel, 1822).

Treatment	Metabolizable Energy(kcal/kg)
Unfermented Rice bran	1494.7632
Fermented Rice bran	1940.9031
Unfermented Palm kernel cake	2881.6124
Fermented Palm kernel cake	2605.659
Unfermented (Rice bran +Palm kernel cake)	3246.7659
Fermented (Rice bran +Palm kernel cake)	2744.0285
Fish feed (standard)	2132.8374

Table 3.3: Feed samples used for feeding trial 3

Ingredient	Standard fish feed	Unfermented rice bran	Fermented rice bran	Unfermented palm kernel cake	Fermented palm kernel cake
Fish meal	25.00	25.00	25.00	25.00	25.00
Soya bean meal	17.78	28.17	26.65	21.07	12.15
Ground nut cake	25.00	25.00	25.00	25.00	25.00
Maize bran	25.04	0.00	0.00	0.00	0.00
Red palm oil	2.00	2.00	2.00	2.00	2.00
Starch	2.00	2.00	2.00	2.00	2.00
Bone meal	1.50	1.50	1.50	1.50	1.50
Premix	1.00	1.00	1.00	1.00	1.00
Methionine	0.20	0.20	0.20	0.20	0.20
Lysine	0.20	0.20	0.20	0.20	0.20
Table salt	0.25	0.25	0.25	0.25	0.25
Vitamin C.	0.03	0.03	0.03	0.03	0.03
Unfermented Rice bran	0.00	21.83	0.00	0.00	0.00
Fermented Rice bran	0.00	0.00	23.37	0.00	0.00
Unfermented Palm kernel cake	0.00	0.00	0.00	28.93	0.00
Fermented Palm kernel cake	0.00	0.00	0.00	0.00	37.85

Table 3: 4: Feed samples substituted with fungal fermented substrates used for feeding trial 4

Ingredient	Fish feed	Rice bran M.E (2406.6000kcal/kg)		Palm kernel cake M.E (2825.3482kcal/kg)	
		10%	20%	10%	20%
Fish meal (FM) (Clupeid)	25	25	25	25	25
Soya bean meal (defatted) (SBM)	17.78	19.66	21.56	20.30	17.80
Groundnut cake (GNC)	25	25	25	25	25
Maize bran (MB)	25.04	13.16	1.26	12.52	5.02
Red Palm oil	2.00	2.00	2.00	2.00	2.00
Starch	2.00	2.00	2.00	2.00	2.00
Bone meal (BM)	1.50	1.50	1.50	1.50	1.50
Premix	1.00	1.00	1.00	1.00	1.00
Methionine	0.20	0.20	0.20	0.20	0.20
Lysine	0.20	0.20	0.20	0.20	0.20
Table salt	0.25	0.25	0.25	0.25	0.25
Vitamine C	0.03	0.03	0.03	0.03	0.03

Control: - Fish feed compounded at N.I.F.F.R, New- Bussa served as the control feed treatment in all the feeding trial experiments (Tables above).

Fermentation with starter cultures

Spores from pure plates of each of the fungi isolates were washed with sterile distilled water into sterile beakers. One litre of suspension of the spores of each fungi isolate was thoroughly mixed with 50g of rice bran and palm kernel cake separately. Each was allowed to ferment for 30days inside sterile nylon bags. Proximate composition of each was carried out. The fermented samples with the highest protein content out of the RB and PKC were incorporated into the components of the standard in 10% and 20% proportions. Rice bran fermented with the spores of *Aspergillus clavatus* with 6.5% protein and 2406.6 kcal/kg metabolizable energy and Palm kernel cake fermented with suspension of *A.tamarii* spores with 17.9% protein and 2825.4 kca/kg were used for the fish feeding trial experiment 4 (Table 4 above).

3.1.9.2. Growth and Nutrient Utilization Parameters

Weight gain, Feed fed, Specific growth ratio, Feed conversion ratio, Nitrogen metabolism Survival and Mortality ratios were the growth and nutrient utilization parameters of the *Claris gariepinus* fingerlins were determined.

3.1.9.2.1. Mean Weight Gain (WTG)

$WTG = W_2 - W_1$ where

W_1 = the initial mean weight at stocking (T_1)

W_2 = the final mean weight of fish at the last day of the experiment (T_2)

3.1.9.2.2. Feed fed (FF)

The addition of daily mean feed fed the fish in each treatment for the experimental period.

3.1.9.2.3. Specific Growth Rate (SGR %)

This was calculated according to the method described by Brown (1975).

$$SGR\ (\%) = \frac{\text{Log } W_2 - \text{Log } W_1}{T_2 - T_1} \times 100$$

W_2 = Weight of fish at time T_2 days

W_1 = Weight of fish at time T_1 days

3.1.9.2.4. Feed Conversion Ratio (FCR)

$$\textbf{FRC} = \frac{\text{Feed fed (g)}}{\text{Weight gain (g)}}$$

3.1.9.2.5. Nitrogen Metabolism (NM)

NM was estimated using the method described by Zeitoun *et al* (1976).

Where 0.549 is the factor used for daily endogenous nitrogen losses in ruminants (Titus, 1927). However this value is much as twice the value in non- ruminant such as *C.gariepinus* which FAO/ADCP (1980) described as an ammonotelic omnivore, hence the denominator factor 2.

b= final body weight of fish (g)

a = initial body weight of fish (g)

g= the number of experimental days

3.1.9.2.6. Survival Rate (%SR) (Omitoyin, 1995)

The survival rate expressed in percentage was calculated from the relationship:

$$\%SR = \frac{\text{Initial number of fish stocked} - \text{mortality}}{\text{Initial number of fish stocked}} \times 100$$

3.1.10. Water Quality Analysis.

3.1.10.1. Water Temperature.

The water temperature for each of the treatments was measured using a thermometer calibrated from $0^{o}C$ – $110^{o}C$ on a daily basis. Measurement was carried out by gently immersing the thermometer into the water at vertical position and left for about 1 -2 minutes to pick up the temperature of the water. It was then quickly moved near the surface of the water and read.

3.1.10.2. Dissolved Oxygen (DO)

The Dissolved Oxygen (DO) concentration in each of the treatments was determined by the electrometric method using oxygen-detecting electrode called Dissolved Oxygen meter (Omitoyin, 1995).

3.1.10.3. Hydrogen Ion Concentration (pH)

The pH was measured using an electrometric method of a combination of glass electrode with a reference potential provided by a standard calomel electrode.

CHAPTER FOUR

4.0 RESULTS

A total of one hundred and twenty four (124) fungal isolates were obtained from the unfermented and fermented substrates (Rice Bran (RB), Palm Kernel Cake samples (PKC)) collected from New Bussa and Ibadan, Niger State and Oyo State respectively. The fungal isolates include 6 strains of *Aspergillus* (*Aspergillus niger*, *A. flavus*, *A. terreus*, *A. clavus*, *A. versicolor*, *A. tamarii*), *Rhizopus oryzae*, *Rhizopus stolonifer* and *Trichoderma* sp. The isolates were identified based on their cultural and morphological observation as shown in Table 4.1

Table 4.2 shows the frequency of occurrence of the fungal isolates obtained from the categorized spontaneously fermented and non-fermented substrates. The most predominant fungal strain isolated was *Aspergillus flavus* (32.25%) followed in order by *R. stolonifer* (16.13%) and *A. niger* (14.52%) while *Trichoderma* sp. (2.42%) was the least. The percentage frequency of occurrence of the isolates is shown in Figure 4.1 in which *A. flavus* had the highest frequency of occurrence (32.25%) followed in order by *R. stolonifer* (16.13%) while *Trichoderma* sp. had the lowest (2.42%).

The temperature and pH change during spontaneous fermentation of the substrates is shown in Table 4.3. The temperature ranged from 31- 38ºC. Wet Palm Kernel Cake Sludge ($PKCS_w$) and Deoiled Cake (DOC) samples had the lowest while Palm Kernel Cake sample from a Local Feed Mill ($LFMPKC_1$) had the highest (38ºC) at day 5 after fermentation. The pH changes ranged from 4.18- 6.46 in which the highest was recorded in sample RB_1 at day 1. The lowest was recorded in sample RB_2 at day 5 of fermentation. There was a significant difference in pH changes during the period of fermentation.

Table 4.1:- Morphological Characteristies of the fungi isolated from categorized fermented and non-fermented lignocellulosic substrates

ISOLATES CODE	Appearance on Agar (PDA)	Microscopic spore shape	Examination of Hyphae	FUNGAL IDENTIFIED.
1BK.	Flat and black on the surface. Orange to creamish-yellow on the reverse.	Stipe is long, smooth, brown; vesicle globose, thick walled.	Conidia globose, rough dark brown which later turned black.	*Aspergillus niger*
2GR.	Green on the surface. Greenish yellow on the reverse.	Stipe is long, varicose and hyaline.	Dome shaped vesicle. Conidia globose, rough and yellowish green.	*Aspergillus flavus.*
3BR	Yellowish brown, brown on the reverse.	Columnar head, Stipe is short and smooth.	Vesicle is hemispherical; Globose Conidia	*Aspergillus terreus*
4BL.	Dull green to Bluish, creamish yellow on the reverse	Microscopically, long and smooth stripe and clavate towards the tip.	Vesicle is hemispherical, conidia globose	*Aspergillus clavatus*
5OR.	Smooth, orange on the surface, creamy on the reverse.	Long and smooth stipe.	Small, globose vesicle; smooth, globose conidia.	*Aspergillus versicolor*
10DB.	Rusty brown on the surface, light brown on the reverse	Long, rough stipe	Globose vesicle; conidia thick, rough and globose.	*Aspergillus tamarii*
6GY.	Growth is grayish, fast growing and about 10mm high.	Stolon smooth, light brown	Sporangiophores arise from aerial hyphae in groups globose. Sporangia globose	*Rhizopus oryzae*
7DW.	Dull white, fast growing, about 20mm high,	Stolon smooth opposite rhizoids.	Sporangia globose, black, Sporangiophore brown..	*Rhizopus stolonifer.*
8WT.	Whitish, fast growing, more than 20mm high	Long smooth stolon	Sporangia black.	*Rhizopus sp.*
11PK.	Brownish pink on the surface and on the reverse.	Short and dense stripes, vesicles small and numerous	Conidia small, globose.	*Trichoderma sp.*

The code and names of the fungi isolated from lignicellulostic substrates (Rice Bran (RB) and Palm Kernel Cake (PKC)) as shown in Table 4:1.

TABLE: 4.2:- Frequency of occurrence of fungi isolated from categorized fermented and non-fermented lignocellulosic substrates

Substrate	Isolate	No of colonies	Frequency of occurrence (%)
RB1	*A. niger*	1	10
	A. flavus	4	40
	A. clavatus	1	10
	A. versicolor	1	10
	R. stolonifer	2	20
	Rhizopus sp	1	10
RB2	*A. niger*	2	16.66
	A. flavus	5	41.66
	A.versicolor	2	16.66
	R. stolonifer	2	16.66
	Rhizopus sp	1	8.33
$LFMPKC_1$	*A. flavus*	5	41.66
	A. terreus	1	8.33
	A. clavatus	1	8.33
	A. tamarii	1	8.33
	R. oryzae	1	8.33
	R. stolonifer	2	16.66
	Rhizopus sp	1	8.33
$LFMPKC_2$	*A. niger*	4	28.57
	A. flavus	3	21.42
	A. terreus	1	7.14
	A. clavatus	1	7.14
	A. tamarii	1	7.14
	R. oryzae	1	7.14
	R. stolonifer	2	14.28
	Rhizopus sp	1	7.14
$IndPKC_1$	*A. niger*	3	25
	A. flavus	3	25
	A. clavatus	1	8.33
	A. tamarii	1	8.33
	A. vesicolor	1	8.33
	R. stolonifer	1	8.33
	Rhizopus sp.	1	8.33
	Trichoderma sp	1	8.33
$IndPKC_2$	*A.niger*	3	27.27
	A. flavus	2	18.18
	A. clavatus	1	9.09
	A. versicolor	1	9.09
	A. tamarii	1	9.09
	R. stolonifer	1	9.09
	Rhizopus sp.	1	9.09
	Trichoderma sp.	1	9.09
$PKCS_D$	*A. niger*	3	33.33
	A. flavus	2	22.22
	A. clavatus	1	11.11
	R. stolonifer	2	22.22
	Rhizopus sp.	1	11.11
$PKCS_W$	*A. flavus*	1	20

	A. clavatus	1	20
	A. vesicolor	1	20
	R. oryzae	1	20
	R. stolonifer	1	20
$LPKC_1$	*A. flavus*	5	50
	A. clavatus	1	10
	A.vesicolor	2	20
	R. stolonifer	1	10
	Rhizopus sp.	1	10
	Trichoderma sp.	1	10
$LPKC_2$	*A. flavus*	4	50
	R. stolonifer	2	25
	Rhizopus sp.	2	25
$LPKC_3$	*A. flavus*	4	36.36
	A. terreus	1	9.09
	A.vesicolor	1	9.09
	A. tamarii	1	9.09
	R. stolonifer	2	18.18
	Rhizopus sp.	2	18.18
DOC	*A. niger*	2	20
	A. flavus	2	20
	A. clavatus	2	20
	A. tamarii	1	10
	R. stolonifer	1	10
	Rhizopus sp.	2	20

Legend: - RB=Rice bran, LPKC=Palm Kernel Cake (PKC) from local oil mill, LFMPKC=PKC from local feed mill, IndPKC=PKC from industry, $PKCS_D$= PKC sludge (dried), $PKCSW_=$ PKC sludge (wet), DOC= Deoiled cake.

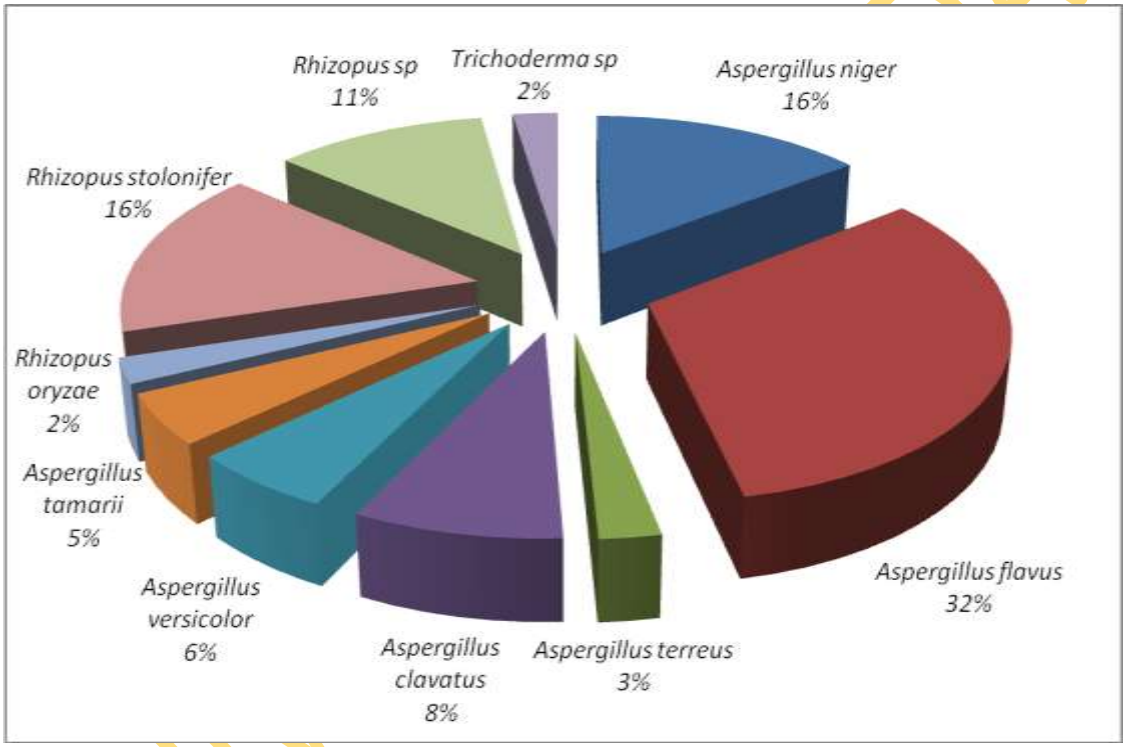


Figure 4.1:- Percentage frequency of occurrence of the fungal isolates from the lignocellulosic substrates

Table 4.3:- Temperature (^{o}C) and pH changes during Spontaneous fermentation of the lignocellulosic substrates.

Lignocellulosic Sample Code	Incubation period (days)/Temperature (^{o}C)					Incubation period (days) / pH				
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 1	Day 2	Day 3	Day 4	Day 5
RB1	34^{a*}	34^{b}	34^{b}	34^{c}	33^{f}	6.46^{a}	5.90^{b}	5.60^{a}	6.01^{b}	6.02^{a}
RB2	34^{a}	33^{c}	33^{d}	32^{e}	33^{f}	5.14^{f}	4.80^{g}	4.50^{g}	5.00^{j}	4.18^{i}
LKPC1	34^{a}	33^{c}	33^{d}	33^{d}	34^{e}	5.41^{cd}	5.98^{a}	5.45^{b}	5.38^{h}	4.45^{h}
LPKC2	34^{a}	33^{c}	33^{d}	32^{e}	34^{e}	5.48^{c}	5.65^{d}	4.80^{f}	5.45^{g}	4.64^{g}
LPKC3	34^{a}	34^{b}	34^{b}	33^{d}	35^{c}	5.40^{d}	5.64^{d}	4.95^{e}	5.84^{d}	4.87^{e}
LFMPKC1	34^{a}	35^{a}	36^{a}	37^{a}	38^{a}	5.27^{e}	5.91^{b}	5.59^{a}	5.80^{d}	5.70^{b}
LFMPKC2	34^{a}	33^{c}	32^{d}	32^{e}	34^{e}	5.27^{e}	5.80^{c}	5.11^{d}	5.50^{f}	4.70^{f}
IndPKC1	34^{a}	34^{b}	32^{d}	33^{d}	33^{f}	5.30^{e}	5.07^{f}	5.28^{c}	5.44^{g}	4.72^{f}
IndPKC2	32^{c}	32^{d}	32^{d}	33^{d}	32^{g}	5.47^{bcd}	5.87^{b}	5.10^{d}	5.73^{e}	5.46^{d}
PKCSD	32^{c}	32^{d}	31^{e}	32^{e}	33^{f}	5.50^{b}	5.89^{b}	5.26^{c}	5.96^{c}	5.55^{c}
PKCSw	32^{c}	32^{d}	31^{e}	32^{e}	33^{f}	4.45^{g}	5.13^{e}	4.45^{h}	5.11^{i}	4.46^{h}
DOC	33^{b}	33^{c}	31^{e}	34^{c}	33^{f}	5.50^{b}	5.78^{c}	5.29^{c}	6.65^{a}	6.04^{a}

*Values with the same letters are not significantly different using Duncan's multiple RangeTest

Key: RB = Rice Bran, LPKC = Local Palm Kernel Cake, IndPLC = Industrial Palm Kernel Cake, LFMPKC = Palm Kernel Cake from Local feed mill, $PKCS_W$ = Sludge of Palm Kernel Cake, PKCSD = Dry Sludge of Palm Kernel Cake DOC = Deoiled Cake.

The Acid detergent Fibre (ADF), Neutral Detergent Fibre (NDF), Acid Detergent Lignin (ADL), Cellulose and metabolizable energy of the unfermented (UF) and fermented (F) substrates is shown in Table 4.4. There was significant difference in ADF, NDF, ADL, Cellulose and ME. The ADF of the unfermented and fermented substrates ranged from 30.00 – 69.00% in which the highest ADF of 69.00% was recorded in fermented RB2 followed in order by unfermented RB1 with 66.00%, fermented LPKC2 and fermented $PKCS_D$ with 58.00%. The least value of 30.00% was recorded in fermented LPKC2.

The Neutral Detergent Fiber (NDF) of the unfermented and fermented substrates ranged from 54.00 - 83.00%. The highest, 83.00%, was recorded in the unfermented RB2 followed in order by unfermented DOC with 80.00%, fermented IndPKC1 with 78.00% and unfermented RB1 with 77.00%. The least, 54.00%, was recorded in fermented Industrial Palm Kernel Cake $(IndPKC)_2$. The Acid Detergent Lignin (ADL) ranged from 20.30 - 51.17% in which the highest was value of 51.17% was obtained from fermented IndPKC1 followed by 42.00% obtained in unfermented $PKS_{W,}$ 38.00% in fermented LPKC3 and 35.00% in fermented LFMPKC1. The 20.30% obtained in the unfermented $IndPKC_1$ was the least. The cellulose of the unfermented and the fermented samples ranged from 5.00 - 47.00% in which the unfermented Palm Kernel Cake from a Local oil mill $(LPKC)_1$ had the highest value of 47.00% followed in order by fermented RB1 and fermented RB2 with 45.00%, fermented IndPKC2 with 31.00%. The unfermented $PKCS_w$ had the lowest value of 5.00%. The metabolizable energy of the substrates ranged from 1440.9893 - 6177.8593kcal/kg. Fermented LPKC2 had the highest value of 6177.8593kcal/kg, followed in order by 6011.7971kcal/kg in fermented IndPKC1, 5978.4847kcal/kg in fermented $PKCS_D$ .The least value, 1440.9893kcal/kg, was in the unfermented DOC.

Table 4.5 shows the proximate composition (%) of the unfermented (UF) and spontaneously fermented (F) substrates. Their protein content ranged from 5.15 - 18.92% and 5.68% - 22.53%. The highest was recorded in FLPKC1 while UFRB1 had the least. The Fibre content of the unfermented and fermented samples ranged from 6.10 - 33.90% and 5.00 - 18.00%. The highest was recorded in UFIndPKC2 while the FLPKC1 had the least. The Nitrogen Free Extract of the unfermented and fermented substrates ranged from 33.87 - 61.43% and 6.90 - 2149%. The UFIndPKC1 had the highest while FLFMPKC1 had the least.

Table 4.4:- Acid Detergent Fiber (ADF), Neutral Detergent Fiber (NDF), Acid Detergent Lignin (ADL), Cellulose and Metabolizable energy profile of the lignocellulosic samples

Substrate Code	ADF%		NDF%		ADL%		Cellulose%		Metabolizable energykcal/kg	
	UF	F	UF	F	UF	F	UF	F	UF	F
RB1	41.00^{m*}	66.00^{b}	74.00^{f}	77.00^{d}	32.00^{g}	21.00^{s}	9.00^{o}	45.00^{b}	1772.9585^{u}	4945.3006^{l}
RB2	41.00^{l}	69.00^{a}	83.00^{a}	71.00^{g}	21.95^{s}	24.64^{n}	20.00^{h}	45.00^{b}	1698.6985^{w}	5722.6975^{h}
LPKC1	52.00^{f}	43.00^{k}	75.00^{e}	58.00^{n}	25.00^{m}	21.16^{r}	47.00^{a}	22.00^{g}	2655.8248^{p}	5848.0171^{g}
LPKC2	31.00^{r}	58.00^{c}	66.00^{k}	60.00^{m}	25.48^{l}	31.00^{h}	25.00^{e}	27.00^{d}	2655.297^{q}	6177.8593^{a}
LPKC3	40.00^{n}	45.00^{j}	58.00^{n}	60.00^{m}	32.00^{g}	38.00^{c}	8.00^{p}	6.00^{s}	2613.2278^{s}	5564.2969^{i}
LFMPKC1	33.00^{q}	50.00^{g}	68.00^{h}	70.00^{h}	28.00^{j}	35.00^{d}	5.00^{t}	15.00^{k}	2648.4682^{r}	5047.9387^{k}
LFMPKC2	53.00^{e}	49.00^{h}	57.00^{p}	60.00^{m}	33.00^{f}	29.39^{i}	19.04^{j}	19.70^{i}	2664.4934^{o}	5880.0015^{e}
IndPKC1	33.00^{q}	58.00^{c}	67.00^{j}	78.00^{c}	20.30^{t}	51.17^{a}	12.69^{l}	6.83^{r}	1540.5909^{w}	6011.7971^{b}
IndPKC2	40.00^{n}	53.00^{e}	75.00^{e}	55.00^{r}	28.00^{j}	22.00^{q}	12.00^{m}	31.00^{c}	2393.452^{t}	5478.8509^{j}
$PKCS_D$	30.00^{s}	58.00^{bc}	63.00^{l}	56.00^{q}	23.00^{o}	34.48^{e}	7.00^{q}	23.52^{f}	3699.0456^{m}	5978.4847^{c}
$PKCS_W$	48.00^{i}	34.00^{p}	54.00^{s}	57.00^{o}	42.00^{b}	25.00^{lm}	6.00^{s}	9.00^{o}	3092.3677^{n}	5947.4165^{d}
DOC	37.00^{o}	54.00^{d}	80.00^{b}	60.00^{m}	27.00^{k}	22.96^{p}	10.00^{n}	31.04^{c}	1440.9893^{x}	5853.1657^{f}

* The values with different superscripts are significantly different (P< 0.05) using Duncan Multiple Range Test.
Legend: - The substrates are as in Table 4:3
Key: - UF – Unfermented, F - Fermented

Table 4.5 - Proximate Composition (%) of Biodegraded lignocellulosic samples under spontaneous fermentation.

		Dry matter		Moisture		Fat		Protein		Ash		Fibre		Nitrogen free extract	
Code	Sample	UF	F	UF	F	UF	F	UF	F	UF	F	UF	F	UF	F
RB	RB1	91.85	48.35	8.15	51.65	10.65	50.70	5.15	5.68	17.45	9.05	31.40	18.00	35.35	16.57
	RB2	91.19	38.01	8.81	61.99	5.60	57.50	6.35	9.85	11.15	6.75	24.99	14.60	52.90	17.80
LPKC	LPKC1	94.47	26.73	5.53	73.27	12.60	53.90	16.69	22.53	4.13	4.55	16.60	5.00	50.05	34.17
	LPKC2	90.44	38.84	9.56	61.16	15.75	59.80	16.84	22.09	4.08	5.00	29.30	7.40	34.17	19.59
	LPKC3	92.31	33.31	7.69	66.69	12.70	50.80	16.18	20.01	4.05	4.50	18.90	5.10	52.54	21.49
LFMPKC	LFMPKC1	92.27	43.58	7.73	56.42	11.80	45.50	17.06	17.71	3.95	4.80	15.10	10.50	61.43	6.90
	LFMPKC2	92.41	28.76	7.59	71.24	9.30	57.40	18.92	19.25	4.25	6.35	6.10	10.10	61.43	6.90
IndPKC	IndPKC1	92.72	35.51	7.28	64.49	9.30	58.20	18.92	22.53	4.25	5.05	6.10	10.90	61.43	3.32
	IndPKC2	93.36	25.56	6.64	74.44	8.70	52.80	12.57	17.50	3.45	4.55	33.90	9.00	52.45	21.13
PKCSludge	$PKCS_D$	94.26	34.93	5.74	65.07	29.20	58.80	13.78	15.31	14.95	5.70	8.20	6.70	33.87	15.50
	$PKCS_W$	53.24	48.46	46.76	51.54	20.60	59.00	13.01	13.35	13.00	5.45	10.00	6.70	43.39	15.50
DOC	DOC	99.41	29.51	0.59	70.49	12.70	55.30	18.15	19.81	4.15	5.10	25.20	5.50	39.80	14.29

Key: RB = Rice Bran, LPKC = Local Palm Kernel Cake, IndPLC = Industrial Palm Kernel Cake, LFMPKC = Palm Kernel Cake from Local feed mill, $PKCS_W$ = Sludge of Palm Kernel Cake, $PKCSD_{=}$ Dry Sludge of Palm Kernel Cake DOC = Deoiled Cake

Key: - UF – Unfermented, F - Fermented

Tables 4.6a and b show the amino acid profile of the lignocellulosic samples.

Lysine values of the lignocellulosic samples ranged from 0.32 to 4.35µg/g. FRB1 had the highest lysine contents of 4.3535µg/g while FLFMPKC2 had the least. Histidine values ranged from 0.25 - 2.70µg/g. The histidine content of FLPKC2U was the highest, 2.70µg/g while the least, 0.25 µg/g, was in FLFMPKC2. Arginine values ranged from 0.51 - 12.7µg/g. UFDOC had the highest arginine content 12.7µg/g while the least was recorded in FLFMPKC2.

Aspartic Acid values ranged from 0.78 - 9.43µg/g in which UFLPKC2 had the highest aspartic acid content of 9.43µg/g while the lowest, 0.78 µg/g, was recorded in FLFMPKC2. Theonine values ranged from 0.17 - 3.50µg/g. RB1UF had the highest value of theonine while LFMPKC2F had the least. Serine ranged from 0.21 - 3.62µg/g. FIndPKC1 had the greatest value of 3.62µg/g while the lowest, 0.21 µg/g was recorded in LFMPKC2F.

The values of Glutamic acid ranged from 1.74 - 16.69µg/g. The highest value was found in $UFPKCS_W$ while the least value was found in FLFMPKC2. The Proline contents of the lignocellulosic samples ranged from 0.03 - 3.66µg/g in which UFLPKC1 and UFIndPKC1 have the highest value while FIndPKC1 had the least. Generally the proline values of the undigested samples were higher than those of the digested samples.

Glycine values in the lignocellulosic samples ranged from 0.31 - 5.38µg/g in which UFLPKC2 had the highest value 5.38µg/g of while the least, 0.31 µg/g was recorded in FLFMPKC2. The Alanine contents in the samples ranged from 0.22 - 5.69µg/g in which UFLPKC2 had the highest while 0.22µg/g in FLFMPKC2 was the least. The alanine values of the undigested lignocellulosic samples were higher than the values of the digested samples.

Cystine values ranged from 0.11 - 2.00µg/g in the lignocellulosic samples in which UFRB1 had the highest while the least was in $FPKCS_D$. The Valine contents ranged from 0.66 - 6.24µg/g in the lignocellulosic samples in which UFLPKC1 ranked highest while FLFMPKC2 had the least. It was also observed that the valine contents of the undigested samples were higher than the values of the digested ones. The values of Methionine in the lignocellulosic samples ranged from 0.36 - 2.63µg/g in which FDOCU had the highest while FIndPKC1 had the least. Isoleocine contents of the lignocellulosic samples ranged from 0.41 - 4.29µg/g in which 4.29µg/g in RB1F was the highest while 0.41µg/g in FLFMPKC2 was the least.

The Leucine contents of the lignocellulosic samples ranged from 0.30 - 7.65µg/g in which UFRB2 was the highest while FLFMPC2 was the least. Tyrosine contents of the lignocellulosic samples ranked from 0.32 - 3.06µg/g in which FRB2 had the highest and FLFMPKC2F had the least. The Phenylanine contents of the lignocellulosic samples ranged from 0.42 - 4.62µg/g in which UFRB1 had the highest while 0.42µg/g in FLFMPKC2 was the lowest. From the general observation, the undigested lignocellulosic samples have higher amino acid contents than the digested samples.

Table 4.6 Amino acid profiles analyses of un-fermented and spontaneously fermented lignocellulosic substrates (Rice bran and Palm kernel cake).

Amino acid	Substrate / Amino acid quality (µg/g)											
	RB1		RB2		LKC1		LPKC2		LPKC3		IndPKC1	
	UF	F	UF	F	UF	F	UF	F	UF	F	UF	F
Lysine	3.74^{abc*}	4.35^{a}	4.00^{ab}	3.02^{cde}	3.24^{bcd}	1.29^{gh}	3.67^{abc}	2.31^{ef}	2.54^{de}	1.69^{fg}	3.08^{cde}	0.70^{hi}
Histidine	2.08^{abcd}	2.21^{abc}	1.77^{bcde}	2.32^{abc}	2.52^{ab}	0.60^{hi}	2.71^{a}	1.70^{bcde}	1.96^{abcd}	0.78^{ghi}	2.15^{abc}	0.56^{hi}
Arginine	6.04^{g}	4.56^{j}	5.35^{h}	5.02^{hi}	11.00^{b}	2.15^{m}	9.32^{c}	4.80^{ij}	9.14^{c}	3.06^{l}	8.63^{d}	1.20^{no}
Aspartic Acid	7.32^{cd}	6.62^{de}	8.00^{bc}	9.14^{a}	9.09^{a}	3.18^{gh}	9.43^{a}	5.15^{f}	6.20^{e}	3.78^{g}	8.07^{b}	2.60^{hi}
Theonine	3.50^{a}	2.35^{cde}	3.09^{ab}	2.55^{c}	2.50^{cd}	1.27^{hij}	3.00^{b}	2.09^{cdef}	1.99^{efg}	1.56^{ghi}	2.24^{cde}	0.46^{l}
Serine	2.17^{de}	1.80^{f}	2.27^{de}	2.05^{e}	3.03^{b}	1.30^{g}	3.50^{a}	2.36^{d}	3.15^{b}	1.66^{f}	2.60^{c}	3.62^{a}
Glutanic Acid	10.60^{ef}	12.16^{d}	11.51^{de}	13.36^{c}	11.36^{de}	13.26^{c}	10.90^{ef}	10.03^{fg}	16.81^{a}	9.31^{g}	13.18^{c}	0.00
Proline	2.94^{bc}	2.11^{ef}	2.03^{ef}	1.31^{g}	3.66^{a}	0.61^{ijk}	3.25^{ab}	1.02^{ghi}	2.85^{bc}	0.75^{hi}	3.66^{a}	0.30l
Glycine	4.99^{ab}	3.15^{cdef}	5.33^{a}	4.06^{abcd}	5.04^{ab}	1.86^{fghi}	5.38	3.51^{bcdef}	5.04^{ab}	2.31^{efgh}	4.80^{abc}	1.05^{hi}
Alanine	3.87^{cd}	2.65^{fg}	3.19^{def}	3.41^{cdef}	4.93^{b}	1.10^{jk}	5.69^{a}	2.24^{gh}	3.11^{def}	1.36^{ij}	3.57^{cde}	1.06^{jk}
Cystine	2.00^{a}	1.46^{g}	1.85^{bc}	1.58^{bc}	1.46^{g}	1.58^{bc}	1.52^{f}	1.77^{d}	1.59^{e}	1.90^{b}	1.47^{fg}	0.39^{k}
Valine	5.40^{bc}	3.19^{ef}	5.02^{c}	3.85^{de}	6.24^{a}	1.65^{j}	6.01^{ab}	2.60^{fghi}	3.99^{d}	2.05^{ij}	5.03^{c}	1.67^{j}
Methionine	1.66^{bcdef}	0.84^{fghi}	1.41^{efg}	0.92^{fghi}	2.44^{ab}	0.64^{ghi}	2.44^{ab}	0.99^{fghi}	2.29^{abc}	0.68^{ghi}	2.24^{abcd}	0.36^{i}
Isoleucine	3.55^{d}	4.29^{a}	3.17^{g}	3.79^{c}	3.48^{de}	1.01^{no}	3.42^{ef}	2.36^{h}	3.39^{ef}	0.80^{o}	3.55^{d}	0.60^{p}
Leucine	7.48^{a}	5.60^{c}	7.65^{a}	6.54^{b}	3.38^{f}	1.35^{j}	3.60^{ef}	4.01^{e}	5.03^{d}	2.26^{g}	2.57^{g}	0.57^{kl}
Tyrosine	2.00^{cd}	2.25^{bc}	2.00^{cd}	3.06^{a}	2.22^{bc}	0.86ghi	2.22^{bc}	1.39^{ef}	2.54^{b}	1.03^{fghi}	2.06^{cd}	0.47^{jk}
Phenylanine	4.62^{a}	3.99^{b}	4.00^{b}	3.75^{bc}	3.25^{de}	2.03^{fg}	3.25^{de}	2.28^{f}	1.97fg	2.03fg	3.09^{e}	0.59^{kl}

*Values with the same superscripts are not significantly different ($p<0.05$) using Duncan's Multiple Range Test.

Legend: - as in Table 4.3.

Table 4.6: Amino acid profiles of un-fermented and spontaneously fermented lignocellulose substrates (Rice bran and Palm kernel cake)

Amino acid	Substrate / Amino acid quality (µg/g)											
	IndPKC2		LFMPKC1		LFMPKC2		$PKCS_W$		$PKCS_D$		DOC	
	UF	F	UF	F	UF	F	UF	F	UF	F	UF	F
Lysine	3.30^{bcd*}	1.02^{ghi}	2.32^{ef}	1.32^{gh}	2.59^{de}	0.32^{i}	3.30^{bcd}	1.02^{ghi}	2.32^{ef}	1.32^{gh}	2.59^{de}	0.91^{ghi}
Histidine	2.27^{abc}	0.91^{fghi}	1.58^{defg}	1.29^{defg}	2.33abc	0.25^{i}	2.27^{abc}	0.91^{fghi}	1.58^{defg}	1.29^{defg}	2.33^{abc}	0.50hi
Arginine	7.94^{e}	4.05^{k}	8.11^{e}	3.27^{l}	7.94^{e}	0.51^{p}	7.94^{e}	4.05^{k}	8.11^{e}	3.27^{l}	7.94^{e}	1.55^{n}
Aspartic Acid	8.19^{b}	3.41^{g}	6.23^{e}	5.06^{f}	7.01^{d}	0.78^{j}	8.19^{b}	3.41^{g}	6.23^{e}	5.06^{f}	7.01^{d}	3.07^{gh}
Theonine	2.37^{cde}	1.18^{ij}	2.04^{def}	1.13^{ij}	2.15^{cde}	0.17^{l}	2.37^{cde}	1.18^{ij}	2.04^{def}	1.13^{ij}	2.15^{cde}	0.55^{kl}
Serine	2.71^{c}	2.98^{b}	2.17^{de}	0.97^{h}	3.07^{b}	0.21^{j}	2.71^{c}	2.98^{b}	2.17^{de}	0.97^{h}	3.07^{b}	0.51^{i}
Glutanic Acid	13.78^{c}	0.00	14.09^{c}	8.33^{h}	11.36^{de}	1.74^{k}	16.96^{a}	13.78^{c}	14.09^{c}	8.33^{h}	11.36^{de}	4.09^{j}
Proline	2.24^{def}	2.34^{de}	1.83^{f}	1.11^{gh}	2.65^{cd}	0.30^{l}	2.24^{def}	2.34^{de}	1.83^{f}	1.11^{gh}	2.65^{cd}	0.61^{ijk}
Glycine	3.96^{abcde}	1.87^{fghi}	2.95^{defg}	2.16^{fgh}	3.46^{bcdef}	0.31^{i}	3.96^{abcde}	1.87^{fghi}	2.95^{defg}	2.16^{fgh}	3.46^{bcdef}	1.05^{hi}
Alanine	4.03^{c}	1.67^{hij}	3.19^{def}	3.60^{cde}	3.04^{ef}	0.22^{l}	4.03^{c}	1.67^{hij}	3.19^{def}	3.60^{cde}	3.04^{ef}	1.17^{jk}
Cystine	1.46^{g}	0.86^{i}	1.59^{e}	0.86^{i}	1.46^{g}	0.33^{l}	1.46^{g}	0.86^{i}	1.59^{e}	0.86^{i}	1.46^{g}	0.66^{j}
Valine	3.99^{d}	2.34^{ghij}	4.33^{d}	3.66^{de}	3.78^{de}	0.66^{k}	3.99^{d}	2.34^{ghij}	4.33^{d}	3.66^{de}	3.78^{de}	2.88^{fg}
Methionine	2.00^{abcde}	0.67^{ghi}	1.56^{cdef}	1.25^{efgh}	1.46^{def}	0.42^{hi}	2.00^{abcde}	0.67^{ghi}	1.56^{cdef}	1.25^{efgh}	2.63^{af}	0.65^{ghi}
Isoleucine	3.42^{ef}	1.10^{n}	3.36^{f}	1.90^{j}	3.42^{ef}	0.41^{q}	3.42^{ef}	1.10^{n}	3.36^{f}	1.90^{j}	3.91^{ef}	1.33^{m}
Leucine	2.51^{g}	1.33^{j}	2.18^{gh}	1.66^{hij}	2.13^{ghi}	0.30^{l}	2.51^{g}	1.33^{j}	2.18^{gh}	1.66^{hij}	$5.92^{c\ i}$	1.09^{jk}
Tyrosine	2.22^{bc}	1.27^{efg}	1.90^{cd}	1.11^{fgh}	1.90^{cd}	0.32^{k}	2.22^{bc}	1.27^{efg}	1.90^{cd}	1.11^{fgh}	1.90^{cd}	0.63^{ijk}
Phenylanine	2.06^{fg}	0.86^{jk}	1.89^{fg}	1.37^{hi}	1.89fg	0.42l	2.06^{fg}	0.86^{jk}	1.89^{fg}	1.37hi	1.89^{fg}	0.77^{jkl}

*Values with the same superscripts are not significantly different ($p<0.05$) using Duncan's Multiple Range Test.

Legend: - as in Table 4.3.

4.2. Enzymatic studies on the different lignocellulosic substrates

4.2.1. Production of Amylase by the fungal isolates

The ten fungal isolates were tested for amylase production and the best amylase producer was selected for futher studies. The effects of physico-chemical parameters on the production of amylase by the selected amylase producer were determined. The fermentation condition was optimized and the enzyme produced was characterized.

Fig.4.2 shows the screening of the isolates for amylase production. The amylase production ranged from 10.24 - 63.17U/ml with *Aspergillus niger* producing the highest amount of amylase while *Aspergillus flavus* had the least amount. About 50% of the isolates (*Aspergillus niger, A. terreus, R*hizopus sp., *R. oryzae* and *A. tamarii)* displayed good production of amylase while 20% (*A. versicolor* and *Rhizopus stolonifer*) produced amylase moderately and about 30% (*Aspergillus flavus, A. clavatus* and *Trichoderma* sp.) are poor amylase producers. Based on this result, *Aspergillus niger* was selected for further studies.

4.2.1.1. Effect of physico-chemical parameters on amylase production by *Aspergillus niger*

4.2.1.1.1. Effect of carbon sources on amylase production and mycelia growth weight of *Aspergillus niger*

Figures 4.3a (i & ii) show the effect of different carbon sources on amylase production and mycelia dry weight of *Aspergillus niger* at different incubation times. On day 3 amylase production ranged from 0.814 - 235.07U/ml. Galactose supported the highest production followed by sucrose while the least was recorded in wheat bran. Generally, most of the simple carbon sources supported maximum amylase production while complex carbon sources repressed maximum amylase production by *Aspergillus niger*. On day 3 the mycelia dry weight ranged from 0.01 - 0.35g. Sucrose supported the highest growth while Lactose, mellibiose and sugar cane baggase supported the least growth by *Aspergillus niger.*

On day 5 the amylase production ranged from 0.126 – 303.427U/ml. Xylose supported the highest production while Palm kernel cake supported the least. On day 5 the mycelia dry weight ranged from 0.01 - 0.12g. Galactose supported the highest growth while saccharose supported the least growth by *Aspergillus niger.*

On day 7 the amylase production ranged from 1.628 – 179.963U/ml. Starch supported the highest production while Palm kernel cake supported the least. On day 7 the mycelia dry weight ranged from 0.01 - 0.07g. Mannitol supported the highest growth while starch supported the least growth by *Aspergillus niger.*

4.2.1.1.2. Effect of nitrogen sources on amylase production and mycelia dry weight of *Aspergillus niger*

Figure 4.3b (i & ii) shows the effect of nitrogen sources (Ammonium nitrate, Ammonium sulphate, Potassium nitrate, Corn steep liquor, Soy bean meal, Peptone, Yeast extract, Urea and Casein) on amylase produced and mycelia dry weight of *A. niger* incubated for different days.

On day 3 amylase production ranged from 0.303 - 33.00U/ml in which ammonium sulphate supported the production of the highest value of 33.00U/ml while the least value was recorded when Corn steep liquor was used. The inducing capability of the nitrogen sources for amylase production is in the following descending order of NH_4SO_4 >Peptone >Yeast extract > NH_4NO_3 >Soy Meal > Urea > KNO_3 > Casein > Corn steep liquor. On day 3 the mycelia dry weight ranged from 0.01 - 0.08g. NH_4NO_3 supported the highest growth while KNO_3 supported the least growth by *Aspergillus niger.*

On day 5 amylase productions ranged from 0.088 – 49.29U/ml in which NH_4NO_3 supported the highest production while the least was recorded in Urea. The inducing capability of the nitrogen sources for amylase production is in descending order of NH_4NO_3 > KNO_3 > Peptone > NH_4SO_4 > Yeast extract > Corn steep liquor > Soy Meal > Casein > Urea. On day 5 the mycelia dry weight ranged from 0.01 - 33.00U/ml 0.04g. KNO_3, Corn steep liquor, Soya meal and Peptone supported the highest growth while Urea and Casein supported the least growth by *Aspergillus niger.*

On day 7 amylase productions ranged from 2.935 – 57.207U/ml in which CSL supported the highest production while the least was recorded when SBM was used.

The inducing capability of the nitrogen sources for amylase production is in descending order of CSL > NH_4SO_4 > NH_4NO_3 > KNO_3 >Yeast extract > Casein > Urea > Peptone >Soy Meal.

On day 7 the mycelia dry weight ranged from 0.02 - 0.05 g. NH_4NO_3, Corn steep liquor, Soya meal supported the highest growth while Yeast extract supported the least growth by *Aspergillus niger.*

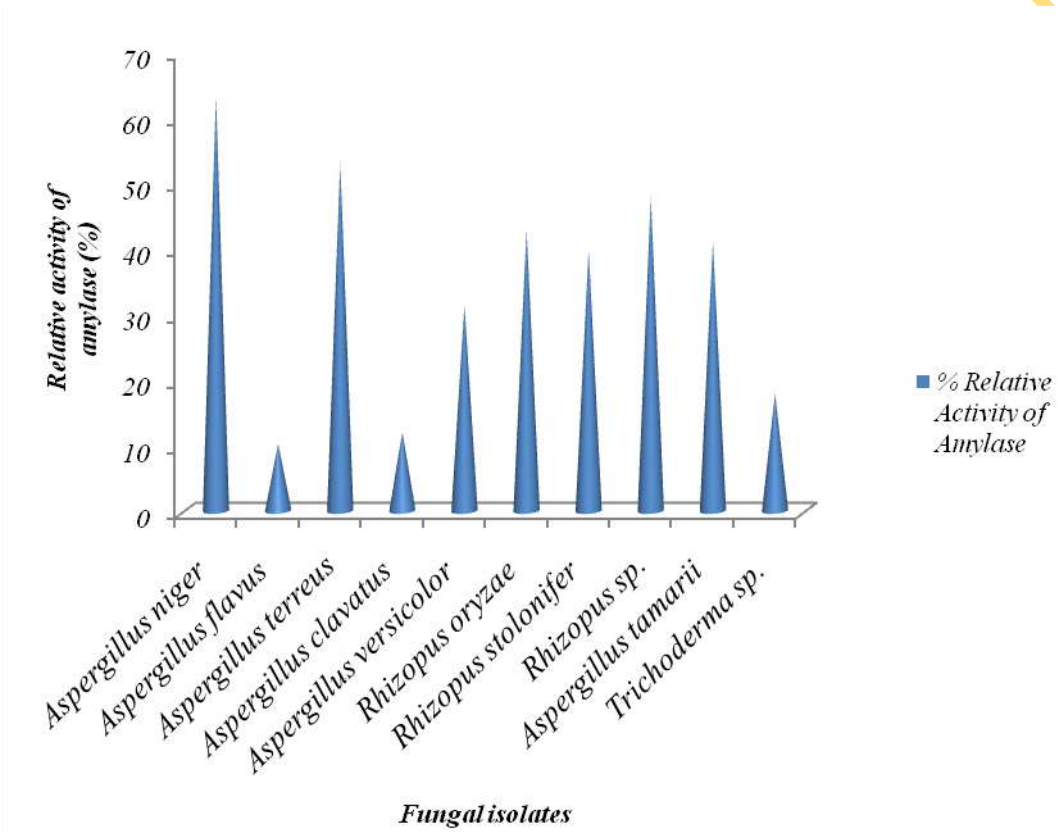
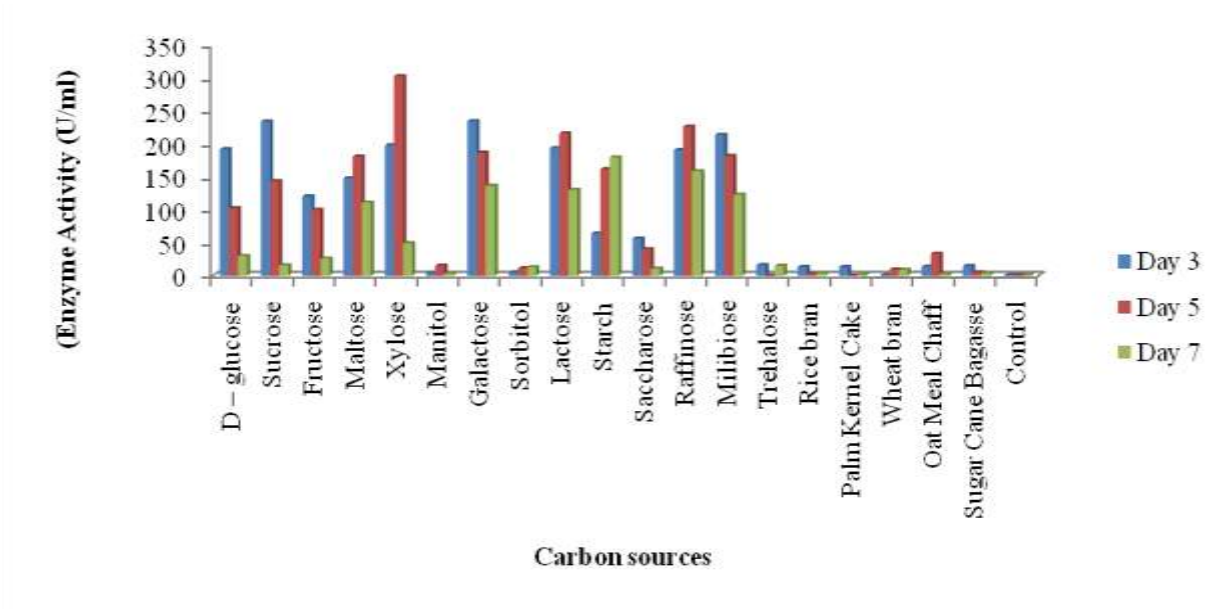
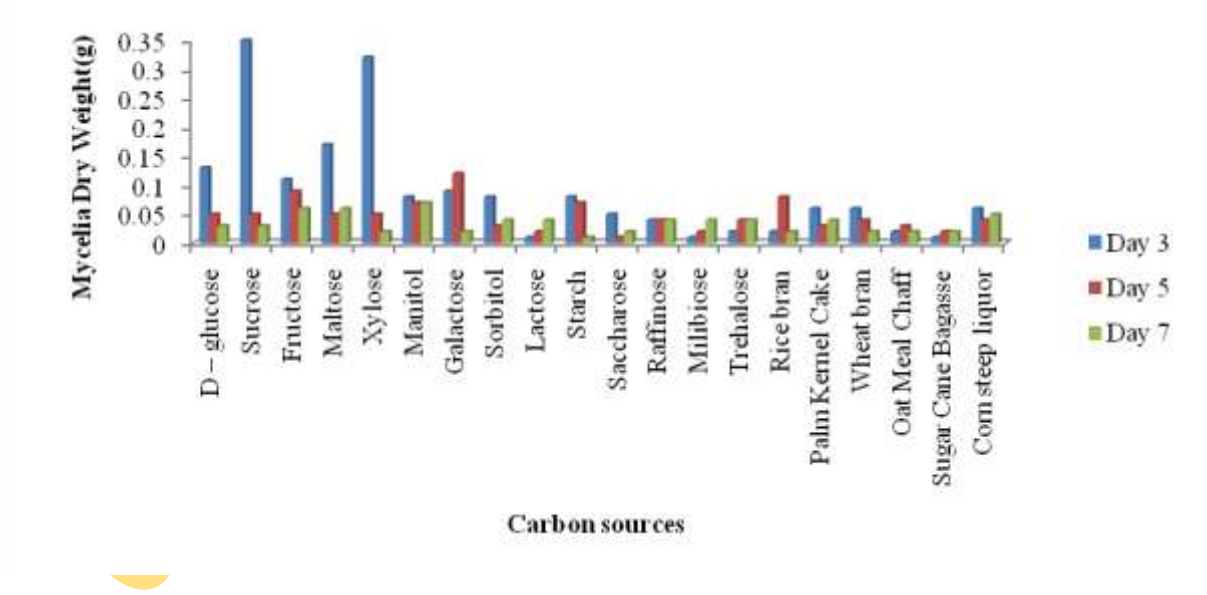


Fig.4:2. Amylase production by the fungi isolated from the lignocellulosic substrates

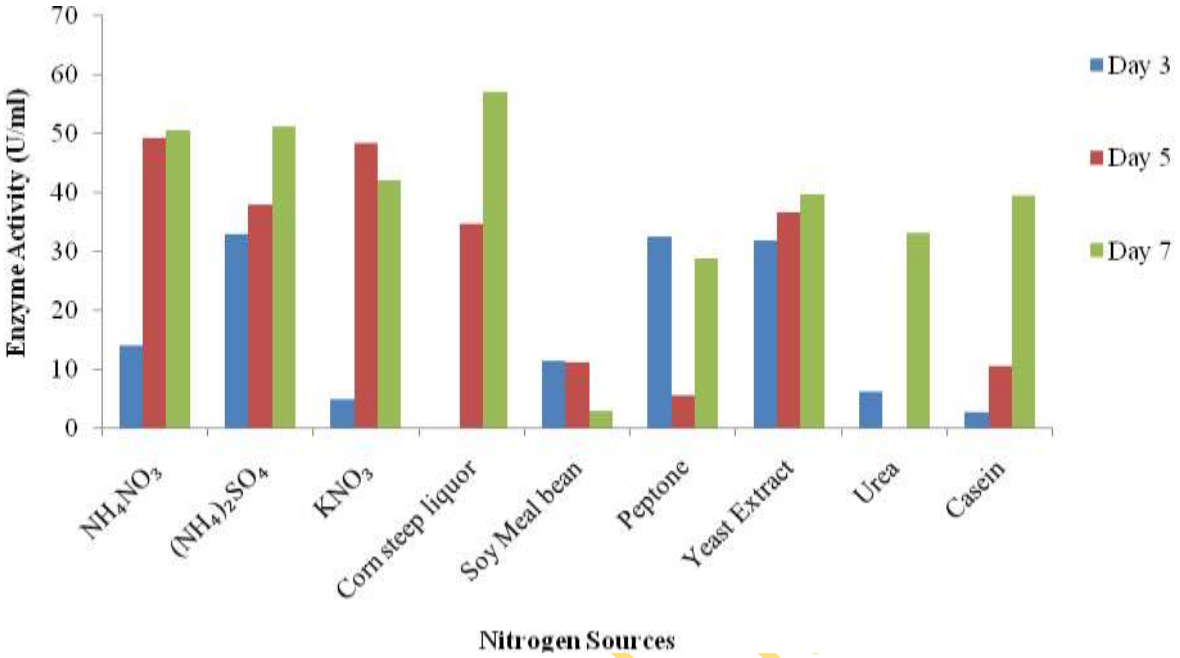


(i)

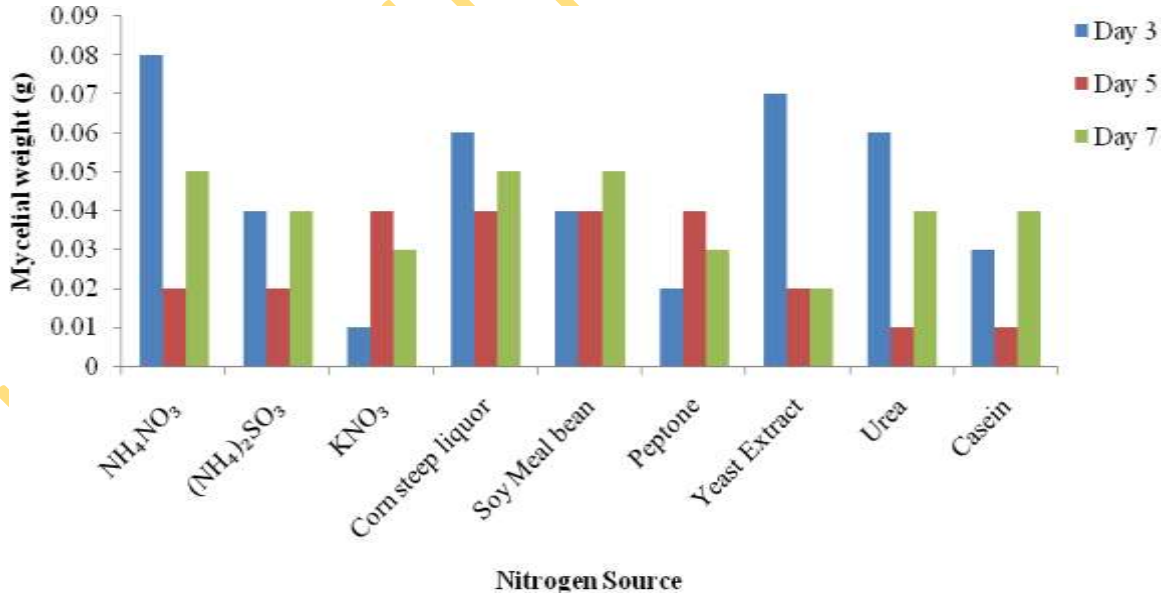


(ii)

Fig.4.3a (i & ii). Effect of different carbon sources on Amylase production and mycelia dry weight of *Aspergillus niger*. .



(i)



(ii)

Fig. 4.3b (i & ii). Effect of nitrogen sources on Amylase production and mycelia dry weight (g) of *A. nger*.

4.2.1.1.3. Effect of pH on amylase production and mycelia dry of *Aspergillus niger*

Figures 4.3c (i & ii) show the effect of pH (4, 5, 6, 7, 8, 9 and 10) on amylase production and mycelia dry weight by *A. niger*. On day 3, the amylase production ranged from 1.83 - 173.033U/ml in which pH 6 supported the highest while the production at pH 8 supported the lowest. On day 3 the mycelia dry weight ranged from from 0.02 - 0.41g. pH 10 supported the highest growth while pH 8 supported the least growth by *Aspergillus niger*.

At day 5 amylase productions ranged from 0.372 - 229.23U/ml in which production at pH 7 supported the lowest while the production at pH6 induced the highest. On day 5 the mycelia dry weight ranged from 0.02 - 0.11g. pH 4 supported the highest growth while pH supported the least growth by *Aspergillus niger*.

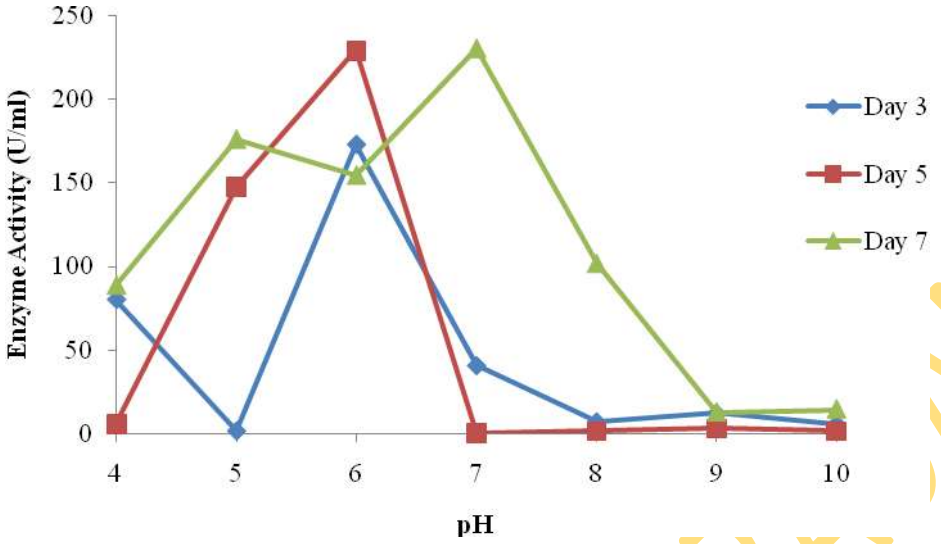
On day 7 amylase productions ranged from 12.85 - 230.644U/ml in which pH 9 and 10 supported the lowest while pH 7 induced the highest. On day 7 the mycelia dry weight ranged from 0.02 - 0.21 g. pH 9 supported the highest growth while pH 8 supported the least growth by *Aspergillus niger*.

4.2.1.1.4. Effect of inoculums size on amylase production and mycelia dry weight of *Aspergillus niger*

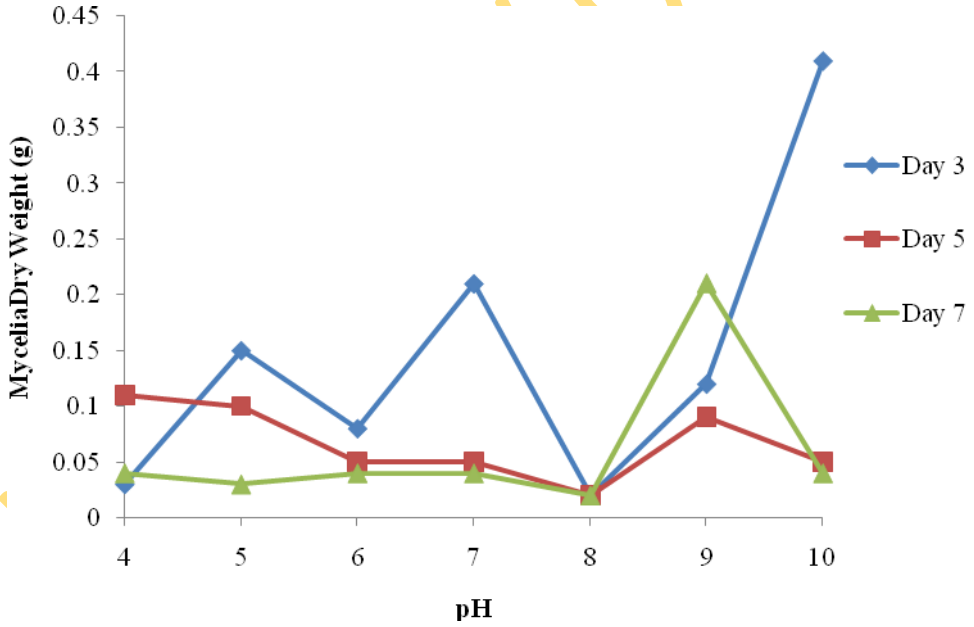
Effect of inoculums size (1ml, 2ml, 3ml or 4ml) on amylase production by *A. niger* incubated for different days at 45^{o}C and pH 6.0 is shown in Figure 4.3d (i & ii). On day 3, amylase production ranged from 0.00 – 20.512 U/ml in which the highest was produced when 4ml of inoculums load was used. At lower inoculums load of 1 - 3ml there was no enzyme production. Mycelia dry weght ranged from 0.05 – 0.7g in which 3ml had the highest growth while 2ml had the least.

On day 5 amylase activities ranged from 0.00 - 222.319U/ml. in which the highest was produced in 1ml inoculums load while the least was produced in 2ml inoculums load. The mycelia dry weight by day 5 ranged from 0.03 – 0.7 in which 4ml had the highest growth while 1ml produced the least.

On day 7, amylase production ranged from 75.857 - 189.841U/ml in which production with 2ml had the lowest followed in order by productions with 3ml and 1ml while amylase production using 4ml inoculums load was the highest. Mycelia dry weight produced ranged from 0.2 – 0.4g in which 1 and 4ml produced the highest while 3ml had the least.

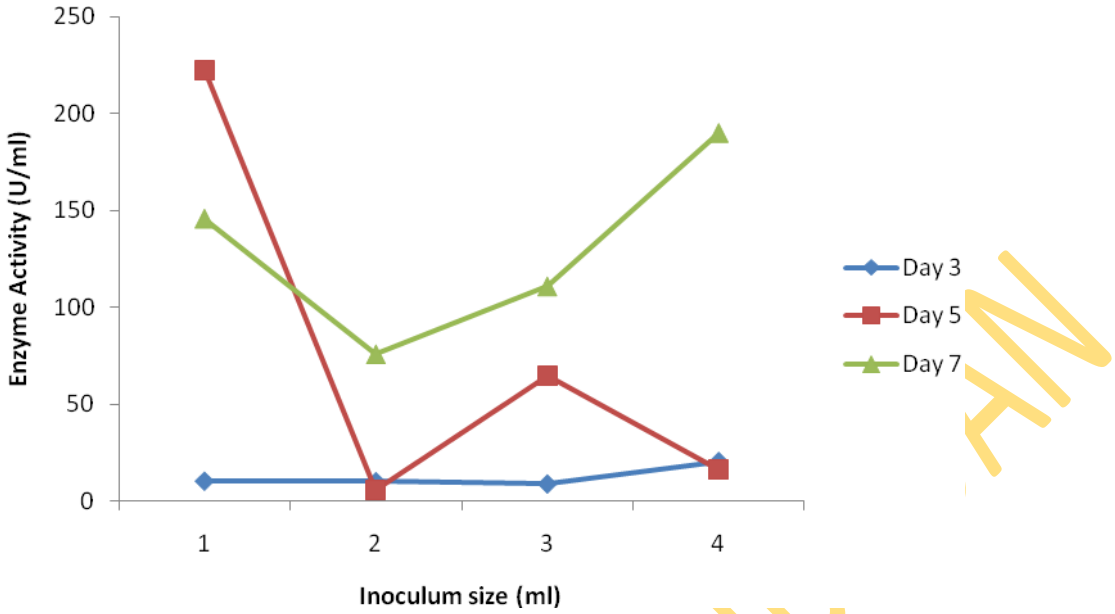


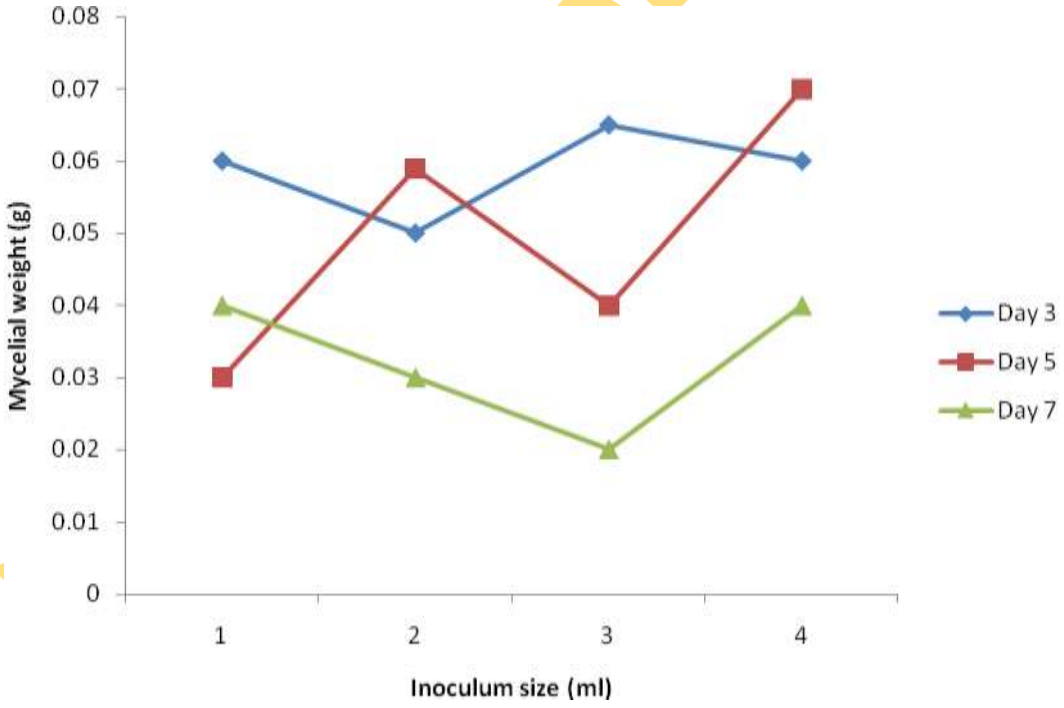
(i)



(ii)

Fig.4.3c (i & ii). Effect of pH on Amylase production and mycelia dry weight (g) of *A.* niger.





(ii)

Fig. 4.3d (i & ii) : Effect of Inoculum size (ml) of *A.niger* on amylase production and its mycelia dry weight (g).

4.2.1.1.5. Effect of temperature on amylase production and mycelia dry weight of *Aspergillus niger*

Figure 4.3e (i & ii) shows the effect of temperature (20^{o}C - 45^{o}C) on amylase production and mycelia dry weight of *A. niger* at different incubation times at pH6.0 with 1ml inoculums size.

On day 3 amylase productions though poor, ranged from 4.73 - 19.99U/ml in which production at 45^{o}C was the highest while production at 25^{o}C was the least.

On day 3 the mycelia dry weight ranged from from 0.01 - 0.03g. 35^{o}C supported the highest growth while 20^{o}C, 40^{o}C and 45^{o}C supported the least growth by *Aspergillus niger.*

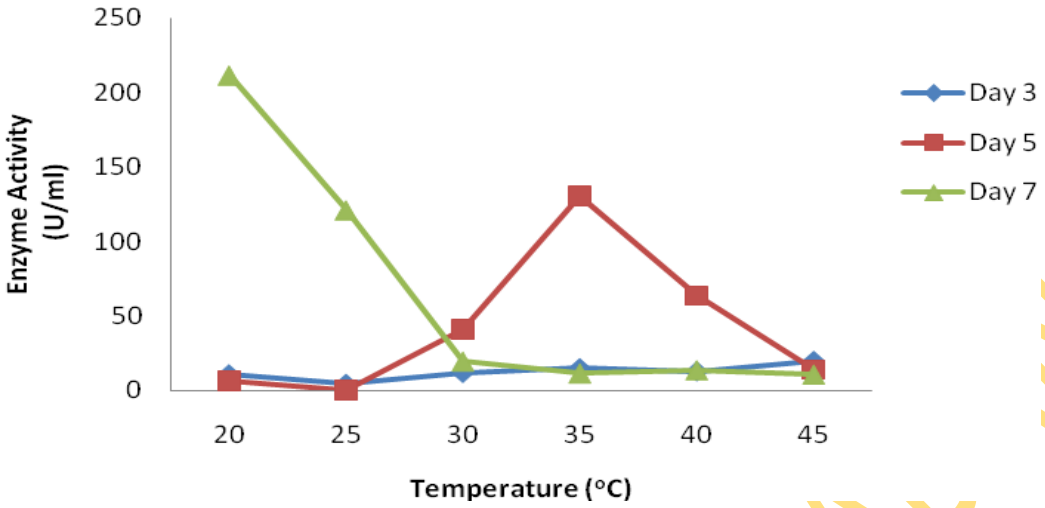
By day 5, amylase production ranged from 0.00 - 130.861UI/ml in which 35^{o}C induced the highest while 25^{o}C induced the least. On day 5 the mycelia dry weight ranged from 0.02 - 0.06 g. The highest growth was supported at 35^{o}C and 40^{o}C while 25^{o}C and 45^{o}C supported the least growth by *Aspergillus niger*

On day 7 amylase productions ranged from 0.00 - 211.59U/ml in which production at 20^{o}C induced the highest while production at 45^{o}C was the least. On day 7 the mycelia dry weight ranged from 0.01 -0.5g 30^{o}C and 40^{o}C supported the highest growth while 20^{o}C and 45^{o}C supported the least growth by *Aspergillus niger.*

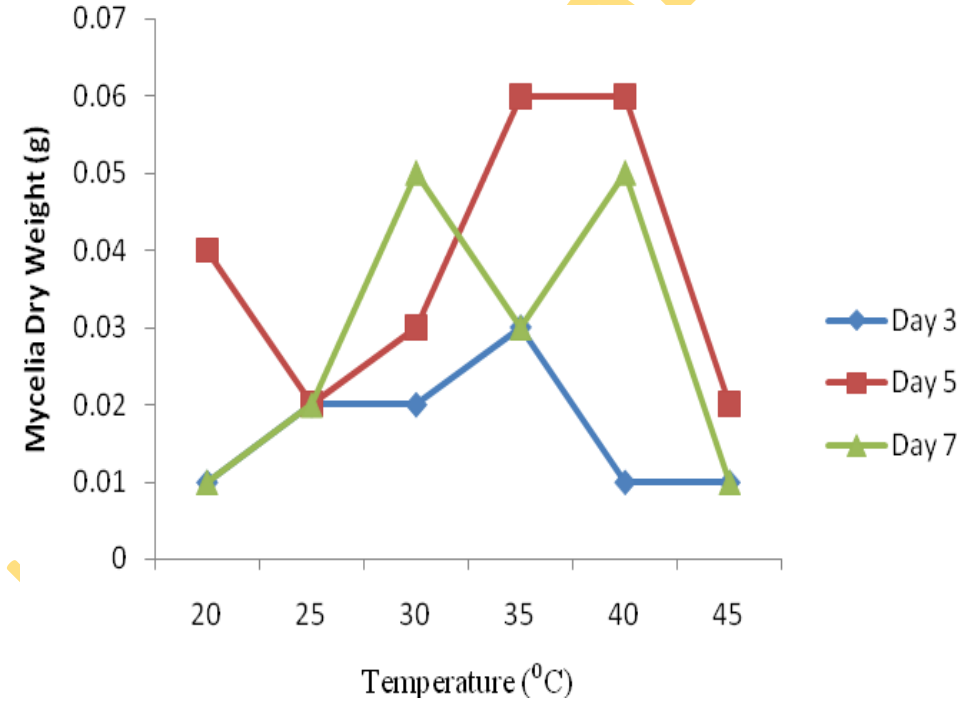
4.2.1.1.6. Effect of incubation time on amylase production and mycelia dry weight of *Aspergillus niger*

Figure 4.3f (i & ii) shows the effect of incubation time (3, 7, 14 and 21 days) on amylase production and mycelia dry weight of *Aspergillus niger* with 1ml inoculum size at pH6.0 and 45^{o}C. Amylase production ranged from 41. 693 - 123.419U/ml in which day 7 supported the highest production while lowest while that of day 21.

The mycelia dry weight after 3 to 21 days icubation ranged from 0.01 - 0.19g. The growth was highest at day 3 of incubation while incubation for 21 days supported the least growth by *Aspergillus niger.*

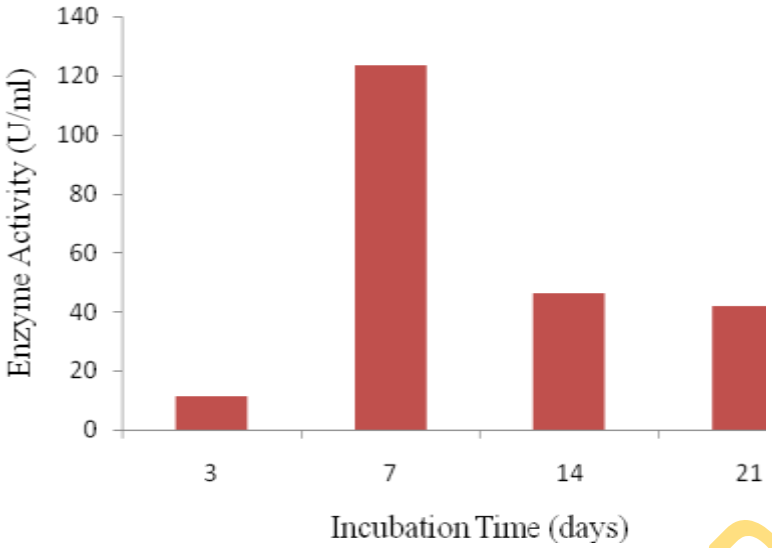


(i)

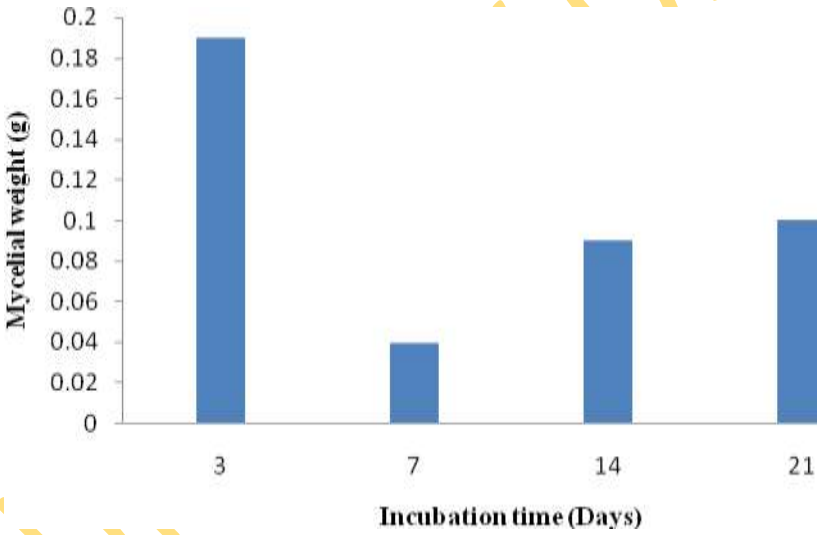


(ii)

Fig. 4.3e (i & ii). Effect of temperature on amylase production and mycelia dry weight (g) of *A. niger*



(i)



(ii)

Fig. 4.3f (i & ii): Effect of incubation time (day) on the production of amylase and mycelia dry weight (g) of *A.niger*.

4.2.1.2. Optimization of Amylase production by *Aspergillus niger*

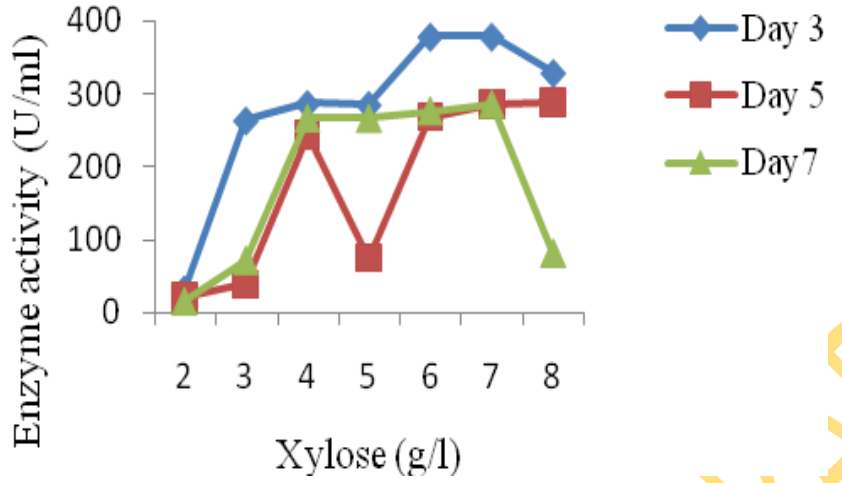
Optimization of production parameters for amylase production by *Aspergillus niger* was investigated. Optimization of the best carbon sources (Xylose and Oat Meal Chaff (OMC)), Nitrogen source (Corn steep liquor and Yeast extract), pH (5.5 - 8.5), inoculum load (1-7ml), temperature (10 - 60^{O}C) and incubation time (2 - 12 days) was determined.

4.2.1.2.1. Amylase production and mycelia dry weight of *Aspergillus niger* under different concentrations (2 - 8g/l) of xylose and Oatmeal Chaff (OMC)

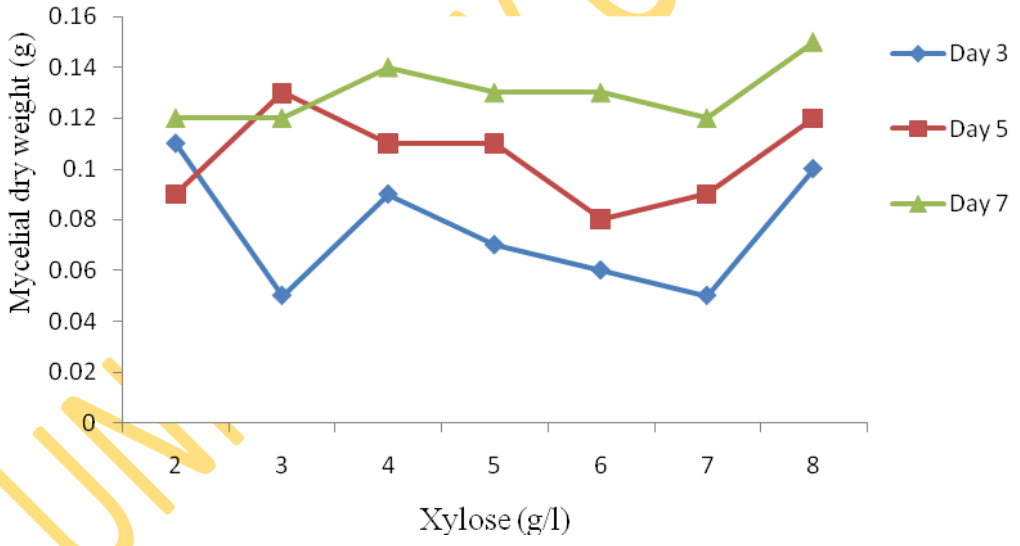
Figures 4.4a (i & ii) and 4.4b (i &ii) show the amylase production and mycelia dry weight of *Aspergillus niger* under different concentrations (2 - 8g/l) of xylose and OMC. On day 3 amylase production on different concentrations of xylose and OMC ranged from 31.166 – 378.684 U/ml and 27.322 – 36.991 U/ml. The highest amylase produced was in 7 and 8g/l xylose and 7g/l OMC. The least production of amylase was recorded in 2g/l and 4g/l of xylose and OMC. On day 3 mycelial dry weights of *Aspergillus niger* under different concentrations of xylose and OMC ranged from 0.05 – 0.11g and 0.08 – 0.11g in which 2g/l and 7g/l xylose and OMC supported the highest mycelia dry weght respectively. The least mycelia dry weight was produced by 7g/l of xylose and 5g/l and 8g/l concentration of OMC.

On day 5, amylase production on different concentrations of xylose and oat meal chaff ranged from 21.244 – 288.456U/ml and 16.763 – 286.418U/ml. The highest amylase, 288.456U/ml and 286.418U/ml, was produced by 8g/l and 6g/l of xylose and OMC respectively while the least amylase, 21.244 and 16.763U/ml, were produced by 2g/l and 4g/l of xylose and OMC respectively. On day 5 mycelial dry weights of *Aspergillus niger* under different concentrations of xylose and OMC ranged from 0.08 – 0.13g and 0.10 – 0.15 g. The highest mycelia dry weight was produced by 3g/l of xylose and 6g/l and 7g/l of oatmeal chaff. The least mycelia dry weight was produced by 6g/l of xylose and 2g/l of OMC.

On day 7, amylase production on different concentrations of xylose and OMC by *Aspergillus niger* ranged from and 18.808 – 37.307 U/ml. The highest amylase was produced by 7g/l of xylose and OMC, while the least amylase production was recorded in 2g/l concentration of xylose and OMC. On day 7 mycelial dry weights of *Aspergillus niger* under different concentrations of xylose and OMC ranged from 0.12 – 0.15g and 0.13 – 0.17g. The highest mycelia dry weight was soupported by 8g/l of xylose and 8g/l of OMC while 2g/l, 3g/l and 7g/l of xylose and 3g/l and 4g/l concentrations of OMC produced the least mycelia dry weight.

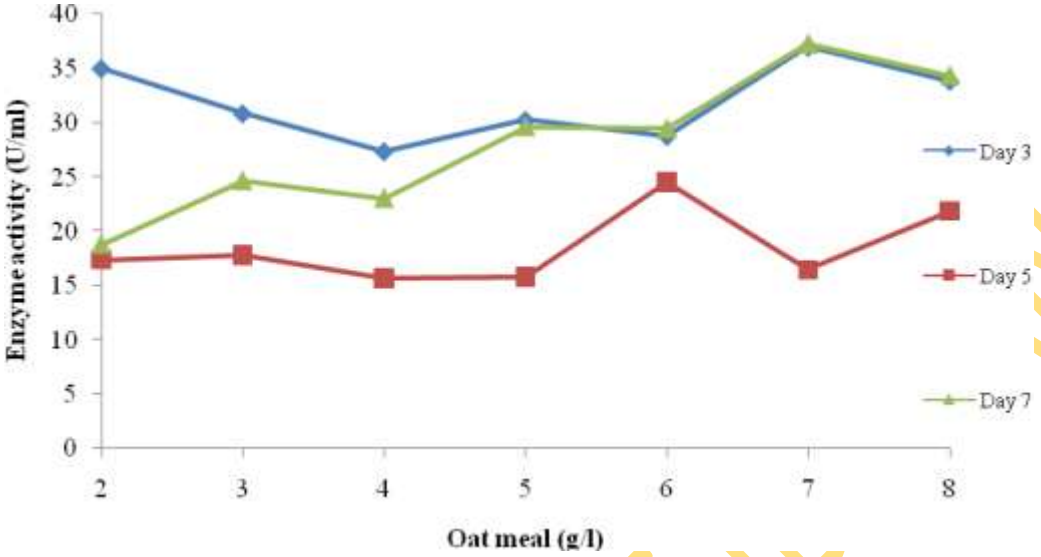


(i)

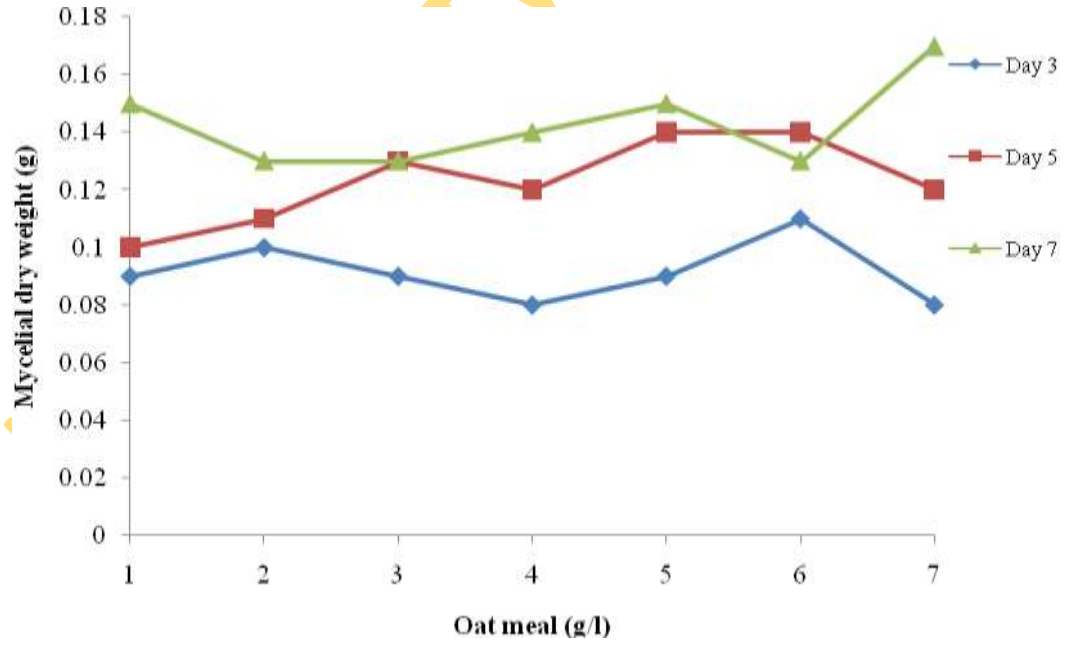


(ii)

Fig. 4. 4a (i & ii): Amylase production and Mycelia dry weight (g) of *A. niger* under different concentration (g/l) of Xylose.



(i)



(ii)

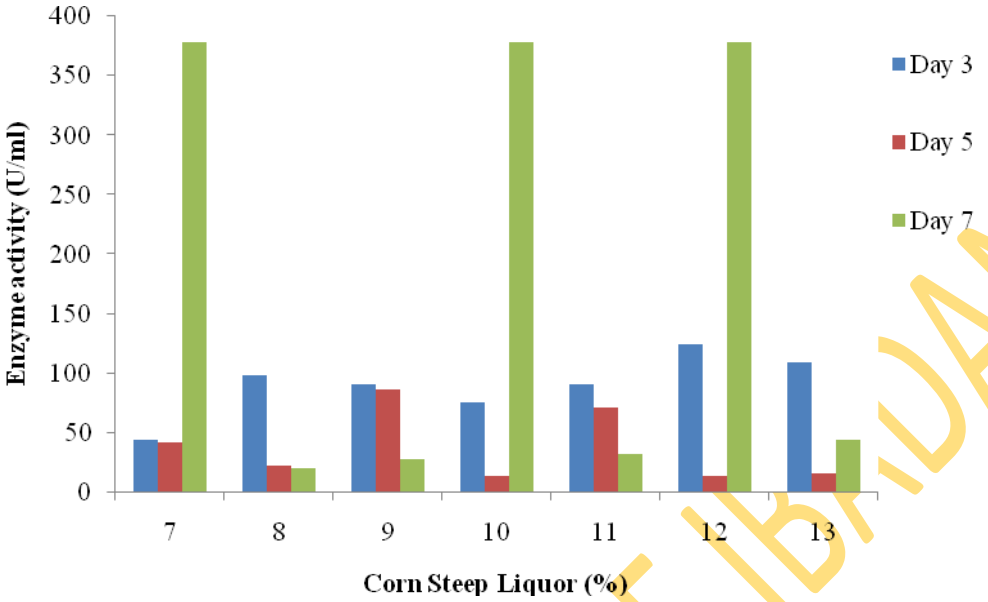
Fig. 4.4b (i & ii): Amylase production and Mycelia dry weight (g) of *A. niger* under different concentration (g/l) of Oat meal chaff.

4.2.1.2.2. Amylase production and mycelia dry weight of *Aspergillus niger* under different concentrations of Corn steepliquor (7-13 % ^{v}/v) and yeast extract (0.5 -3.5g/l)

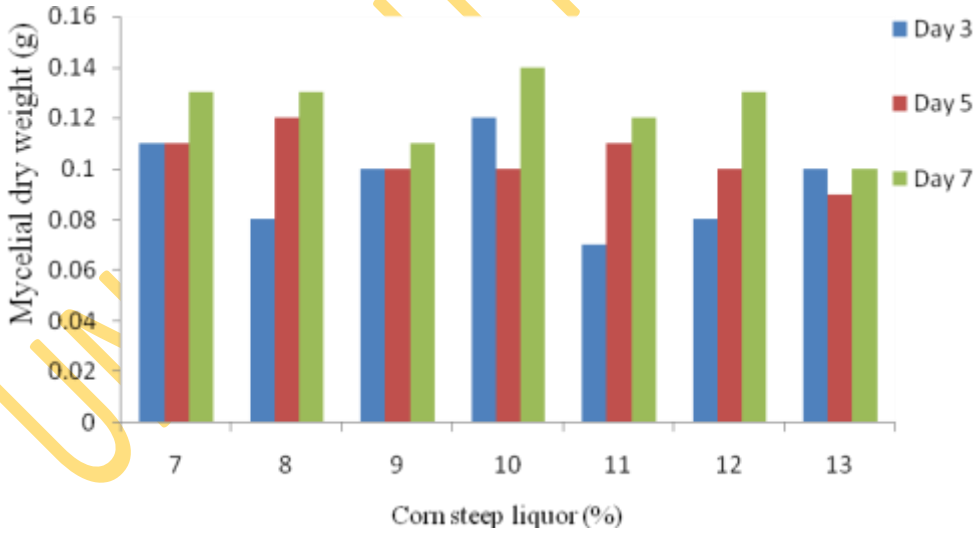
Figures 4.4c (i & ii) and 4.4d (i & ii) show the amylase production and mycelia dry weight of *Aspergillus niger* under different concentration (7 - 13 % ^{v}/v and 0.5 - 3.5g/l) of Cornsteep liquor and yeast extract respectively. On day 3 amylase production on different concentrations of Cornsteep liquor and yeast extract ranged from 44.773 – 124.966U/ml and 25.561 – 58.5571U/ml. The highest amylase production was supported by 12% ^{v}/v Cornsteep liquor and 0.5g/l yeast extract while 7% ^{v}/v and 3.5g/l Yeast extract produced the least production of amylase by *Aspergillus niger*. On day 3 mycelial dry weight of *Aspergillus niger* under different concentrations of Corn steep liquor and Yeast extract chaff ranged from 0.08 – 0.12g and 0.08 – 0.12g. The highest mycelia dry weight was produced by 10% ^{v}/v and 1 -2.5g/l Corn steepliquor and yeast extract respectively while 8% ^{v}/v and 1.5g/l of Cornsteep liquor and yeast extract yielded the least mycelia dry weight.

On day 5 amylase production on different concentrations of Cornsteep liquor and yeast extract ranged from 13.759 – 86.214U/ml and 12.187 – 28.603U/ml. The highest amylase production was supported by 9% ^{v}/v and 3g/l of Cornsteep liquor and yeast extract while 10 % ^{v}/v and 1 - 1.5g/l Cornsteep liquor and yeast extract produced the least production of amylase by *Aspergillus niger*. On day 5 mycelial dry weight of *Aspergillus niger* under different concentration of Cornsteep liquor and yeast extract ranged from 0.09 – 0.12g and 0.11 – 0.16g. The highest mycelia dry weights, 0.12g and 0.16g were obtained in 8% ^{v}/v and 2.0g/l Corn steep liquor and yeast extract respectively. The least mycelia dry weights, 0.09g and 0.11g were produced by 13% ^{v}/v and 0.5g and 3.5g/l of Cornsteep liquor and yeast extract respectively.

On day 7, amylase production on different concentrations of Cornsteep liquor and yeast extract by *Aspergillus niger* ranged fom 21.0011 - 378.68U/ml and 18.196 – 51.066U/ml. The highest amylase production was by 12% ^{v}/v and 1.5g/l of Cornsteep liquor and yeast extract respectively while 8% ^{v}/v and 0.5g/l concentrations of Cornsteep liquor and yeast extract yielded the least amylase production by *Aspergillus niger*. On day 7 mycelial dry weight of *Aspergillus niger* under different concentrations of Cornsteep liquor and yeast extract ranged from 0.10 – 0.14g and 0.13 – 0.14g. The highest mycelial dry weights were produced by 10% ^{v}/v and 0.5 - 2.5g/l Cornsteep liquor and yeast extract respectively while 13% ^{v}/v of Cornsteep liquor and 1g/l, 1.5g/l, 3.0g/l and 3.5g/l of Yeast extract yielded the least mycelia dry weight.

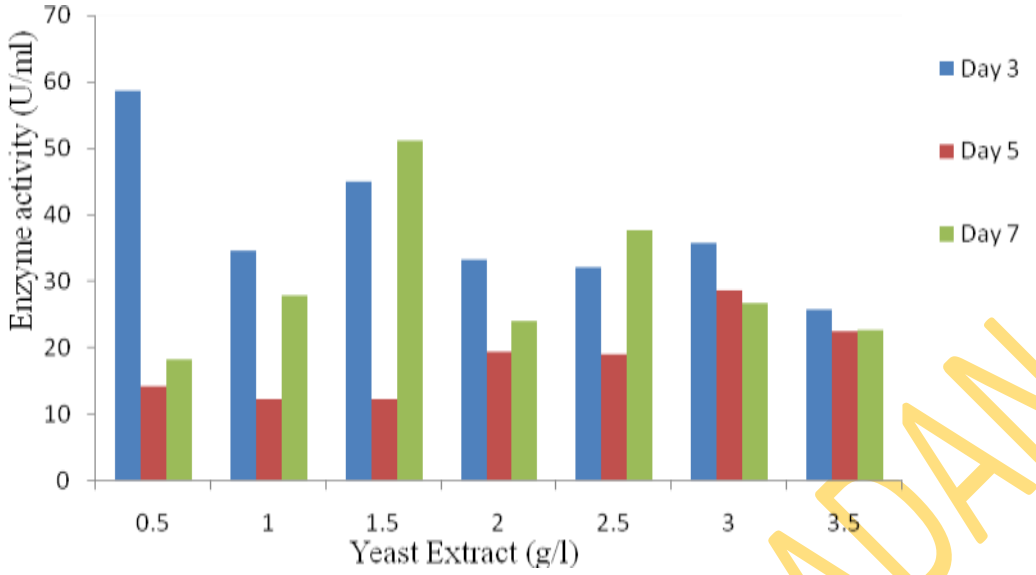


(i)

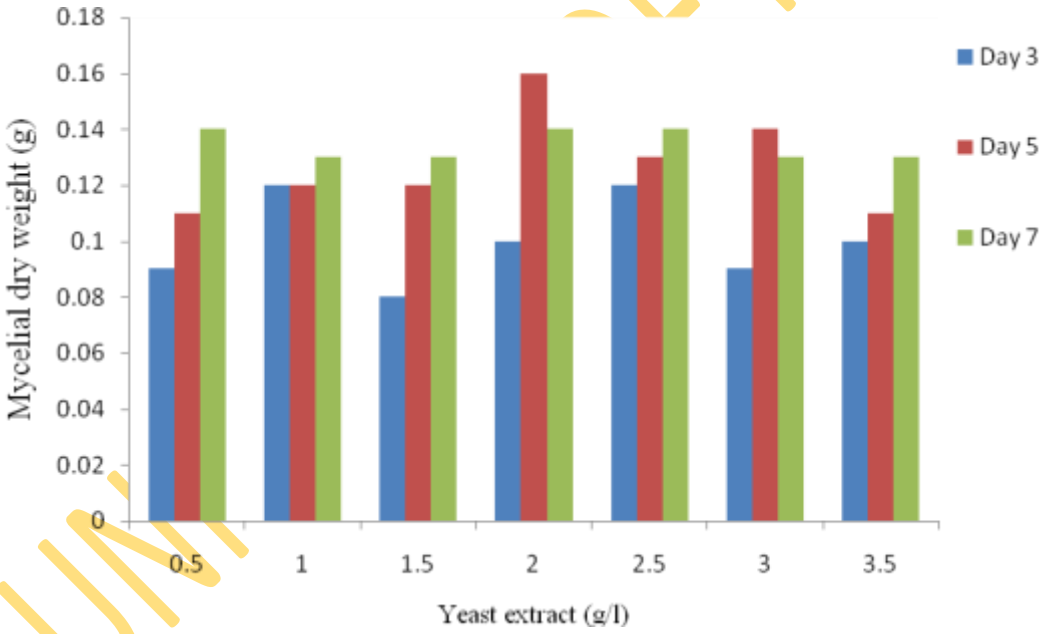


(ii)

Fig.4.4c (i & ii): Amylase production and mycelia dry weught (g) of *A. niger* under different concentration (g/l) of Corn steep liquor.



(i)



(ii)

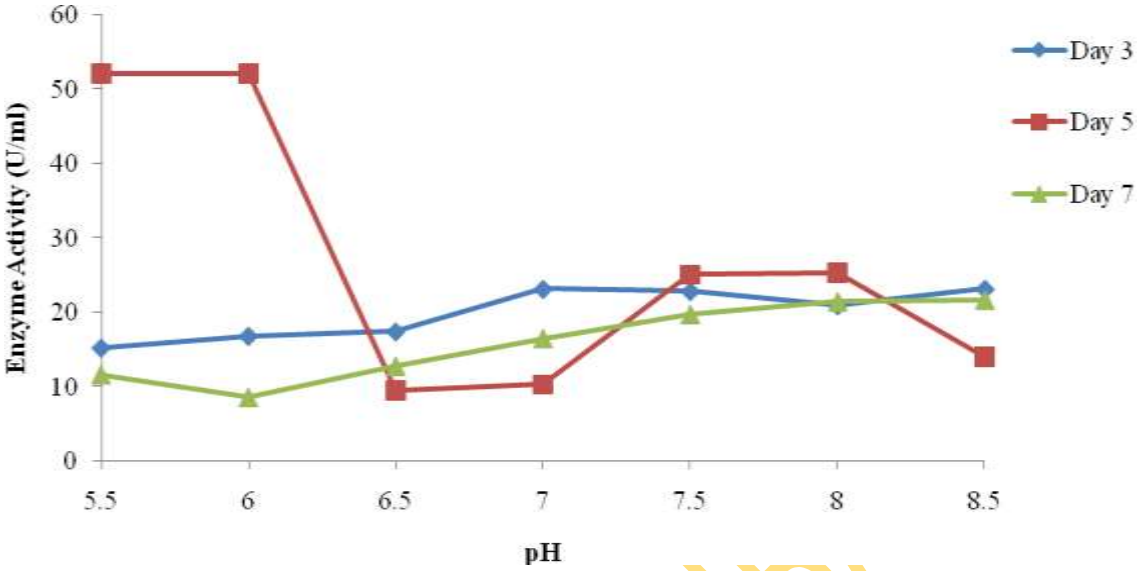
Fig. 4.4d (i & ii): Amylase production and Mycelia dry weight (g) of *A. niger* under different concentration(g/l) of Yeast Extract.

4.2.1.2.3. Amylase production and mycelia dry weight of *Aspergillus niger* under optimized pH (5.5 -8.5)

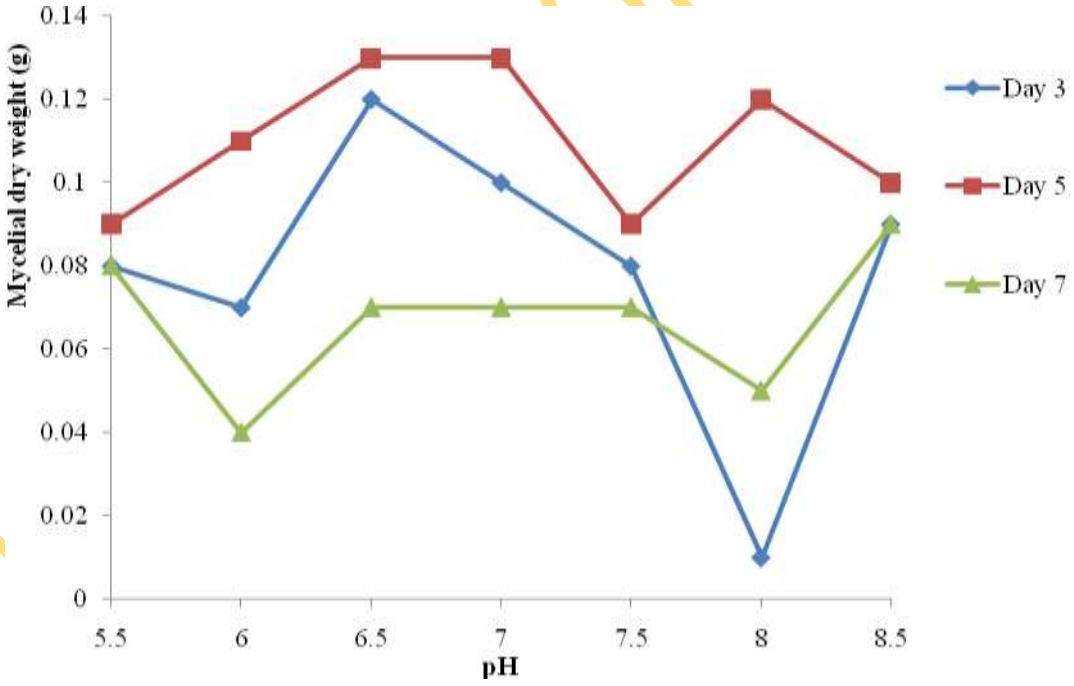
Figures 4.4e (i & ii) show the amylase production and mycelia dry weight of *Aspergillus niger* under different pH (5.5 - 8.5). On day 3 amylase productions under optimized pH (5.5 - 8.5) ranged from 15.267 – 23.201 UI/ml and pH 8.5 supported the highest amylase production while the least amylase production by *Aspergillus niger* was at pH 5.5. On day 3 mycelia dry weight of *Aspergillus niger* under optimized pH (5.5 - 8.5) ranged from 0.01 – 0.12g. pH 6.5 supported the highest mycelia dry weight while pH 8.0 produced the least mycelia dry weight by *Aspergillus niger*.

On day 5 amylase productions under optimized pH (5.5 - 8.5) ranged from 9.448 – 52.183 U/ml. At pH 6.0 the highest amylase, 52.183U/ml, was produced while at pH 6.5 the least amylase production by *Aspergillus niger*, 9.448U/ml, was obtained. On day 5 mycelia dry weight of *Aspergillus niger* under optimized pH (5.5 - 8.5) ranged from 0.09 – 0.13g. pH 6.5 and pH 7.0 supported the highest mycelia dry weight production while the least mycelia dry weight production by *Aspergillus niger* was at pH 5.5.

On day 7 amylase production under optimized pH (5.5 - 8.5) ranged from 8.57 – 21.66 U/ml. pH 8.5 supported the highest amylase production while at pH 6.0, the least amylase production by *Aspergillus niger* was produced. On day 7 mycelia dry weight of *Aspergillus niger* under under optimized pH (5.5 - 8.5) ranged from 0.04 – 0.09g. pH 8.5 yielded the highest mycelia dry weight of 0.09g while pH 6.0 produced the least mycelia dry weight of 0.04g by *Aspergillus niger*.



(i)



(ii)

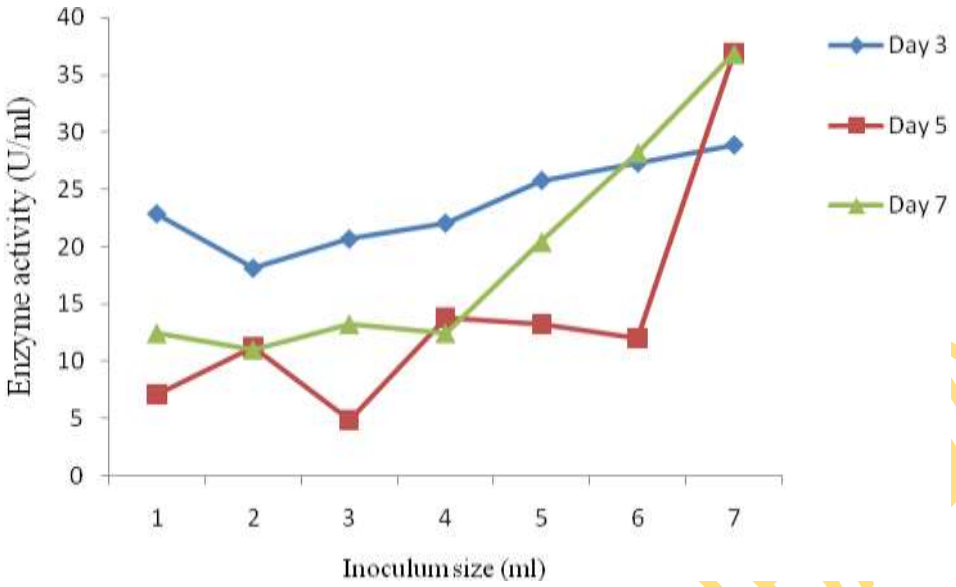
Fig. 4.4e (i & ii): Amylase production and Mycelia dry weight of *A. niger* under different pH.

4.2.1.2.4. Amylase production and mycelia dry weight of *Aspergillus niger* under optimized inoculums size (1-7ml)

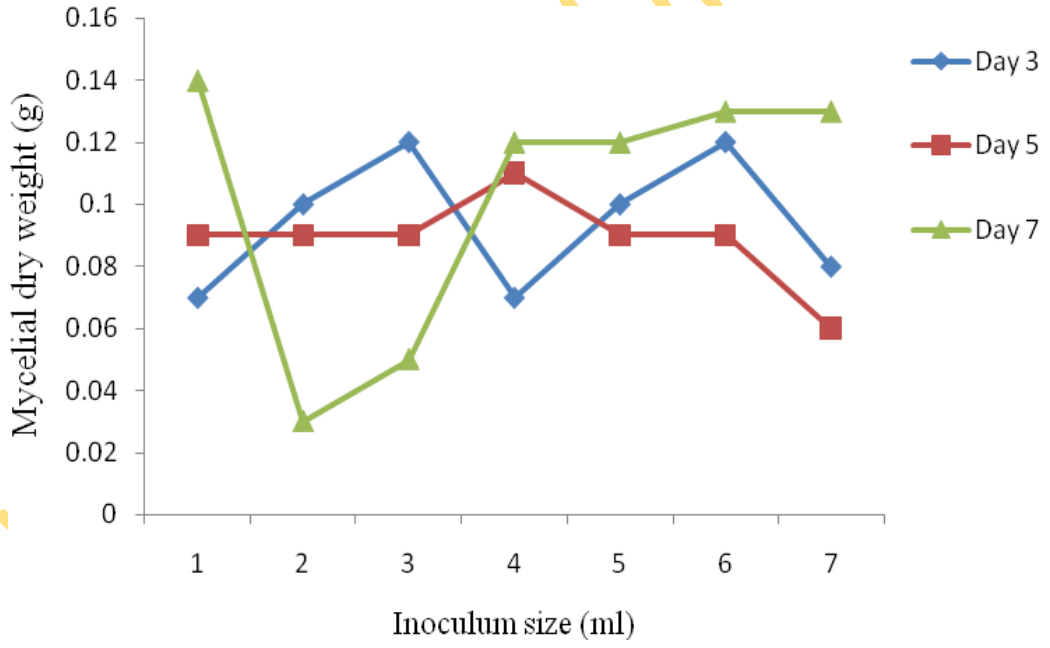
Figures 4.4f (i & ii) show the amylase production and mycelia dry weight of *Aspergillus niger* under different inoculum size (1 - 7ml). On day 3 amylase productions under optimized inoculum size (1-7ml) ranged from 18.167 – 28.91U/ml. The highest amylase was produced by 7ml inoculum size while 2ml inoculum size supported the least production by *Aspergillus niger*. On day 3 mycelia dry weight of *Aspergillus niger* under optimized inoculum size (1 - 7ml) ranged from 0.07 – 0.12 g. The highest mycelia dry weight was produced by 3ml and 6ml inoculum size while 1ml and 4ml inoculum size supported the least production by *Aspergillus niger*.

On day 5 amylase productions under optimized inoculum size (1 - 7ml) ranged from 4.86 – 36.88U/ml. The highest amylase was produced by 7ml inoculum size while 3ml inoculum size yielded the least production by *Aspergillus niger*. On day 5 mycelia dry weight of *Aspergillus niger* under optimized inoculum size (1 - 7ml) ranged from 0.06 – 0.11g. The highest mycelia dry weight was produced by 5ml inoculum size while 7ml inoculum size yielded the least mycelia dry weight by *Aspergillus niger*.

On day 7 amylase productions under optimized inoculum size (1 - 7ml) ranged from 10.98 – 36.86U/ml. The highest amylase was produced by 7ml inoculum size while 2ml inoculum size supported the least production by *Aspergillus niger*. On day 7 mycelia dry weight of *Aspergillus niger* under under optimized inoculum size (1-7ml)) ranged from 0.03 – 0.4g. The highest mycelia dry weight was supported by 1ml inoculum size while 2ml inoculum size supported the least production by *Aspergillus niger*.



(i)



(ii)

Fig. 4.4f (i & ii): Amylase and Mycelia production of *A. niger* under different inoculum size (1- 7ml.).

4.2.1.2.2.5. Amylase production and mycelia dry weight of *Aspegillus niger* under optimized temperature (10 – 60°C)

Figures 4.4g (i & ii) show the amylase production and mycelia dry weight of *Aspergillus niger* under different temperatures (10 – 60°C)

On day 3 amylase productions ranged from 16.90 - 45.73 U/ml. Amylase was optimally produced at 25°C while the least production was at 40°C by *Aspergillus niger*. On day 3 mycelia dry weight of *Aspergillus niger* ranged from 0.00 – 0.18g. The highest production of mycelia dry weight was at 50°C (0.18g) while 30 °C yielded zero mycelia dry weght by *Aspergillus niger*.

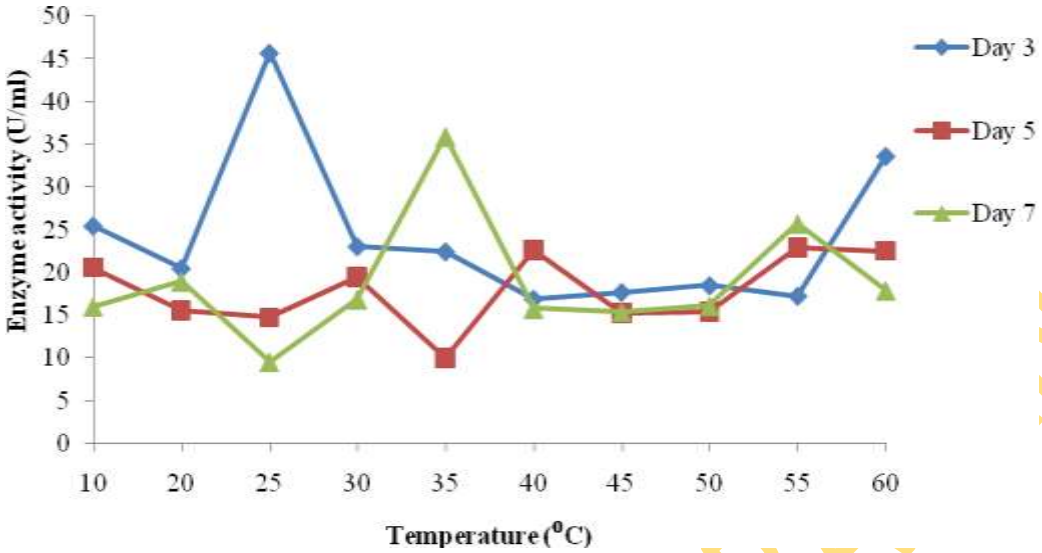
On day 5 amylase productions under optimized temperature (10 – 60°C) ranged from 14.73 – 22.97 U/ml. The highest amylase production was at 55°C while 25°C produced the least amylase by *Aspergillus niger*. On day 5 mycelia dry weight of *Aspergillus niger* under under optimized temperature (10 – 60°C) ranged from 0.07 – 0.14g. At 20°C and 55°C, the highest amylase production (0.14g) was recorded while least mycelia dry weight by *Aspergillus niger* was at 10°C.

On day 7 amylase productions under optimized temperature (10 – 60°C) ranged from 9.46 – 35.94 U/ml. The highest amylase production was at 35°C while at 25°C the least amylase production by *Aspergillus niger* was recorded. On day 7 mycelia dry weight of *Aspergillus niger* under under optimized temperature (10 – 60°C) ranged from 0.06 – 0.18 g. At 20°C and 45°C the highest mycelia dry weight was obtained while the least production by *Aspergillus niger* at 30°C.

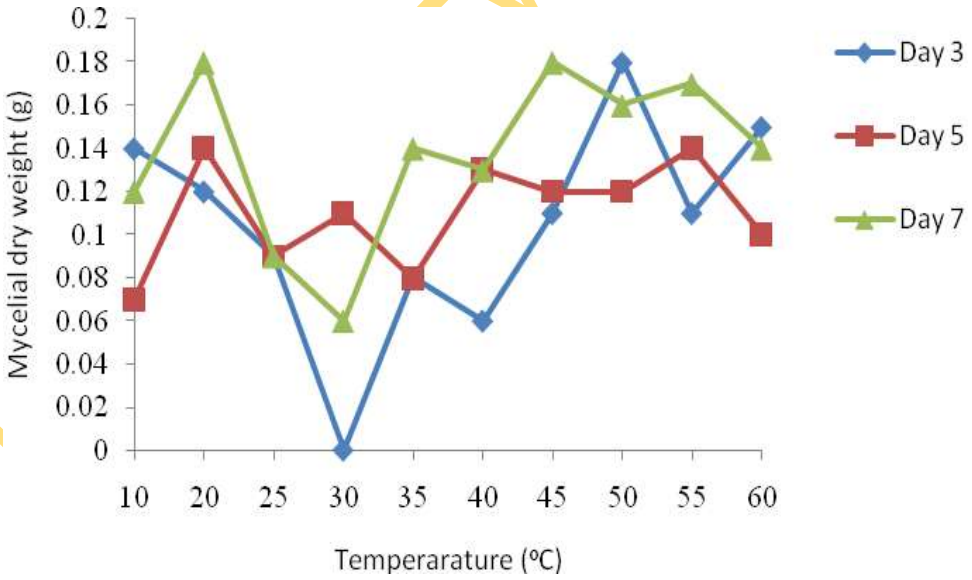
4.2.1.2.6. Amylase production and mycelia dry weight of *Aspergillus niger* under optimized incubation time (2 -12 days)

Figures 4.4h (i & ii) show the amylase production and mycelia dry weight of *Aspergillus niger* under different incubation time (2 - 12 days)

Amylase production under optimized incubation time (2 - 12 days) ranged from 7.33 - 24.65 UI/ml. At day 7 the highest amylase was produced while the least amylase production by *Aspergillus niger* was at day 11. Mycelia dry weight of *Aspergillus niger* under optimized Incubation time (2 - 12 days) ranged from 0.05 – 0.29g. The highest production was at day 12 while the least production of mycelia dry weght by *Aspergillus niger* was at day 7.

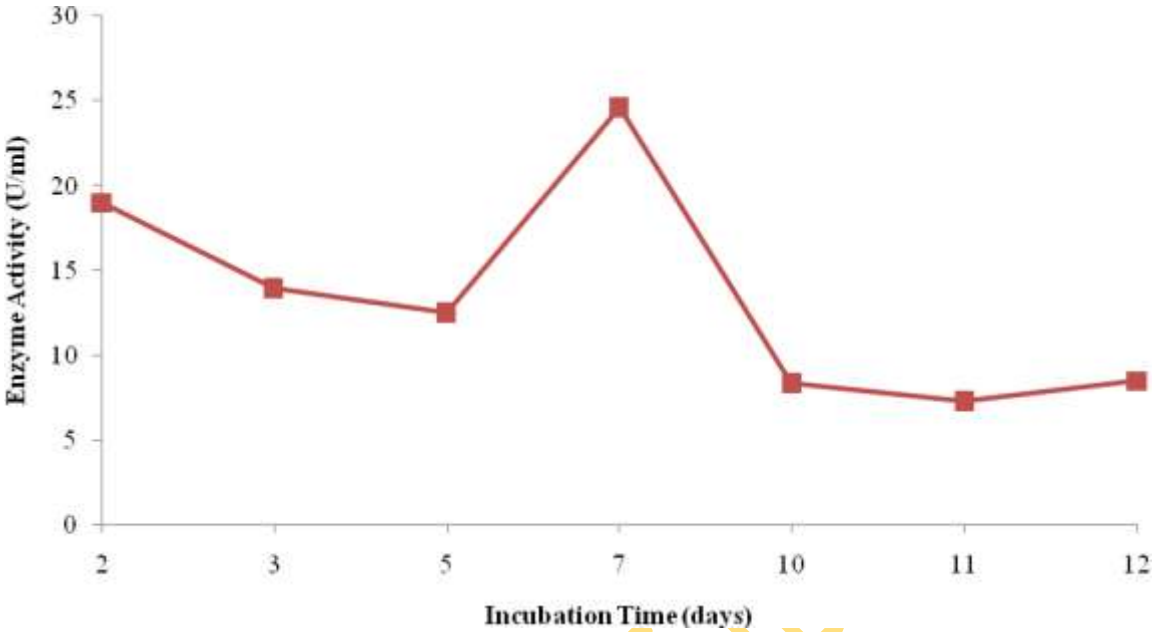


(i)

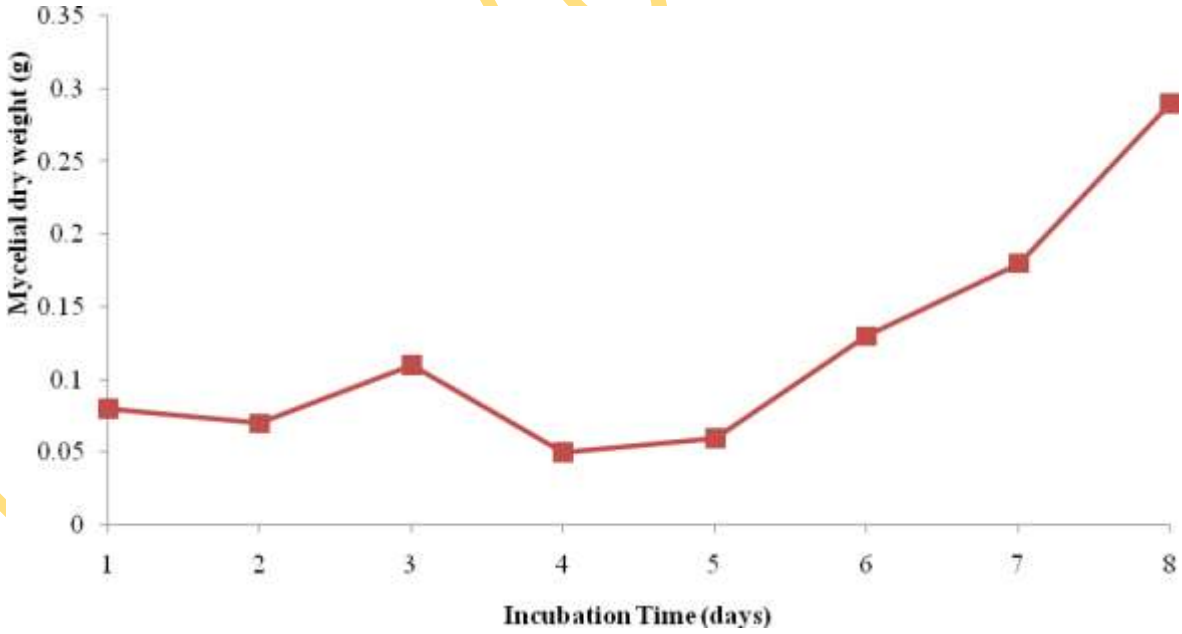


(ii)

Fig. 4.4g (i & ii): Amylase production and Myceliadry weight of *A. niger* under different temperature.



(i)



(ii)

Fig. 4.4h (i & ii): Amylase production and Mycelia dry weight (g) of *A. niger*r under different Incubation time (days).

4.2.1.2.3. Characterization of Amylase produced by *Aspergillus niger*

Amylase produced by *Aspergillus niger* was characterized by observing its activities under the following parameters - temperature, temperature stability, pH and pH stability, Cations ($CaCl_2$, $MgSO_4$, $MnSO_4$, KNO_3 and $NaCl_2$), Anions($NaNO_3$, $FeSO_4$, $FeCl_2$, $CuSO_4$, and $CaCO_3$), Inhibitors(EDTA, HgCl, FeKCN, Benzoic acid and Urea), Time (minutes), Substrate (Starch) concentrations and Enzyme (Amylase) concentrations.

4.2.1.3.1. Effect of different incubation temperatures (10^oC – 60^oC) on enzyme activity and stability

Effect of different incubation temperature (10^oC – 60^oC) on activity and stability of *Aspergillus niger* amylase was studied as shown in Figure 4.5a. The activities of the amylase ranged from 3.04 – 12.32U/ml in which the highest activity, 12.32U/ml was recorded at 50^oC while the least was at 20^oC. The stability of the enzyme activity produced by *Aspergillus niger* ranged from 2.68 – 14.35UI/ml in which 50^oC supported the highest while the least was recorded at 20^oC.

4.2.1.3.2. Effect of different pHs (3 - 12) on enzyme activity and stability

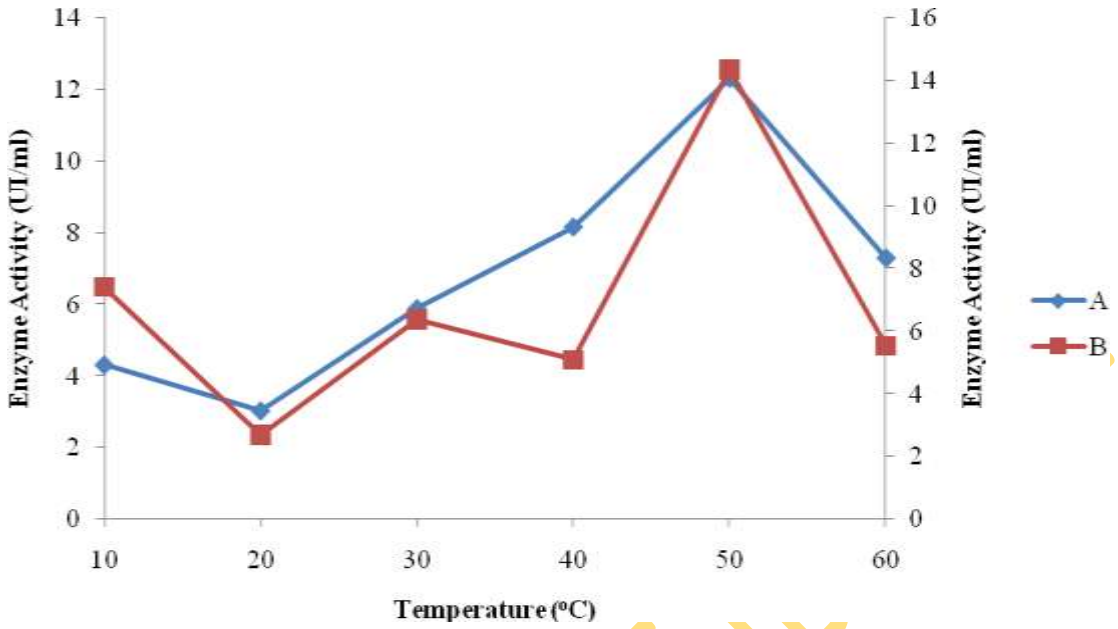
Effect of different pH (3 - 12) on activity and stability of *Aspergillus niger* amylase was studied as shown in Figure 4.5b. The activity ranged from 2.27 - 16.85U/ml in which pH 12 supported the highest while the least was recorded at pH 9. *Aspergillus niger* amylase stability ranged from 1.96 – 18.04U/ml in which the highest was at pH 3 while the least was recorded at pH 9.

4.2.1.3.3. Effect of different concentrations (0.1M – 1M) of cations on enzyme activity

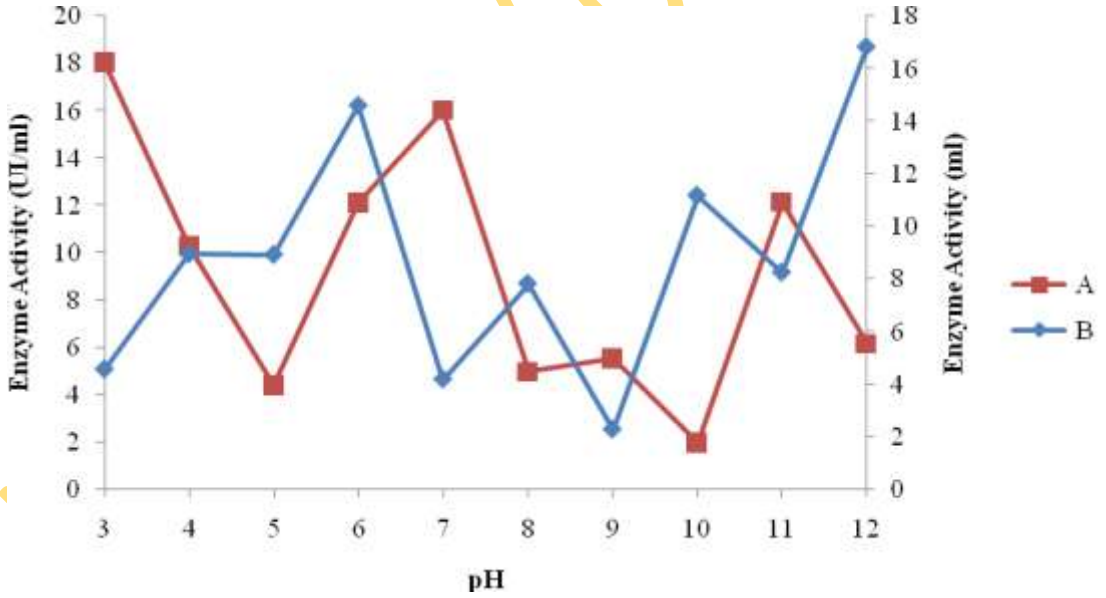
Effect of different concentrations (0.1M to 1M) of cations (CaCl, $MgSO_4$, $MnSO_4$, KNO_3 and $NaCl_2$) on activity of *Aspergillus niger* amylase was studied as shown in Figure 4.5c. The amylase activity ranged from 0.28 - 10.195U/ml in which 1M KNO_3 had the highest amylase activity of 10.195U/ml while 0.3M KNO_3 had the least amylase activity of 0.28 U/ml.

4.2.1.3.4. Effects of different concentrations of anions on enzyme activity

Effect of different concentrations (0.1M to 1M) of Anions ($NaNO_3$, $FeSO_4$, $FeCl_2$, $CuSO_4$, and $CaCO_3$) on activity of *Aspergillus niger* amylase was studied as shown in Figure 4.5d.The amylase activity ranged from 0.20 - 18.80UI/ml in which 1M $FeSO_4$ supported the highest while 0.1M $CaCO_3$ supported the least.

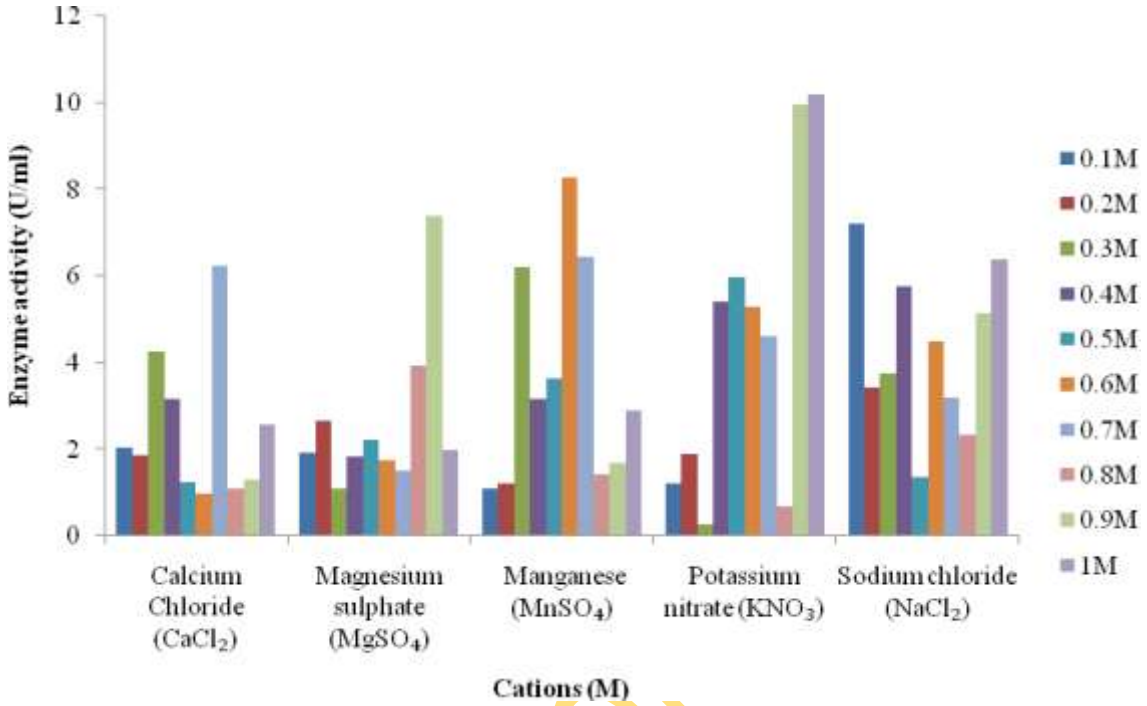


(a)

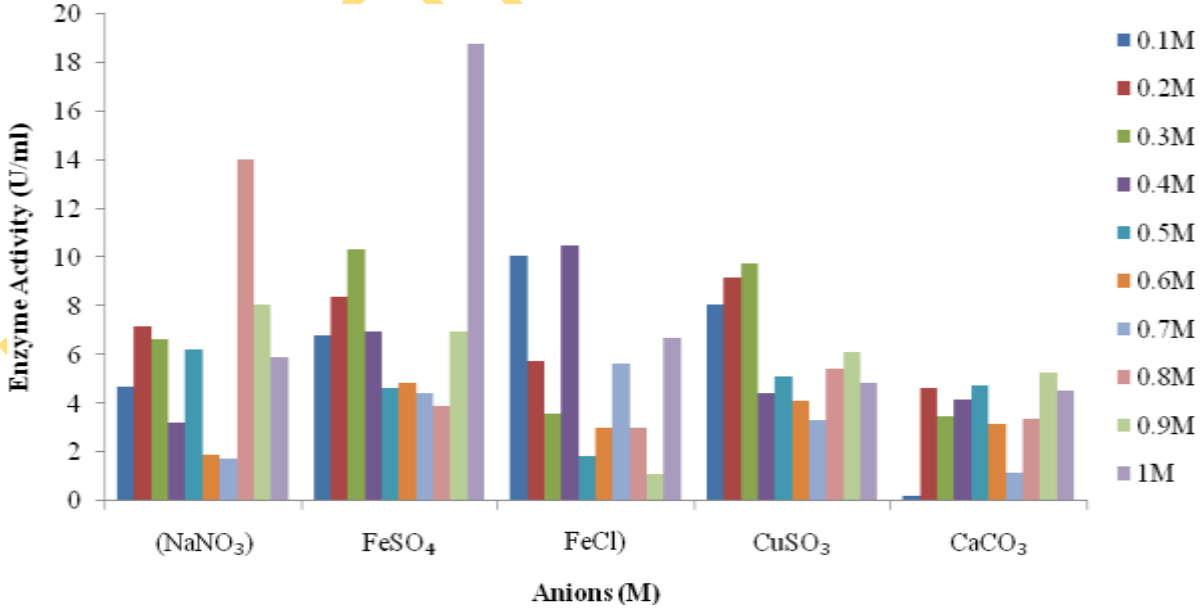


(b)

Fig. 4.5(a & b): Effect of different Temperatures (^{o}c) and pH on activity and stability of *A. niger* Amylase.



(c)



(d)

Fig.4.5(c & d): Effect of different Cations and Anions concentrations on activity of *A.niger* Amylase.

4.2.1.3.5. Effects of different concentrations of inhibitors on enzyme activity

Effect of different concentrations (0.1M to 1M) of inhibitors (EDTA, HgCl, FeKCN, Benzoic acid and Urea) on activity of *Aspergillus niger* amylase was studied as shown in Figure 4.5e

The amylase activity ranged from 0.71 - 13.31UI/ml in which 0.7M FeKCN supported the highest while the least, 0.72U/ml was in 0.3M HgCl.

4.2.1.3.6. Effect of different incubation time on enzyme activity

Effect of different incubation time (10 to 100min) on activity of *Aspergillus niger* amylase was determined as shown in Figure 4.5f. The amylase activity ranged from 5.78 - 20.70U/ml in which the highest activity was recorded after 90min of incubation while the least was recorded after 40 min. of incubation.

4.2.1.3.7. Effect of enzyme concentration on amylase activity

Effect of enzyme concentration (1 – 10% ^{v}/v) on activity of *Aspergillus niger* amylase was studied as shown in Figure 4.5g. The amylase activity ranged from 0.87 - 7.30U/ml in which the highest activity was recorded at enzyme concentration of 8% ^{v}/v while the least was at substrate concentration of 7% ^{v}/v.

4.2.1.3.8. Effect of substrate concentration on amylase activity

Effect of substrate concentration (0.5 – 5.0% ^{w}/v) on activity of *Aspergillus niger* amylase was investigated as shown in Figure 4.5h. The amylase activity ranged from 0.94 - 6.56U/ml in which the highest activity was recorded at substrate concentration of 2.5% ^{w}/v while the least was at substrate concentration of 1.5% ^{w}/v.

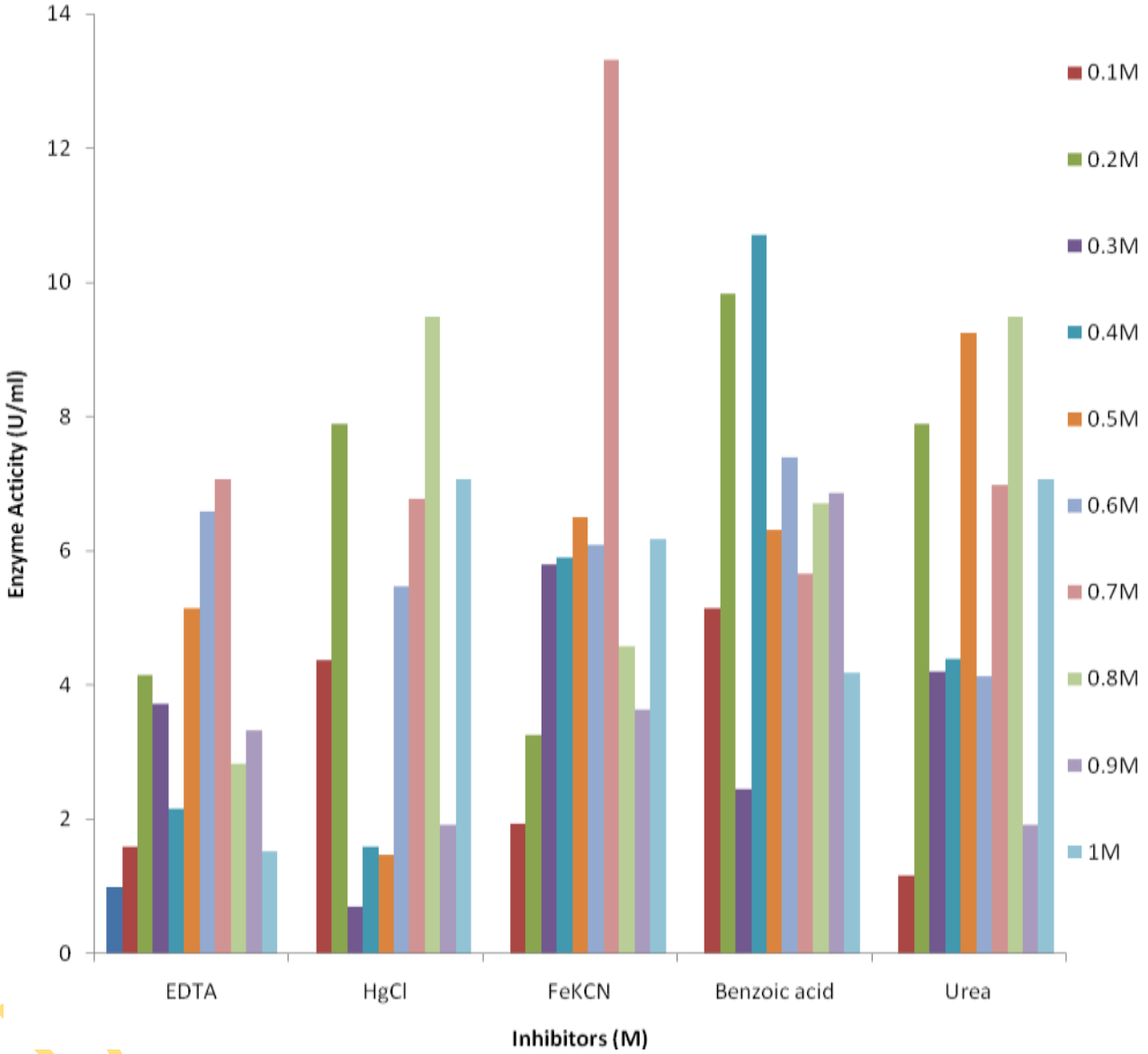
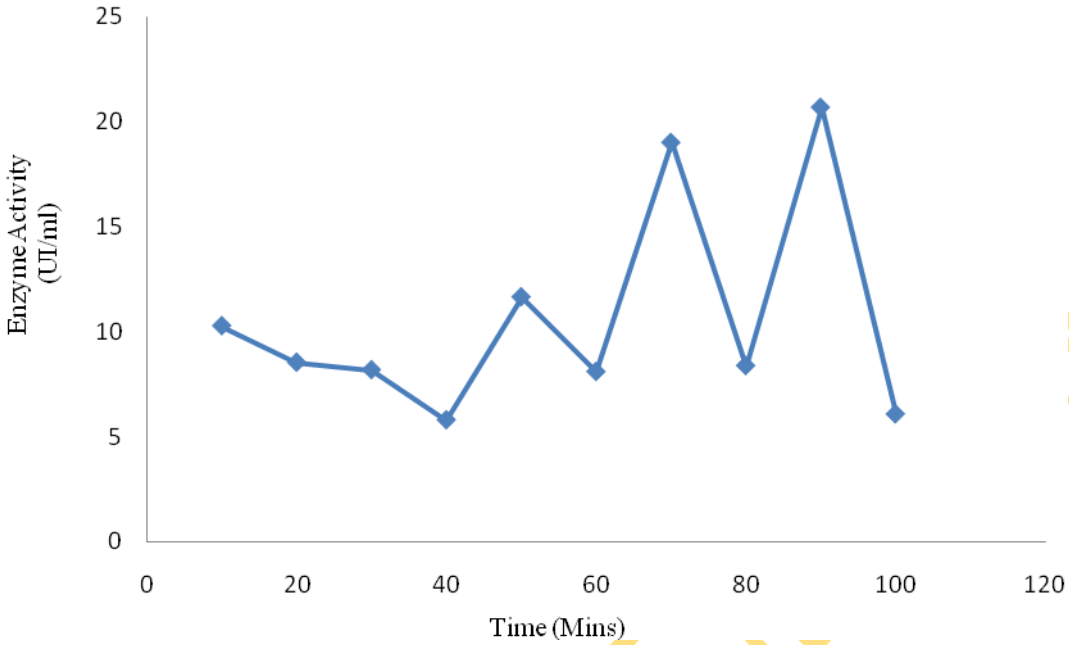
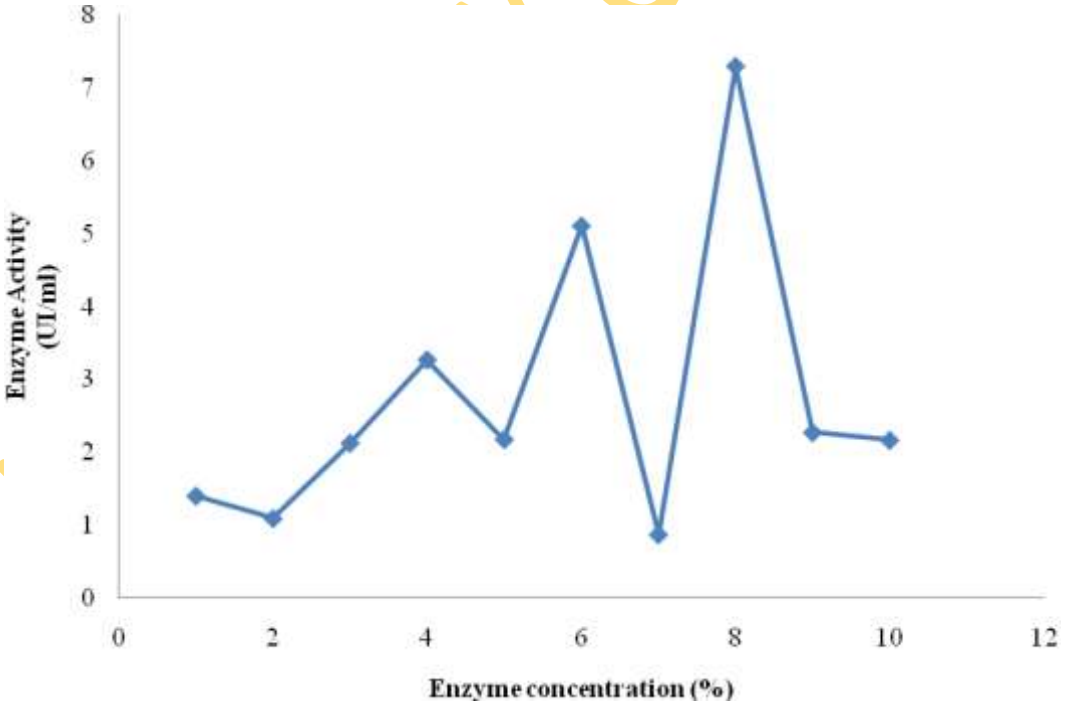


Fig.4.5 (e): Effect of different Inhibitors' concentrations on activity of *A. niger* Amylase.



(f)



(g)

Figs. 4.5(f & g): Effect of Time (mins) and Enzyme concentration (%v/v) on the activity of *A. niger* Amylase.

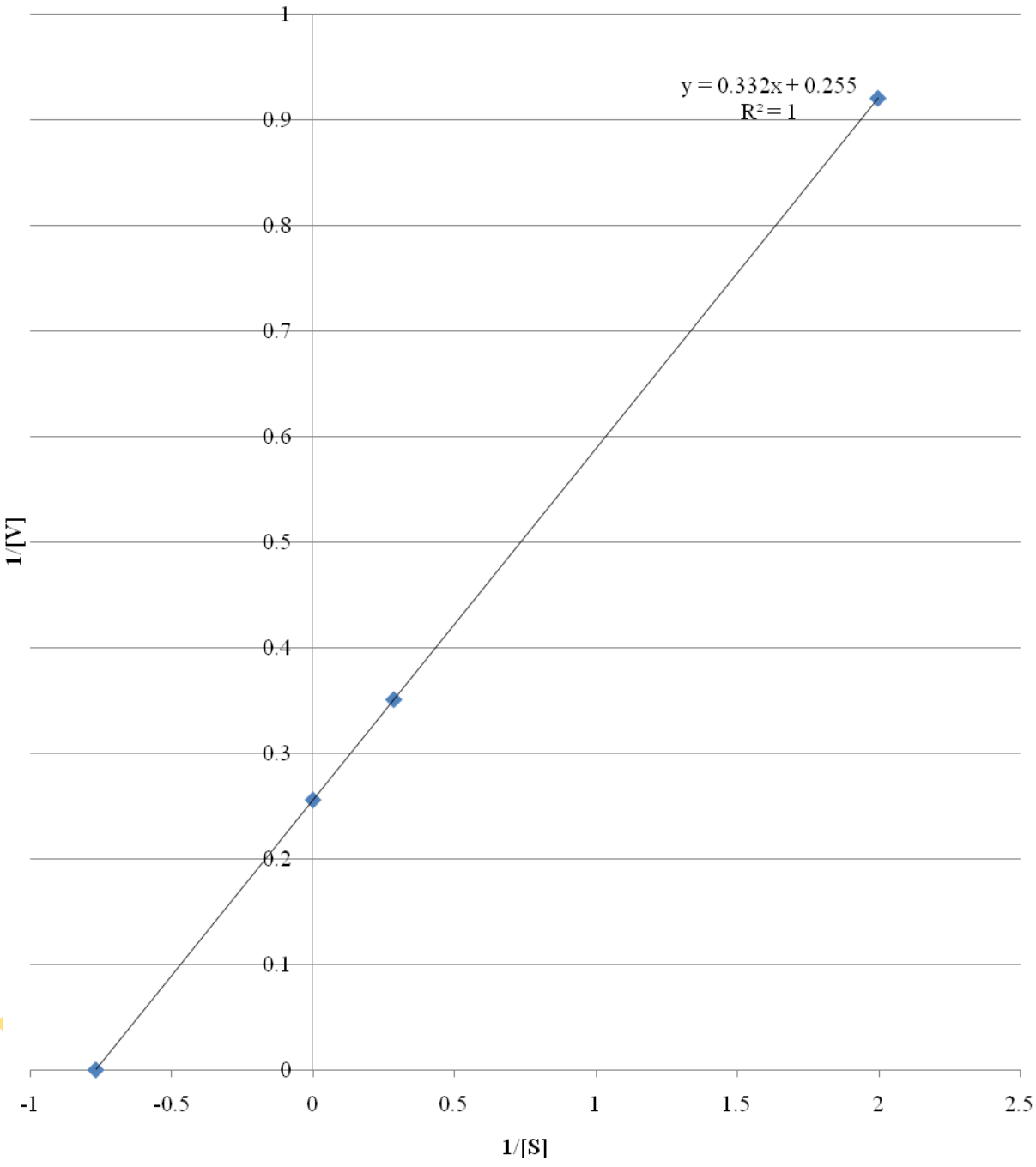


Fig. 4.5 (h): Characterization of Amylase activity at the optimum substrate concentration (starch).

4.2.2. Cellulase production by the fungal isolates

The ability of the fungal isolates obtained from lignocellulosic materials to produce cellulase and mycelia growth using submerged fermentation was tested. The best cellulase producer was selected for further studies. The effects of physico-chemical parameters on the production of cellulase by the selected cellulase producer were determined. The fermentation condition was optimized and the enzyme produced was characterized.

The screening of the fungal isolates for cellulase production is shown in Figure 4.6. The cellulase production ranged from 2.82 - 3.13U/ml in which *Rhizopus oryzae* had the highest while *Rhizopus stolonifer* had the least. About 40% of the isolates (*R. oryzae, Aspergillus versicolor, Rhizopus* sp and *A. terreus)* are good producers of cellulase and 60% (*A. flavus, A. niger, A. clavertus, R. stolonifer, A.tamarii and Trichoderma* sp.+ produced cellulase moderately.

4.2.2.1. Effect of physicochemical parameters on cellulase production by the selected *R. oryzae*

4.2.2.1.1. Effect of carbon sources on cellulase production and mycelia dry weight of *R. oryzae*

The effects of physico-chemical parameters (carbon sources, nitrogen sources, pH, inoculums size, temperature and incubation time) on cellulase production by the selected *R. oryzae* were determined.

Figures 4.7 (a & b) show the effect of different carbon sources on cellulase and mycelia dry weight production by *R. oryzae* at different incubation times. On day 3, cellulase production ranged from 0.47 – 14.10U/ml in which xylose induced the highest production while the least was recorded in palm kernel cake. *R. oryzae* was able to produce cellulase in all the carbon sources used. There was a significant difference in cellulase production by *R. oryzae* under different carbon sources. On day 3 the mycelia dry weight ranged from 0.06 - 0.19g in which Raffinose induced the highest growth while maltose and starch produced the least growth by *R. oryzae.*

On day 5 the cellulase production ranged from 0.01 – 5.18U/ml in which Xylose supported the highest production while sucrose yielded the least. On day 5 the mycelia dry weight

ranged from 0.02 - 0.18g. Xylose supported the highest growth while trehalose produced the least growth by *R. oryzae*.

On day 7 the cellulase production ranged from 0.03 – 5.92U/ml in which Rice bran supported the highest production while Maltose supported the least.On day 7 the mycelia dry weight ranged from 0.06 - 0.18g in which Sugarcane bagasse supported the highest growth while the least mycelia dry weight by *R. oryzae* was *by* saccharose.

4.2.2.1.2. Effect of nitrogen sources on cellulase production and mycelia growth weight of *R. oryzae*

The effect of nitrogen sources (Ammonium nitrate, Ammonium sulphate, Potassium nitrate, Corn steep liquor, Soy bean meal, Peptone, Yeast extract, Urea and Casein) on cellulase production and mycelia dry weight of *R. oryzae* is shown in Figures 4.7 (c & d)

On day 3 cellulase production ranged from 2.01 – 10.15U/ml in which the highest cellulase production was supported by Cornsteep liquor while the least was recorded when Yeast extract was used as the source of nitrogen. On day 3 the mycelia dry weight ranged from 0.07 - 0.13g. Peptone supported the highest growth while NH_4NO_3 gave the least growth by *R. oryzae.*

On day 5, cellulase production ranged from 0.03 – 1.0U/ml in which Casein produced the highest cellulase while the least was recorded in Peptone. On day 5 the mycelia dry weight ranged from 0.06 - 0.15g. Peptone supported the highest growth while Casein produced the least growth by *R. oryzae*.

On day 7 cellulase production ranged from 2.08 – 11.28U/ml in which KNO_3 supported the highest production while the least was recorded when Soya bean supplied nitrogen. On day 7 the mycelia dry weight ranged from 0.06 - 0.14g. The highest growth was in Peptone while the least growth by *R. oryzae* was in Yeast extract.

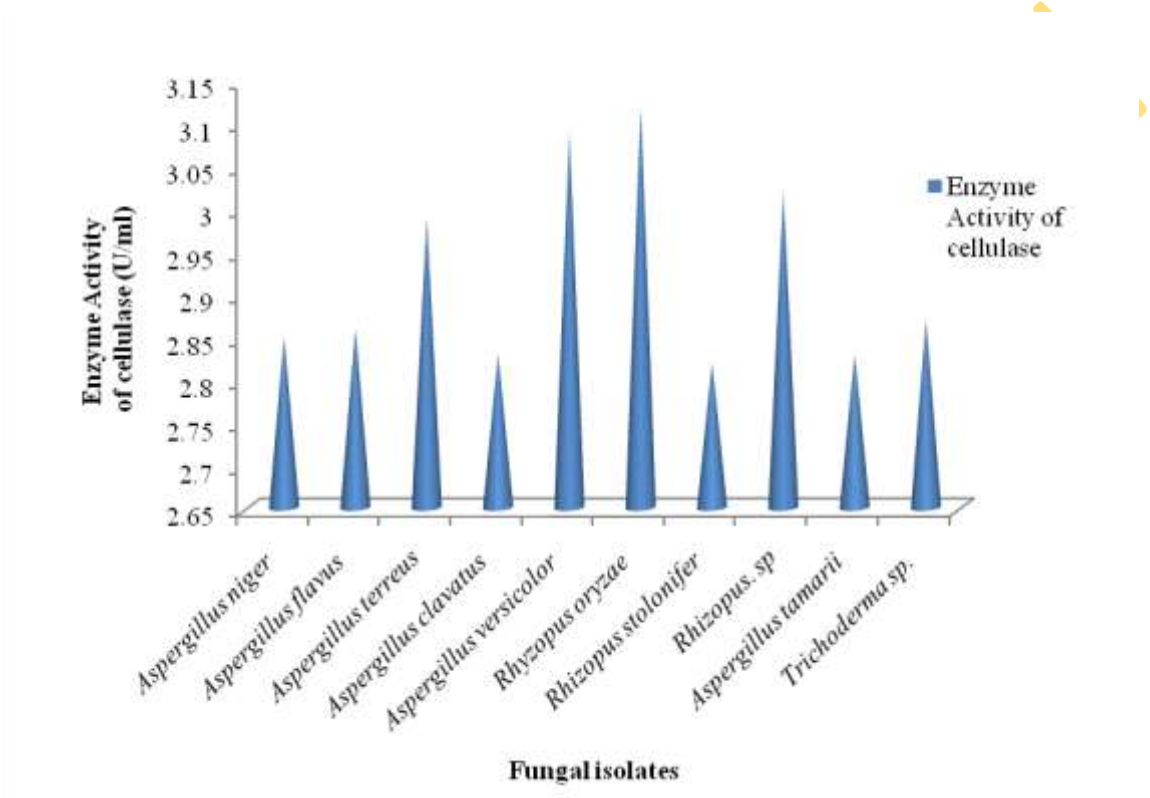
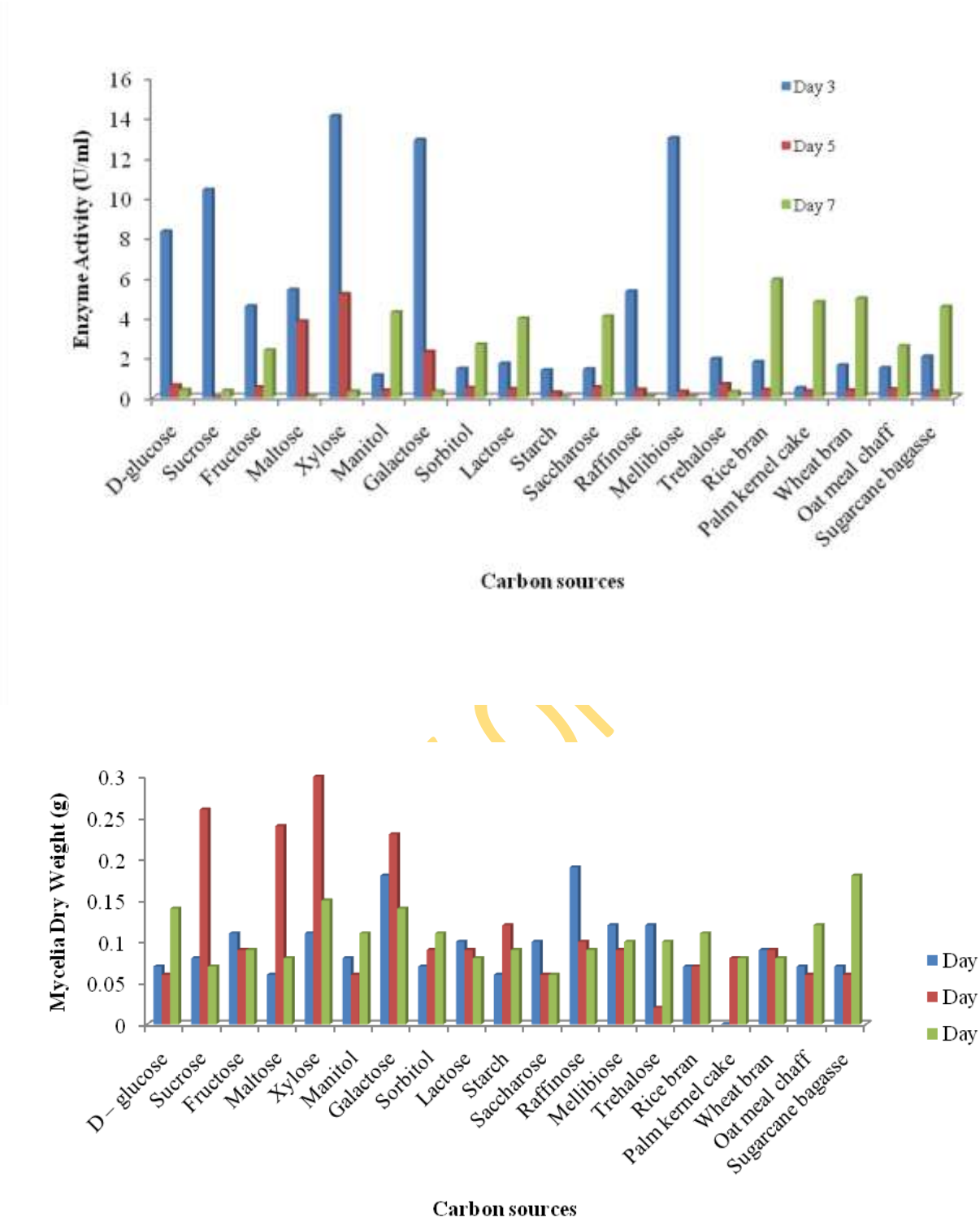
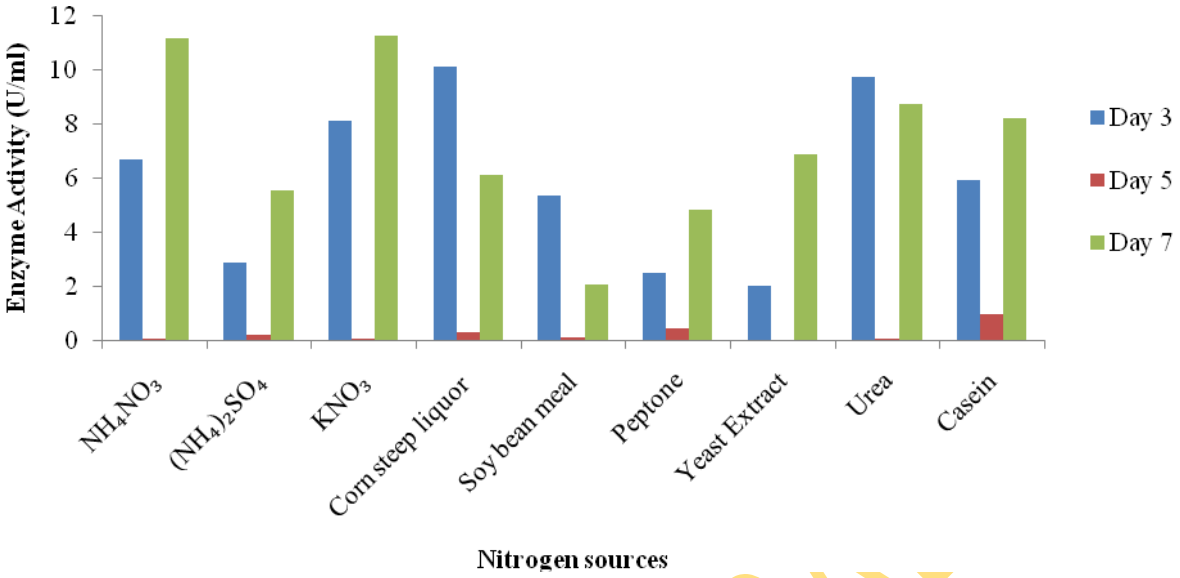


Fig. 4.6: Cellulase production by the fungi isolated from the lignocellulosic substrates.

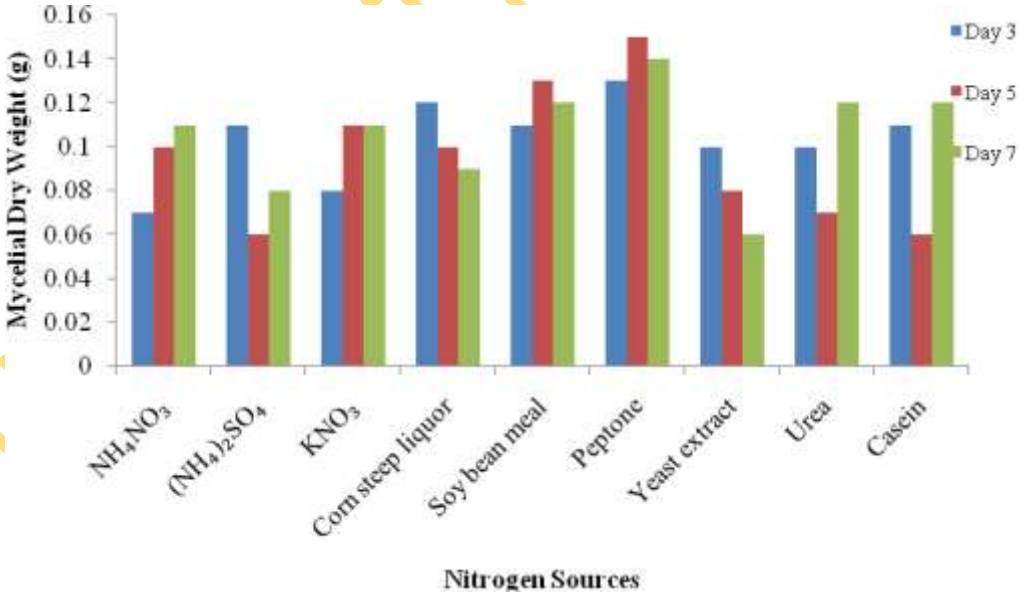


(b)

Fig. 4.7 (a & b): Effect of different carbon sources on Cellulase production and Mycelia dry weight of *Rhizopus oryzae*.



(c)



(d)

Fig. 7 (c & d): Effect of Nitrogen sources on Cellulose production and Mycelia dry weight (g) of *R. oryzae*.

4.2.2.1.3. Effect of pH on cellulase production and mycelia dry weight of *R. oryzae*

Figures 4.7 (e & f) show the effect of pH (4, 5, 6, 7, 8, 9 and 10) on cellulase production at different by *R. oryzae*. On day 3, the cellulase production ranged from 2.365 - 3.718U/ml in which pH 4 supported the highest cellulase while the lowest production was at pH 10. On day 3 the mycelia dry weight ranged from 0.06 - 0.11g. pH 7 supported the highest growth while pH 4 yeilded the least growth by *R. oryzae.*

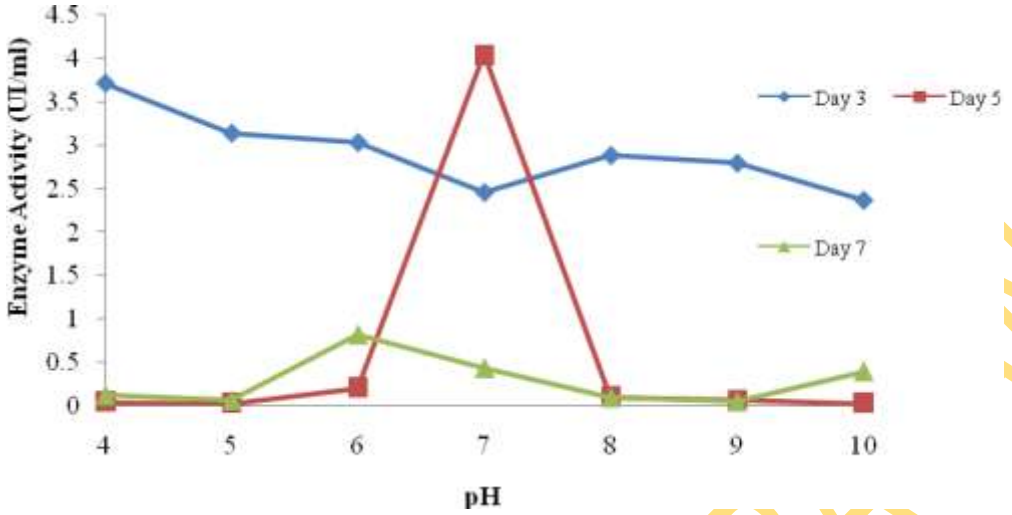
At day 5, cellulase production ranged from 0.03 – 4.05U/ml in which the cellulase production at pH 5 was the lowest while pH 7 induced the highest cellulase production. On day 5 the mycelia dry weight ranged from 0.03 - 0.17g. pH 7 supported the highest growth while at pH 10 the least mycelia dry weight was produced by *R. oryzae.* On day 7, cellulase produced ranged from 0.04 - 0.81U/ml in which pH 9 supported the lowest while pH 6 induced the highest cellulase production. On day 7 the mycelia dry weight ranged from 0.10 - 0.16g. pH 9 supported the highest growth while the least growth by *R. oryzae.*was at pH 4.

4.2.2.1.4. Effect of inoculums size on cellulase production and mycelia dry weight of *R. oryzae*

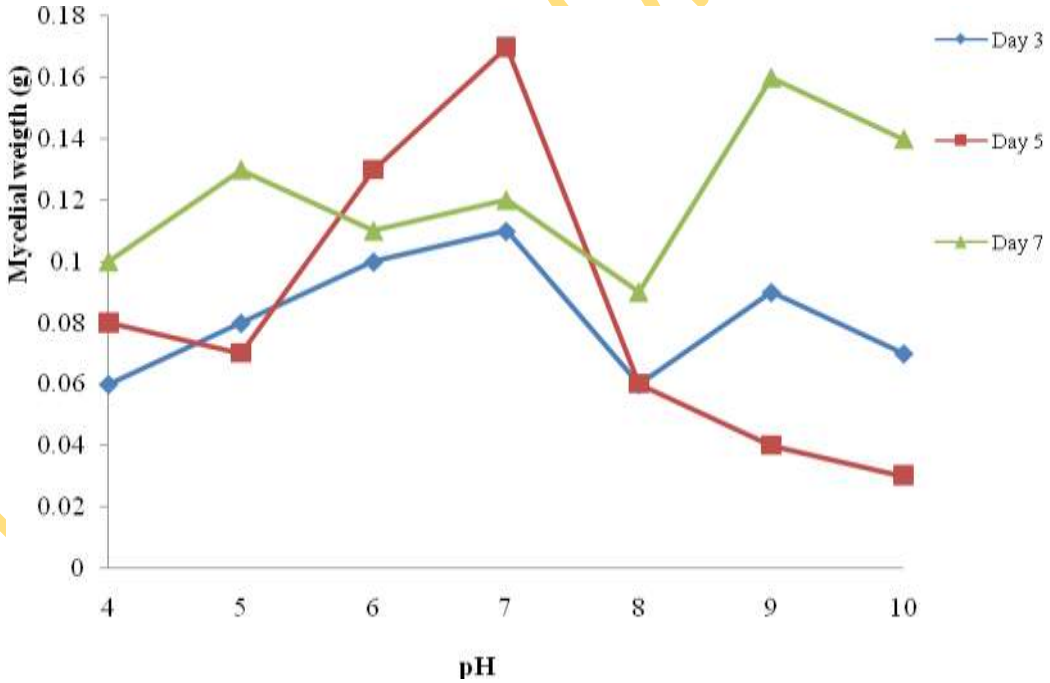
The effect of inoculums size (1ml to 4ml) on cellulase production and mycelia dry weight of *R. oryzae* is shown in Figures 4:7 (g & h). On day 3 Cellulase production by *R. oryzae* ranged from 1.522 - 5.018U/ml in which 4ml inoculum size supported the highest cellulase production while the lowest cellulase production was with 3ml inoculums size. On day 3 mycelia dry weight of *R. oryzae* ranged from 0.03 – 0.33 in which the highest mycelia dry weight was produced by 3ml inoculums size supported while 2ml produced the lowest mycelia dry weight.

On day 5 Cellulase production by *R. oryzae* under different inoculums size (1ml - 4ml) ranged from 0.053 - 2.200U/ml in which the highest cellulase production was by 2ml inoculum size while 3ml yielded the lowest. On day 5 mycelia dry weight of *R. oryzae* under different inoculum size (1ml - 4ml) ranged from 0.09 – 0.25 in which 3ml inoculum size supported the highest mycelia dry weight while 2ml produced the lowest

On day 7 Cellulase production by *R. oryzae* ranged from 0.076 to 0.332U/ml in which 2ml inoculum size produced the highest while 4ml gave the lowest cellulase production. On day 7 mycelia dry weight of *R. oryzae* ranged from 0.12 – 0.15g in which 3ml inoculum size supported the highest while 1ml produced the lowest.

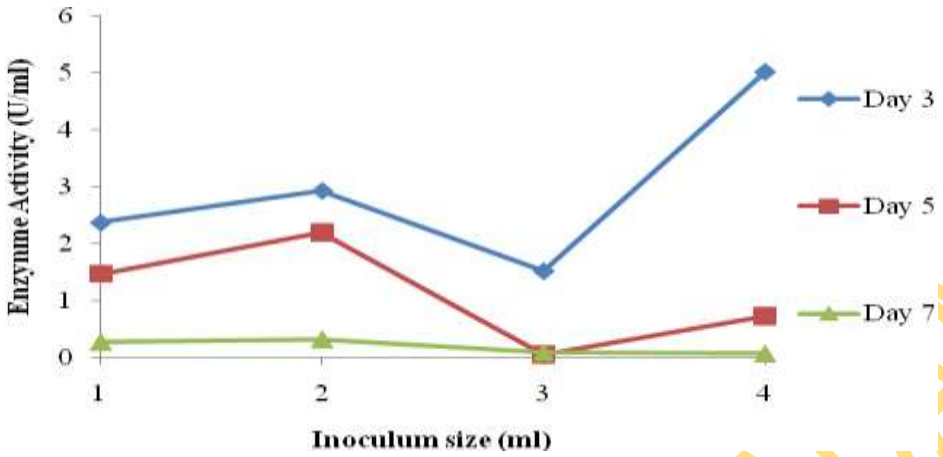


(e)

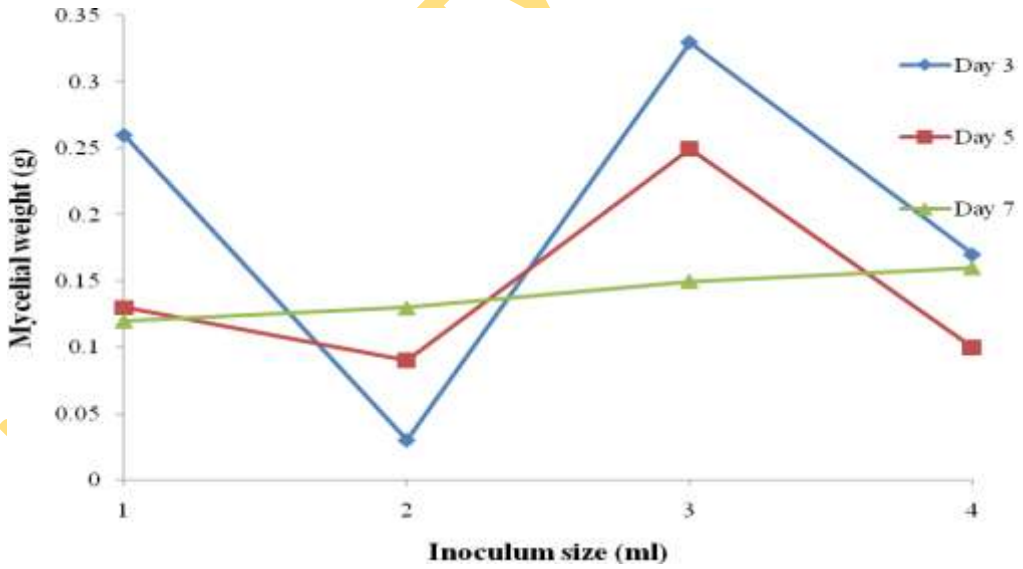


(f)

Fig. 4.7 (e & f): Effects of pH on Cellulase production and Mycelia dry weight (g) by *R. oryzae*.



(g)



(h)

Fig. 4.7 (g & h): Effects of Inoculum size (ml) on cellulase production and Mycelia dry weight (g) by *R.oryzae*.

4.2.2.1.5. Effect of temperature on cellulase production and mycelia dry weight of *R. oryzae*

The effect of different incubation temperatures (20°C, 25°C, 30°C, 35°C, 40°C and 45°C) on cellulase production and mycelia dry weight of *R. oryzae* is shown in Figures 4:7 (i & j).

On day 3 Cellulase productions by *R. oryzae* under different incubation temperatures (20°C, 25°C, 30°C, 35°C, 40°C and 45°C) ranged from 0.051 - 3.278U/ml in which the highest cellulase production of 3.278U/ml was at 20°C while at 40°C the lowest cellulase was produced. On day 3 mycelia dry weight of *R. oryzae* under different incubation temperature (20°C, 25°C, 30°C, 35°C, 40°C and 45°C)) ranged from 0.07 – 0.31g. At 45°C the highest mycelia dry weight was produced while the lowest growth was at 30° C.

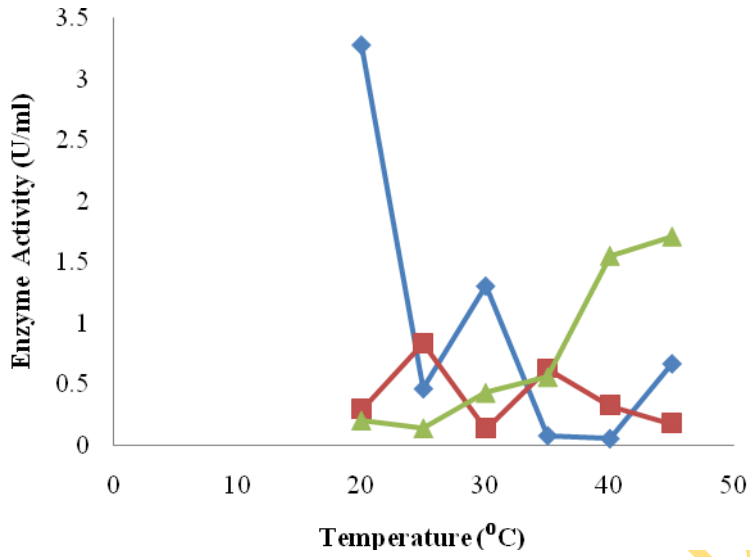
On day 5 Cellulase productions by *R. oryzae* under different incubation temperatures (20°C, 25°C, 30°C, 35°C, 40°C and 45°C) ranged from 0.133 - 0.837U/ml in which the highest cellulase production was at 45°C while at 25°C the lowest cellulase was produced. On day 5 mycelia dry weight of *R. oryzae* under different incubation temperatures (20°C, 25°C, 30°C, 35°C, 40°C and 45°C) ranged from 0.01 – 0.12g in which 20°C and 45°C supported the highest mycelia dry weight while at 30°C the least mycelia dry weight was recorded.

On day 7 Cellulase productions by *R. oryzae* under different incubation temperatures (20°C, 25°C, 30°C, 35°C, 40°C and 45°C) ranged from 0.142 - 1.708U/ml in which 45°C supported the highest while at 25°C there was the lowest cellulase yield. On day 7 mycelia dry weight of *R. oryzae* under incubation temperatures (20°C, 25°C, 30°C, 35°C, 40°C and 45°C) ranged from 0.09 – 0.18g in which 35°C supported the highest while the lowest was produced at 40°C.

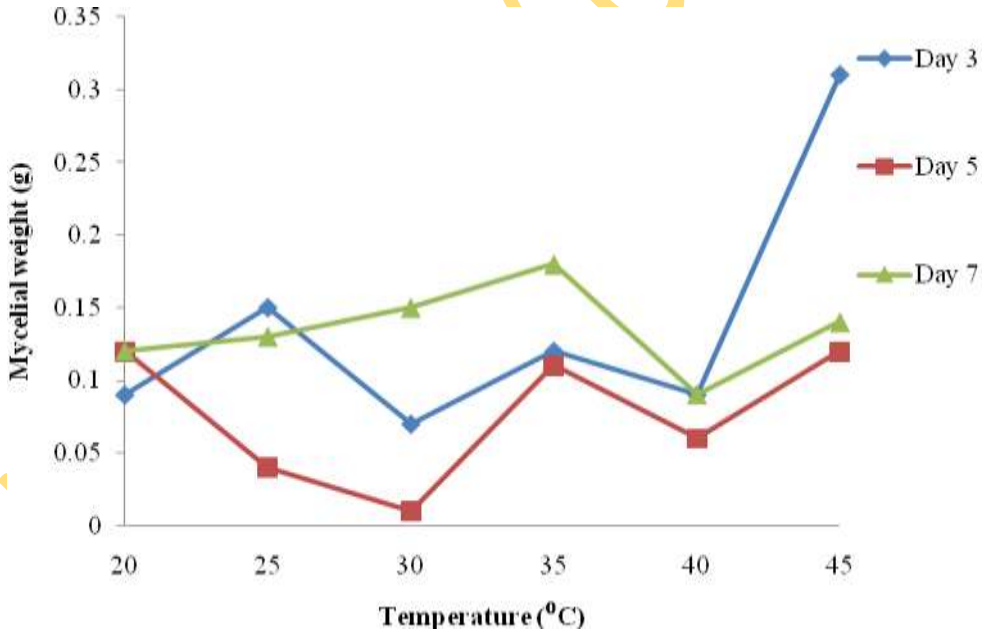
4.2.2.1.6. Effect of incubation time on cellulase production and mycelia dry weight of *R. oryzae*

The effect of different incubation times on cellulase production and mycelia dry weight of *R. oryzae* is shown in Figure 4:7 (k & l)

The Cellulase production and mycelia dry weight of *R. oryzae* under different incubation times ranged from 1.11 – 3.46U/ml and 0.09 – 0.10g respectively. On days 3 and 14, the highest values were obtained while at days 7 and 3 the lowest cellulase production was obtained as shown in Figure 4.7 (k & l).

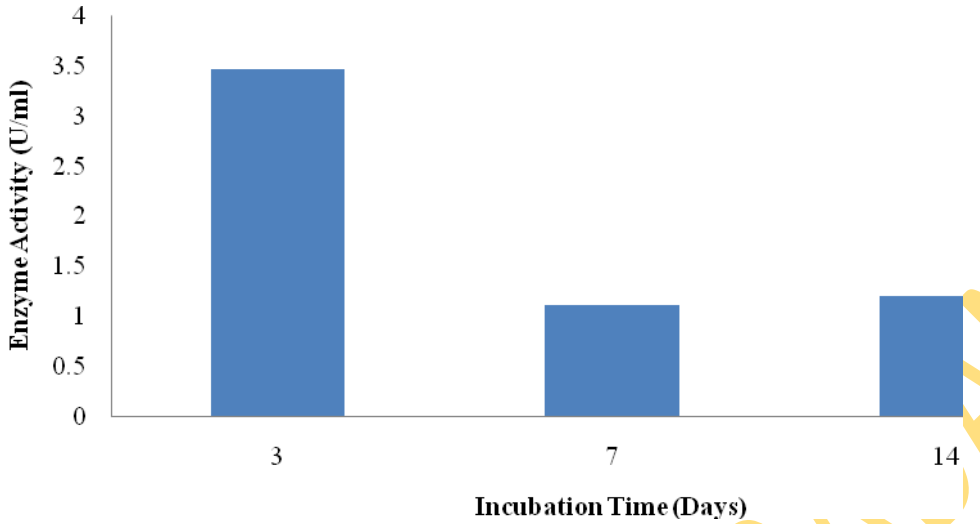


(i)

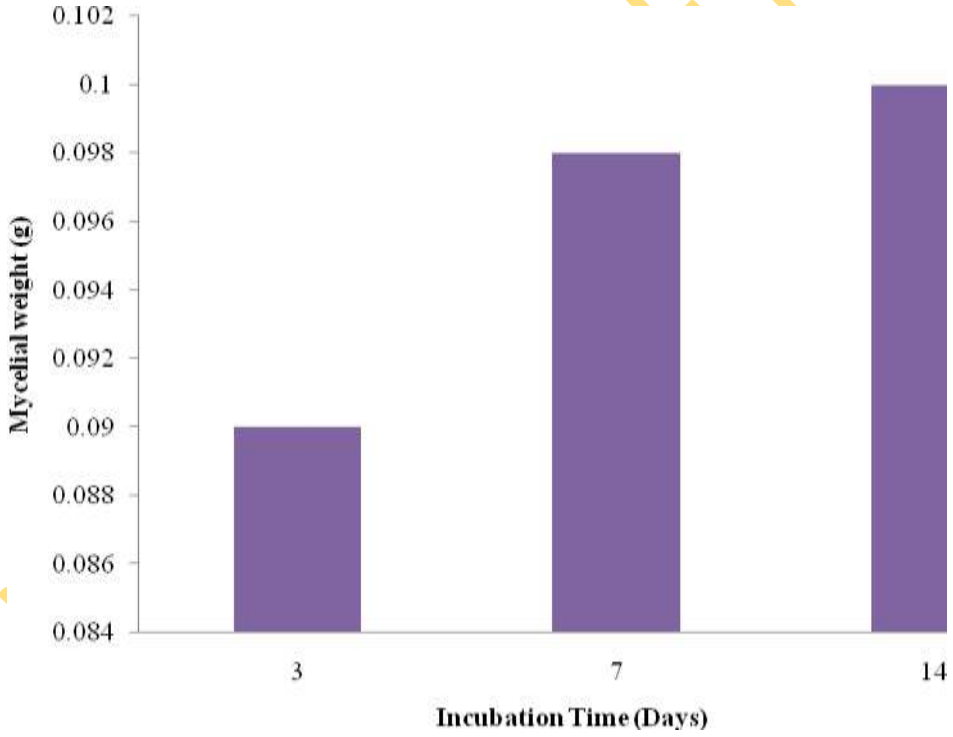


(j)

Fig. 4.7 (i & j): Effects of Temperature (^{o}C) on cellulase production and Mycelia dry weight (g) by *R. oryzae.*



(k)



(l)

Fig. 4.7 (k & l): Effects of Incubation time (days) on the production of Cellulase and Mycelia dry weight (g) by *R. oryzae.*

4.2.2.2 Optimization of cellulase production by *R. oryzae*

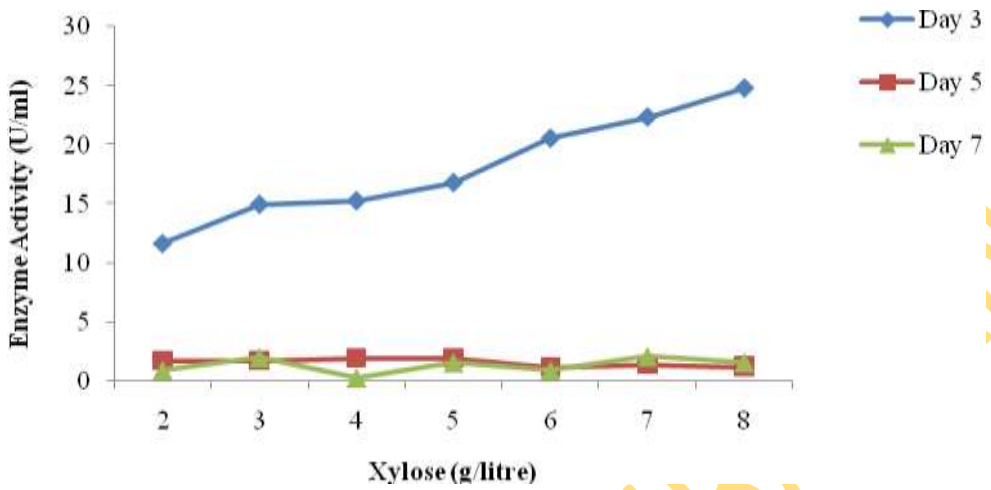
The level of the best carbon sources (Xylose and Corn steep liquor), Nitrogen sources (KNO_3 and Urea), pH (3 - 6), Inoculum load (1-7ml), temperature (10 -60^oC) and Incubation time (2- 12 days) at which cellulase production will be optimum by *R. oryzae* was determined.

4.2.2.2.1. Cellulase production and mycelia dry weight of *R. oryzae* under different concentration (2 -8g/l) of xylose and Corn steep liquor

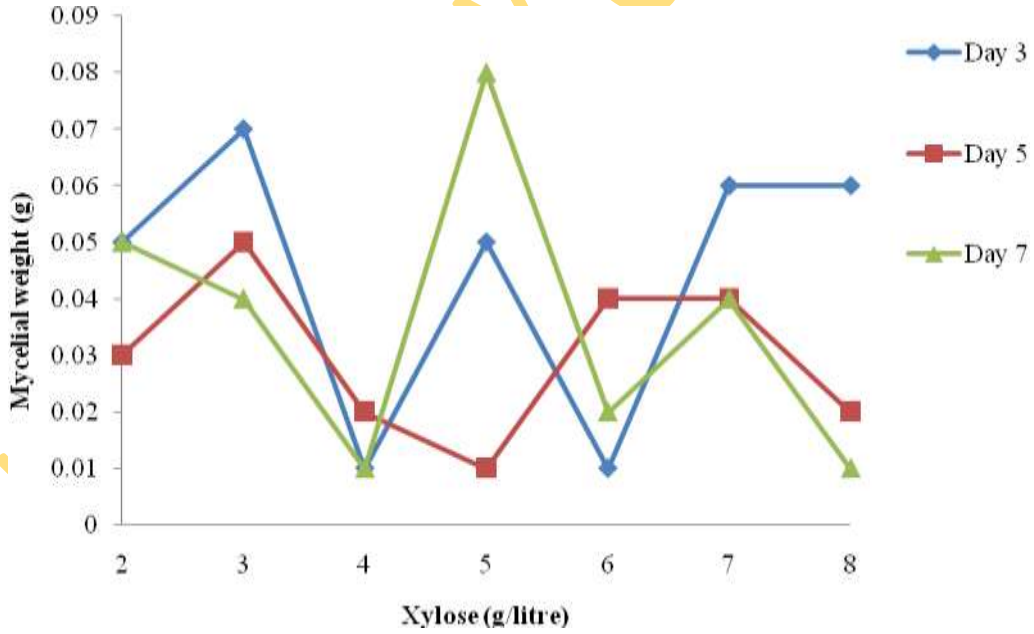
Cellulase production and mycelia dry weight of *R. oryzae* under different concentrations (2 - 8g/l) of xylose and Corn steep liquor (7 – 13 % ^{v}/v) respectively was determined (Figures 4.8a (i & ii) and 4.8b (i & ii)). On day 3 Cellulase production on different concentrations of xylose and Corn steep liquor ranged from 11.647 - 24.827 U/ml and 6.30 – 8.49UI/ml. The highest cellulase production was obtained at 8g/l xylose and 7%^{v}/v Corn steep liquor respectively while 2g/l and 9%^{v}/v of xylose and Corn steep liquor produced the least cellulase. On day 3 mycelia dry weight of *R. oryzae* under different concentrations of xylose and Corn steep liquor ranged from 0.01 – 0.07g and 0.04 – 0.11g in which 3g/l and 12 %^{v}/v xylose and Corn steep liquor supported the highest growth respectively. The least mycelia dry weight was at 4g/l and 6g/l of xylose and 10 %^{v}/v concentration of Corn steep liquor.

On day 5 Cellulase productions on different concentrations of xylose and Corn steep liquor ranged from 1.17 – 1.92U/ml and 1.82 – 2.80U/ml. The highest production was at 4g/l and 10%^{v}/v concentration of xylose and Corn steep liquor while 6g/l and 9%^{v}/v concentration of xylose and Corn steep liquor produced the least. On day 5 mycelial dry weight of *R. oryzae* under different concentrations of xylose and Corn steep liquor ranged from 0.01 – 0.05g and 0.04 – 0.10g. At 3g/l concentration of xylose and 9%^{v}/v concentration of Corn steep liquor, the highest mycelia dry weight was obtained respectively while 5g/l of xylose and 7 and 13 %^{v}/v concentration of Corn steep liquor produced the least mycelia dry weight.

On day 7, Cellulase production on different concentrations of xylose and Corn steep liquor by *R. oryzae* ranged from and 0.22 – 2.07U/ml and 0.25 - 1.48U/ml in which 7g/l of xylose and 12%^{v}/v Corn steep liquor supported the highest production while 4g/l of xylose and 7 %^{v}/v Corn steep liquor yielded the least production by *R. oryzae*. On day 7 mycelial dry weight of *R. oryzae* under different concentrations of xylose and Corn steep liquor ranged from 0.01 – 0.08g and 0.01 – 0.91g. The highest mycelia dry weight was recorded at 5g/l of xylose and 7%^{v}/v of Corn steep liquor respectively while 4 and 8g/l of xylose and 13 %^{v}/v of Corn steep liquor produced the least mycelia dry weight.

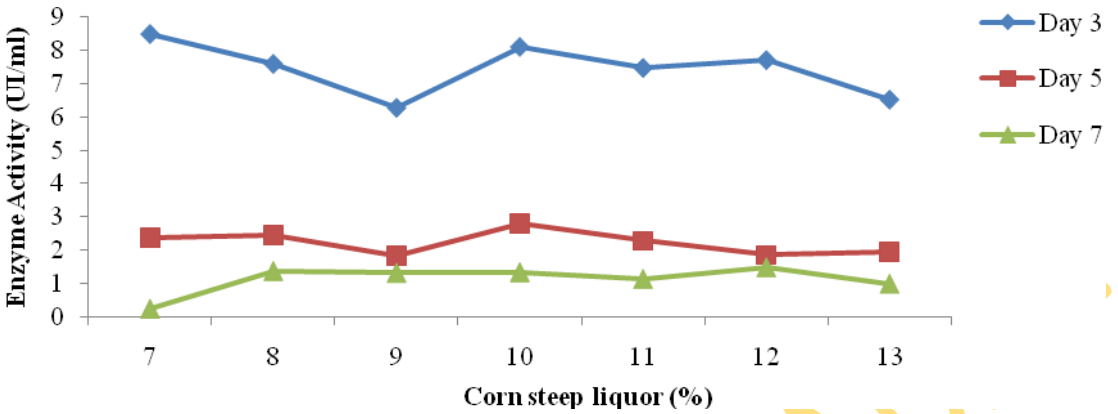


(i)

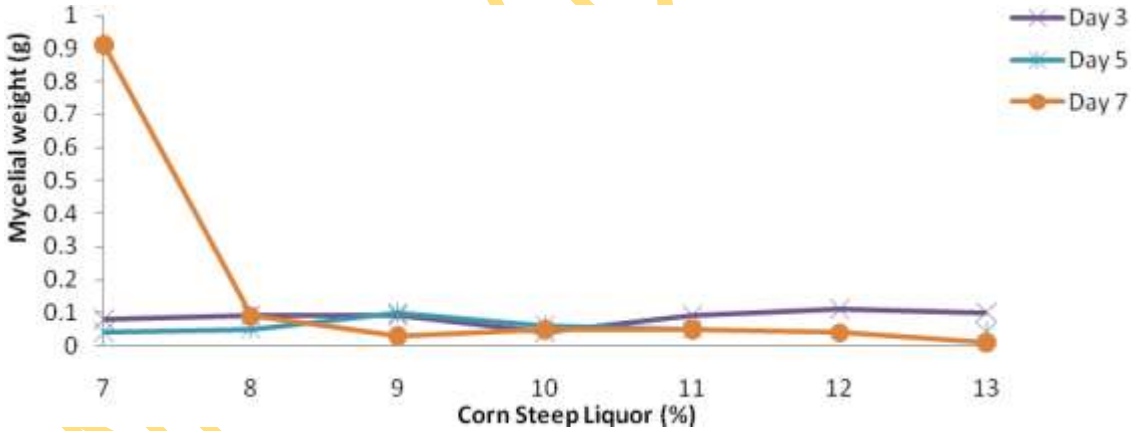


(ii)

Fig. 4.8a (i & ii): Cellulase production and Mycelia dry weight (g) of *R. oryzae* under different concentrations (g/l) of Xylose



(i)



(ii)

Fig.4.8b (i & ii): Cellulase production and Mycelia dry weight (g) of *R. oryzae* under different concentrations (%) of cornsteep liquor.

4.2.2.2.2. Cellulase production and mycelia dry weight of *R. oryzae* under different concentrations of KNO_3 and Urea

Cellulase production and mycelia dry weight of *R. oryzae* under different concentrations of KNO_3 and Urea (2 - 8g/l) respectively was investigated as reported in Figures 4.8c and 4.8d.

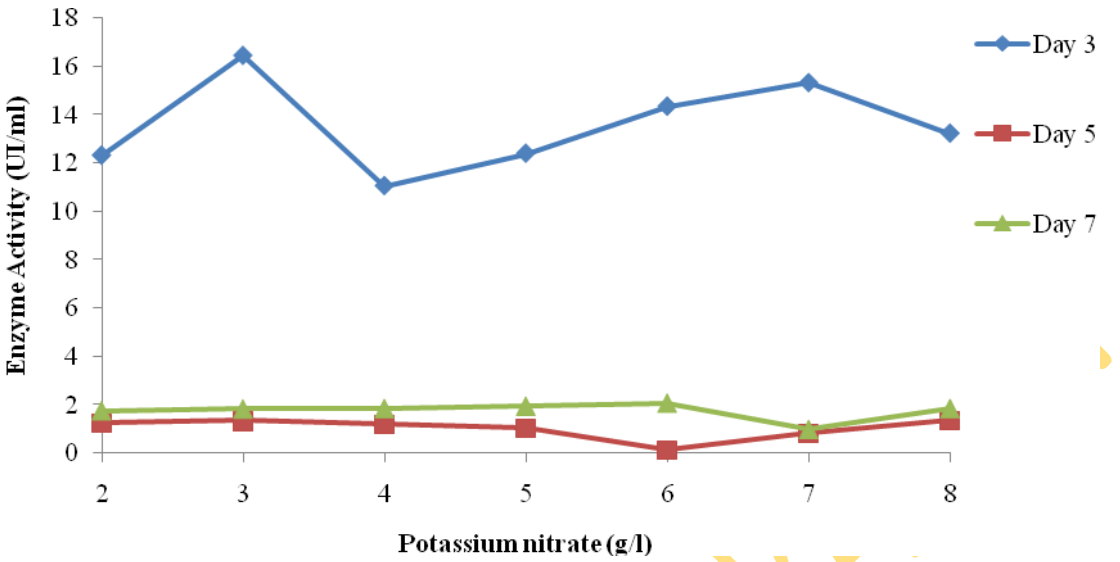
On day 3 Cellulase productions on different concentrations of KNO_3 and Urea ranged from 11.06 – 16.44U/ml and 1.23 – 13.23U/ml. At 3g/l KNO_3 and 5g/l Urea, the highest cellulase was produced respectively while 4g/l and 3g/l of KNO_3 and Urea supported the least. On day 3 mycelial dry weight of *R. oryzae* under different concentrations of KNO_3 and Urea ranged from 0.06 – 0.09g and 0.01 – 0.11g. At 7g/l of KNO_3 and Urea the highest mycelia dry weght was produced respectively while the production of the least mycelia dry weight was by 2, 3, 6 and 8g/l of KNO_3 and 2g/l concentration of Urea supported the least mycelia dry weight.

On day 5 Cellulase productions on different concentrations of KNO_3 and Urea ranged from 0.1 – 1.36U/ml and 0.96 – 1.48U/ml. The highest cellulase was produced at 8g/l and 2g concentration KNO_3 and Urea while 6g/l and 8g/l concentration of KNO_3 and Urea produced the least.

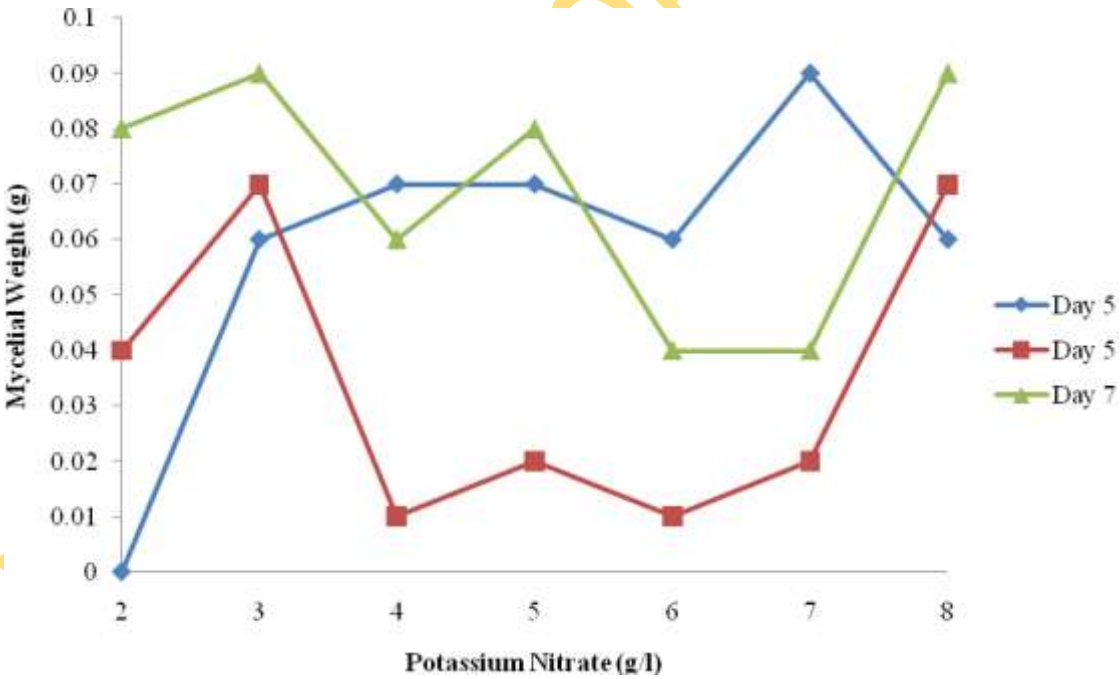
On day 5 mycelial dry weight of *R. oryzae* under different concentrations of KNO_3 and Urea ranged from 0.01 – 0.07g and 0.01 – 0.07g. At 3 and 8g/l of KNO_3 and 2g of Urea the highest mycelia dry weight was obtained while 6g/l of KNO_3 and 5 and 8g/l of Urea supported the least mycelia dry weight.

On day 7, Cellulase production on different concentrations of KNO_3 and Urea by *R. oryzae* ranged from and 0.98 – 2.08U/ml and 0.21 – 9.87U/ml in which 6g/l concentration of KNO_3 and 8g/l Urea supported the highest production. At 7g/l of KNO_3 and 7 g Urea the least cellulase was produced by *R. oryzae*.

On day 7 mycelial dry weight of *R. oryzae* under different concentrations of KNO_3 and Urea ranged from 0.04 – 0.09g and 0.01 – 0.08g in which 3 and 8g/l of KNO_3 and 4g/l of Urea supported the highest growth respectively. At 6 and 7g/l and KNO_3 and 3, 5 and 7g/l of Urea, the least mycelia dry weight was produced.

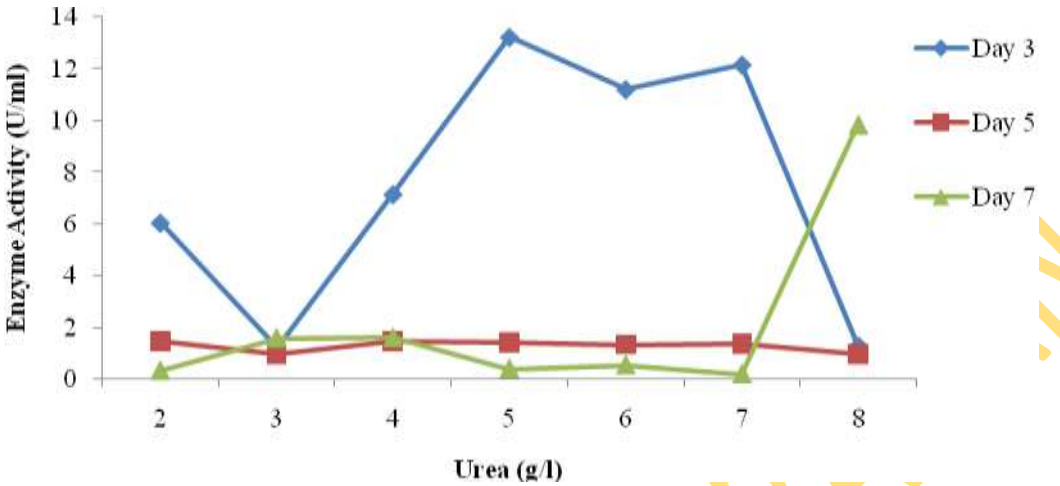


(i)

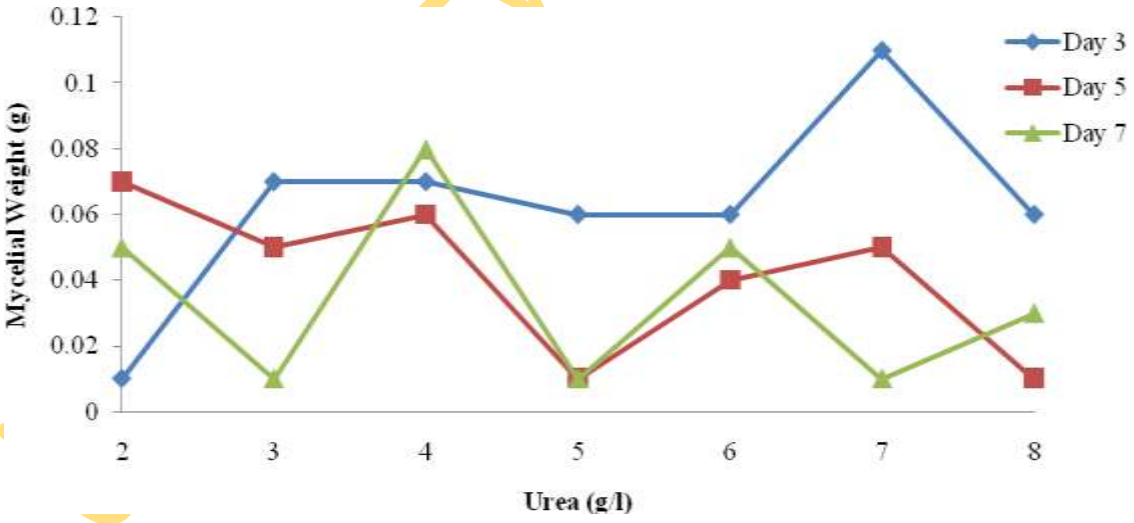


(ii)

Fig. 4.8c (i & ii): Cellulase production and Mycelia dry weight (g) of *R. oryzae* under different concentrations (g/l) of Potassium nitrate.



(i)



(ii)

Fig. 4.8d (i & ii): Cellulase production and Mycelia dry weight (g) of *R. oryzae* under different concentrations (g/l) of Urea.

4.2.2.2.3. Cellulase production and mycelia dry weight of *R. oryzae* under different pHs (3, 3.5, 4, 4.5, 5, 5.5 and 6)

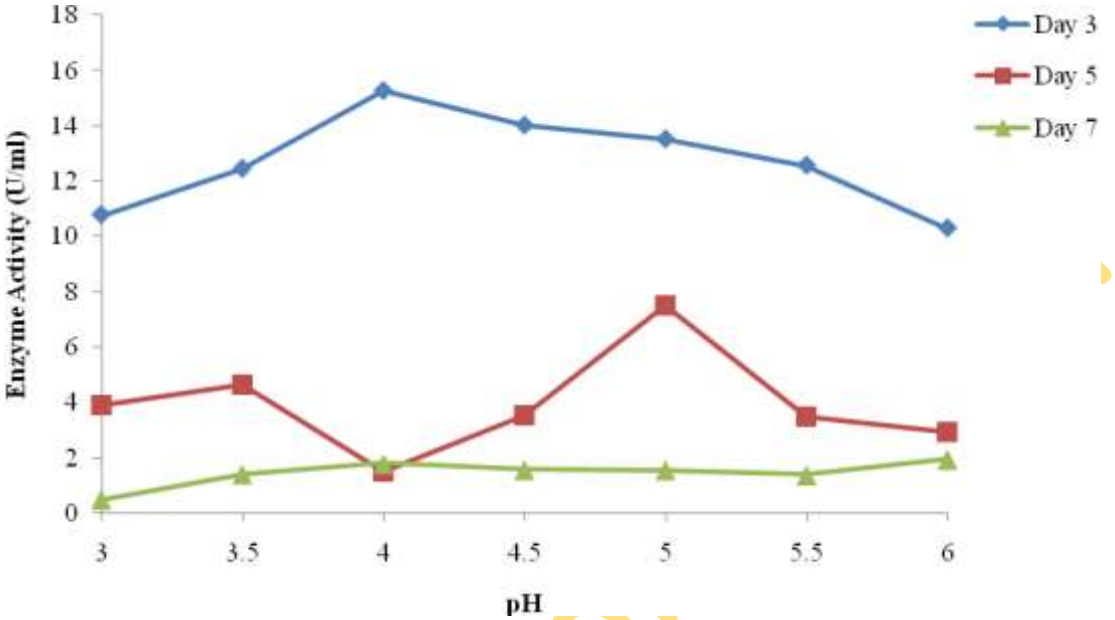
Cellulase production and mycelia dry weight of *R. oryzae* under different pHs is shown in Figures 4.8e (i & ii).

On day 3 cellulase productions at different pHs ranged from 10.292 - 15.268U/ml. pH 4 supported the highest production while pH 6 produced the least cellilase. On day 3 mycelial dry weight of *R. oryzae* under different pH ranged from 0.06 – 0.12g. pHs 3.5 and 4 supported the highest growth while pH 5.5 supported the least mycelia dry weight.

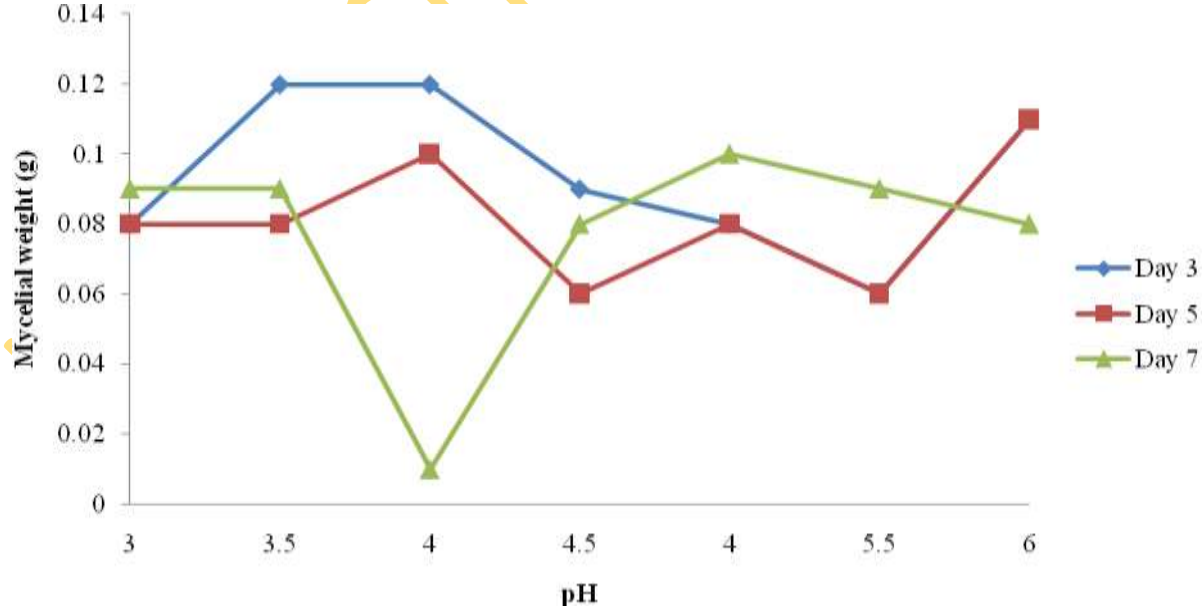
On day 5, cellulase production on different pHs ranged from 1.497 - 7.509UI/ml. pH 5 supported the highest production of cellulase. The least was at pH 4. On day 5 mycelial dry weight of *R. oryzae* under different pHs ranged from 0.06^{d} – 0.11^{a} g. pH 6 supported the highest growth while pHs 4.5 and 5.5 supported the least mycelia dry weight.

On day 7, cellulase production on different pH ranged from 0.490 - 1.936U/ml. pH 6 supported the highest production while at pH 3 the least cellulase was produced.

On day 7 mycelial dry weight of *R. oryzae* under different pHs ranged from 0.01 –0.10g. The highest mycelia dry weight was produced at pH 5 while pH 4 supported the least mycelia dry weight.



(i)



(ii)

Fig.4.8e (i & ii): Cellulase production and Mycelia dry weight (g) of *R. oryzae* under different pHs

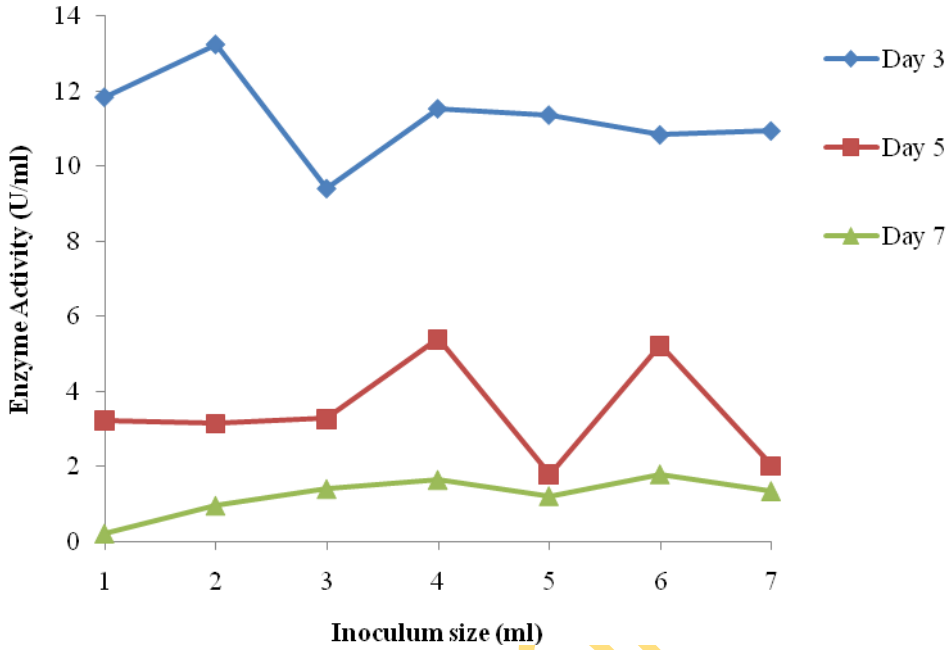
4.2.2.2.4. Cellulase production and mycelia dry weight of *R. oryzae* under different inoculum size (1 -7ml)

Cellulase production and mycelia dry weight of *R. oryzae* under different inoculum size is shown in Figures 4.8f (i & ii).

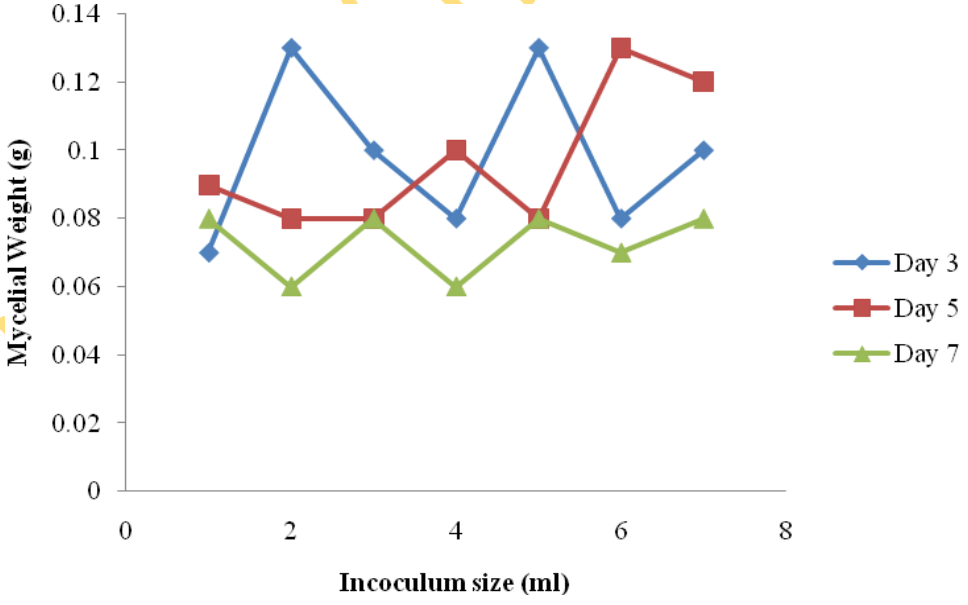
On day 3, cellulase production on different inoculum size ranged from 9.415 - 13.243U/ml in which 2ml Inoculum size supported the highest production. The least cellulase was produced by 3ml inoculums size. On day 3 mycelial dry weight of *R. oryzae* under different inoculum size ranged from 0.07 – 0.13g. With 2 and 5ml inoculum size, the highest growth was produced while 1ml inoculums load yielded the least mycelia dry weight.

On day 5, cellulase production on different inoculum size ranged from 1.775 - 5.403U/ml. The highest cellulase production was by 4ml inoculum size while 5ml inoculum size produced the least. On day 5 mycelial dry weight of *R. oryzae* under different inoculum size ranged from 0.08 – 0.13g in which 6ml inoculums size supported the highest growth while 2, 3 and 5ml inoculum size produced the least mycelia dry weight.

On day 7, cellulase production on different inoculum size ranged from 0.223 - 1.788U/ml. With 6ml inoculum size, the highest cellulase was produced while with 1ml inoculum size the least cellulase production was recorded. On day 7 mycelial dry weight of *R. oryzae* under different inoculum size ranged from 0.06 – 0.80g in which 1, 3, 5 and 7ml inoculum size produced the highest growth while 2 and 4ml inoculum size supported the least mycelia dry weight.



(i)



(ii)

Fig.4, 8f (i & ii): Cellulase production and Mycelia dry weight (g) of *R. oryzae* under different Inoculum size (ml).

4.2.2.2.5. Cellulase production and mycelia dry weight of *R. oryzae* under different temperatures (10 - 60°C)

Cellulase production and mycelia dry weight of *R. oryzae* under different temperatures is shown in Figures 4.8g (i & ii).

On day 3, cellulase production between 10 - 60°C ranged from 0.107 - 13.441U/ml. At 35°C the highest cellulase was produced while 10°C supported the least. On day 3 mycelial dry weight of *R. oryzae* under different temperatures ranged from 0.073 – 0.10g. The highest growth was recorded at 50°C while the least mycelia dry weight was produced at 12°C.

On day 5, cellulase production on different temperatures ranged from 0.043 - 5.880U/ml. At 35°C, the highest cellulase production was obtained while the least cellulase production was at 60°C. On day 5 mycelial dry weight of *R. oryzae* under different temperatures ranged from 0.07 – 0.14g in which 45 and 50°C supported the highest growth while the least mycelia dry weight was at 10, 35 and 60°C.

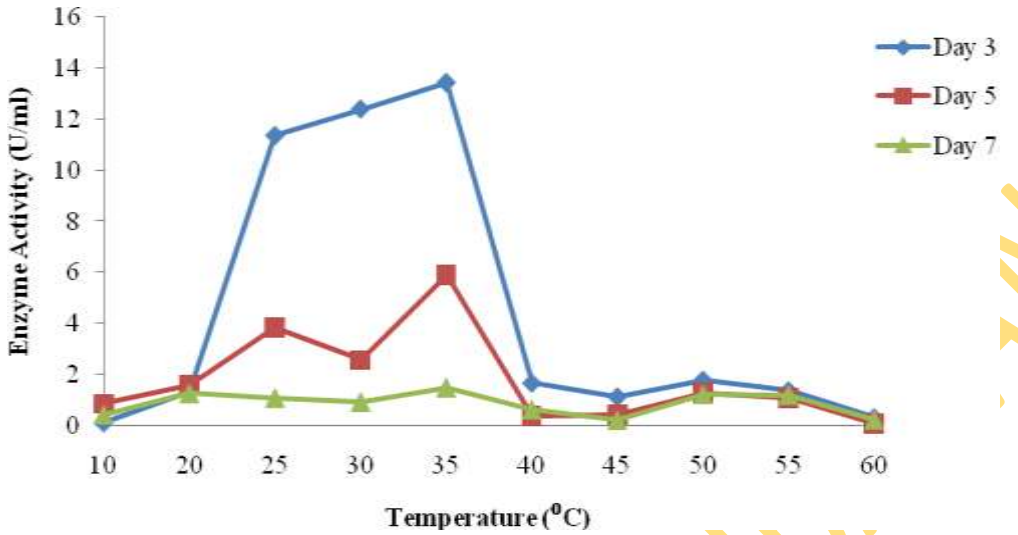
On day 7, cellulase production on different temperatures ranged from 0.193 - 1.474U/ml. The highest cellullase was produced at 35°C while the least cellulase production was at 45°C. On day 7 mycelial dry weight of *R. oryzae* under different temperatures ranged from 0.09 – 0.23g. The highest growth was at 55°C while the least mycelia dry weight was produced at 35°C.

4.2.2.2.6. Cellulase production and mycelia dry weight of *R. oryzae* under different incubation times (2 - 21 days)

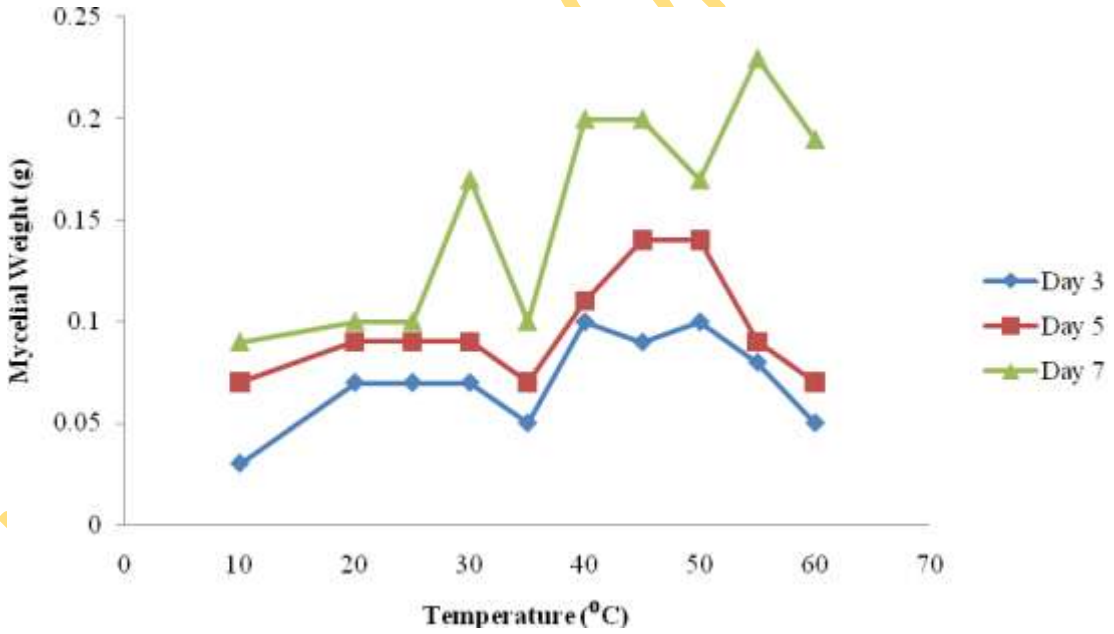
Cellulase production and mycelia dry weight of *R. oryzae* under different incubation times

is shown in Figures 4.8h (i & ii).

Effect of incubation time on cellulase production ranged from 0.984 - 11.168U/ml. The highest production of cellulase was at day 3 while at day 12 the least cellulase was produced. Mycelial dry weight of *R. oryzae* under different incubation times ranged from 0.4 – 0.20g. At day 21, the highest growth was produced while the least mycelia dry weight was produced at day 2

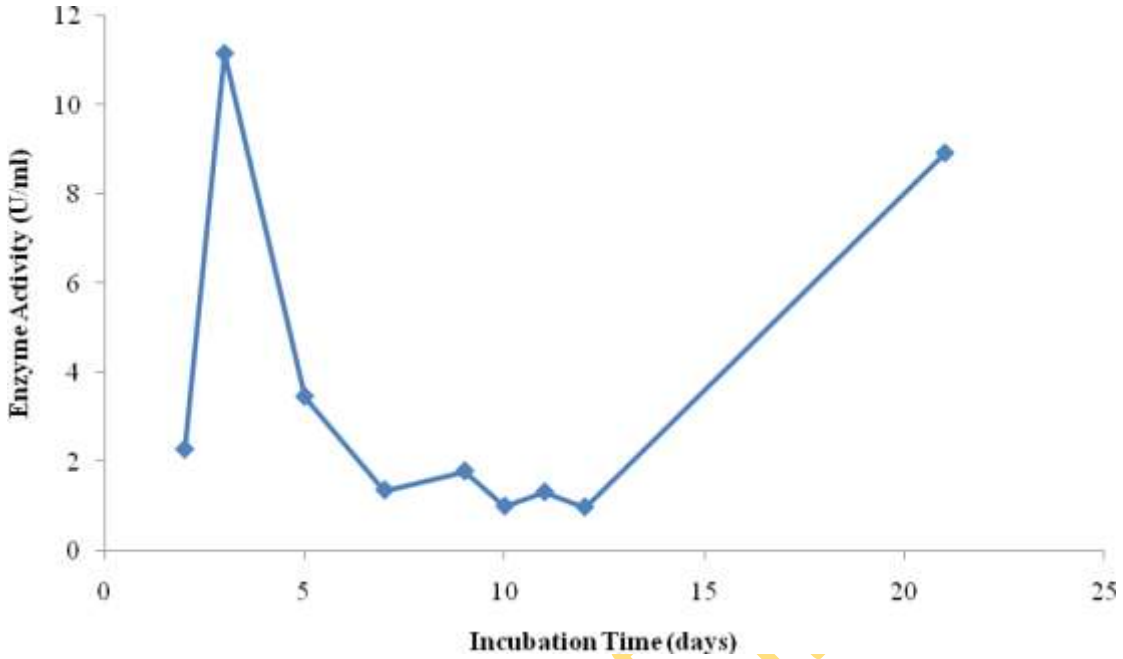


(i)

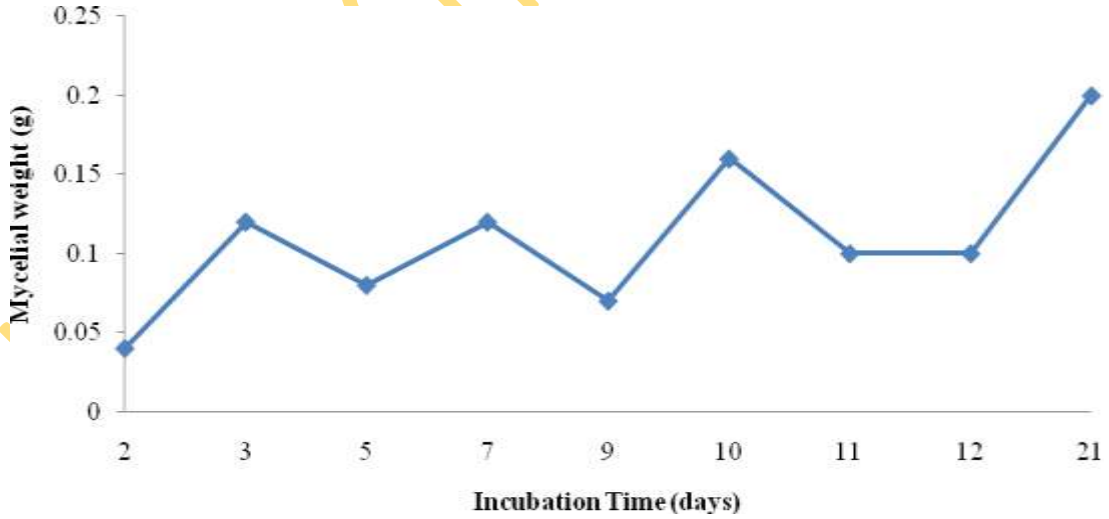


(ii)

Fig. 4.8g (i & ii): Cellulase production and Mycelia dry weight (g) by *R. oryzae* under different Temperatures (^{o}C).



(i)



(ii)

Fig. 4.8h (i & ii): Cellulase and Mycelia dry weight (g) of *R. oryzae* under different Incubation times (days).

4.2.2.3. Characterization of cellulase produced by *Rhizopus oryzae*

Characterization of *Rhizopus oryzae* cellulase activities under the following parameters - temperature, temperature stability, pH and pH stability, Cations ($CaCl_2$, $MgSO_4$, $MnSO_4$, KNO_3 and $NaCl_2$), Anions ($NaNO_3$, $FeSO_4$, $FeCl_2$, $CuSO_4$, and $CaCO_3$), Inhibitors (EDTA, HgCl, FeKCN, Benzoic acid and Urea), Time (minutes), Substrate (Starch) concentrations and Enzyme (Amylase) concentrations is recorded bellow

4.2.2.3.1. Effect of different incubation temperatures (10^oC – 60^oC) on enzyme activity and stability

Effect of different incubation temperatures (10^oC – 60^oC) on activity and stability of *R. oryzae* cellulase is shown in Figure 4.9 (a). The activities of the cellulase ranged from 0.300 - 1.257U/ml in which the highest was recorded at 10^oC while the least was at 30^oC. The stability of the enzyme activity produced by *R. oryzae* ranged from 0.208 - 1.530UI/ml in which 50^oC supported the highest activity while the least was recorded at 30^oC.

4.2.2.3.2. Effect different pHs (3 - 12) on enzyme activity and stability

Effect of different pHs (3 - 12) on activity and stability of *R. oryzae* cellulase is shown in Figure 4.9(b). The activity ranged from 1.683 - 4.415U/ml in which pH 8 supported the highest activity while the least was recorded at pH 4. *R. oryzae* cellulase stability ranged from 0.383 to 1.296UI/ml in which pH 8 supported the highest while the least was recorded at pH 7.

4.2.2.3.3. Effect of different cation concentrations (0.1M – 1M) on enzyme activity

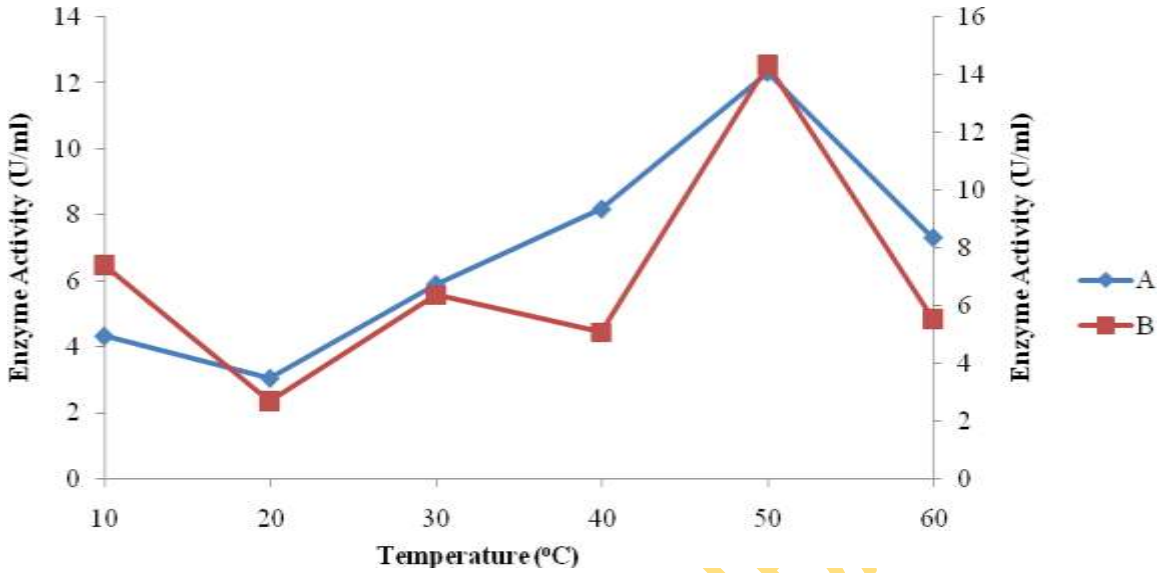
Effect of different concentrations (0.1M - 1M) of cations (CaCl, $MgSO_4$, $MnSO_4$, KNO_3 and $NaCl_2$) on activity of *R. oryzae* cellulase is shown in Figure 4.9(c).

The cellulase activity ranged from 0.20 - 6.90.195U/ml in which 0.7M NaCl had the highest while 0.4M $CaCl_2$ had the least.

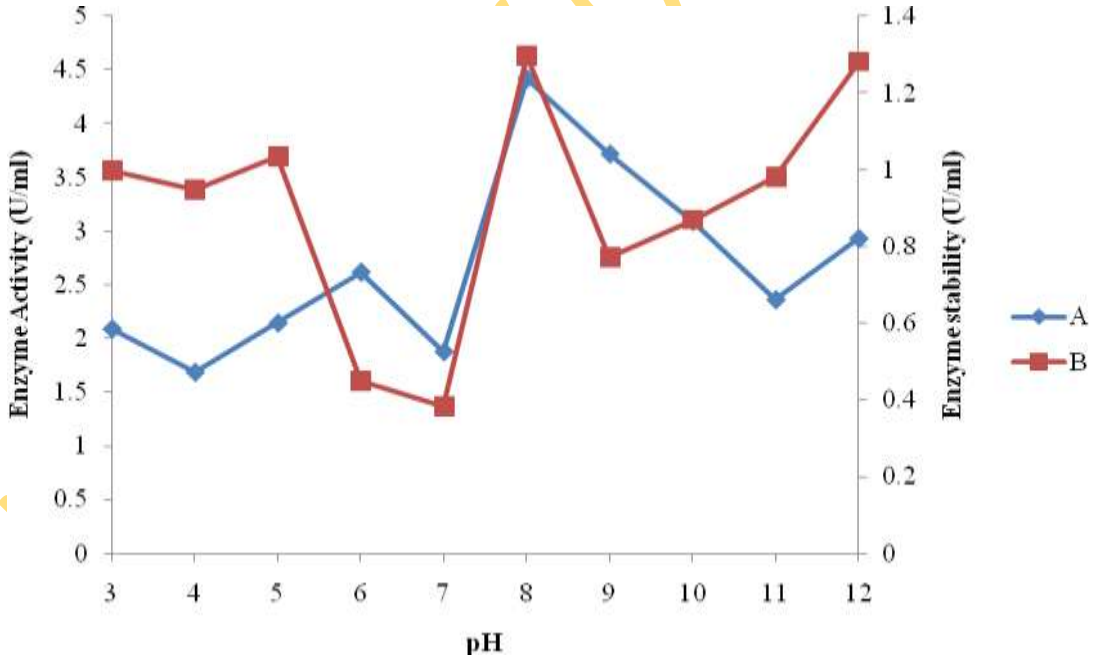
4.2.2.3.4. Effect of different anion concentrations on enzyme activity

Effect of different concentrations (0.1M - 1M) of Anions ($NaNO_3$, $FeSO_4$, $FeCl_2$, $CuSO_4$, and $CaCO_3$) on activity of *R. oryzae* cellulase investigated is shown in Figure 4.9 d).

The cellulase activity ranged from 0.01 - 3.44U/ml in which 0.5M $FeSO_4$ supported the highest while 0.1M $CuSO_4$ supported the least.



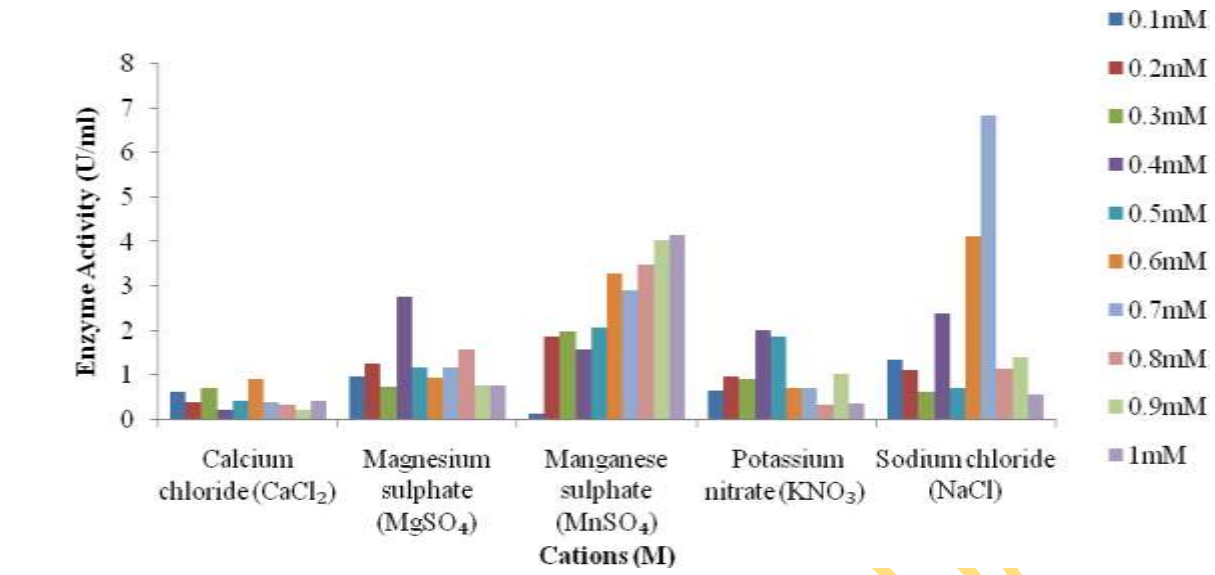
(a)



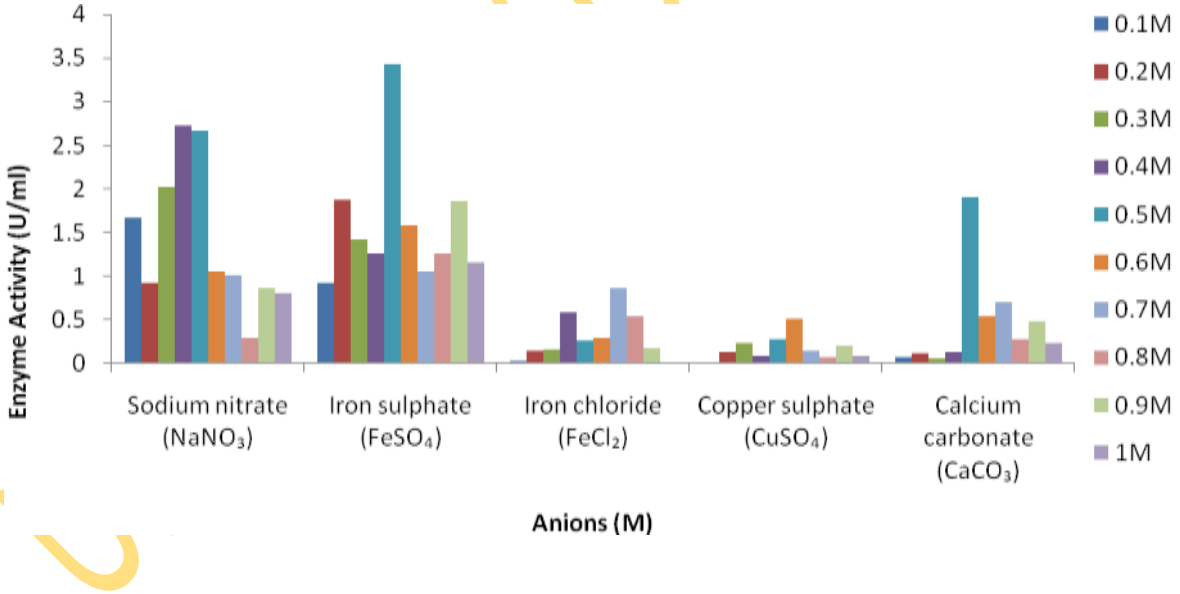
Key: A – Activity, B - Stability

(b)

Fig. 4.9 (a & b): Effect of different Temperatures and pHs on activity and stability of *R. oryzae* Cellulase



(c)



(d)

Fig.4.9(c & d): Effect of different cations and anions on activity of *R. oryzae* Cellulase.

4.2.2.3.5. Effect of different concentrations of inhibitors on enzyme activity

Effect of different concentrations (0.1M - 1M) of inhibitors (EDTA, HgCl, FeKCN, Benzoic acid and Urea) on activity of *R. oryzae* cellulase is shown in Figure 4.9 (e).

The cellulase activity ranged from 0.20 - 2.70U/ml in which 1M Urea induced the highest while 0.9M Benzoic acid supported the least.

4.2.2.3.6. Effect of different incubation times on enzyme activity

Effect of different incubation times (10 - 100min) on activity of *R. oryzae* cellulase is shown in Figure 4.9 (f). The cellulase activity ranged from 0.61 - 2.08U/ml in which the highest activity was recorded after 70min of incubation while the least was recorded after 40 min. of incubation.

4.2.2.3.7. Effect of substrate concentrations on cellulase activity

Effect of substrate concentrations (0.5 – 5.0% ^{w}/v) on activity of *R. oryzae* cellulase studied is shown in Figure 4.9 (g). The cellulase activity ranged from 0.1294 - 0. 61U/ml. The highest activity was recorded at 2.0% ^{w}/v substrate concentration. The least was recorded at substrate concentration of 5% ^{w}/v.

4.2.2.3.8. Effect of enzyme (cellulase) concentrations on cellulase activity

Effect of enzyme concentrations (1 – 10% ^{v}/v) on activity of *R. oryzae* cellulase studied is shown in Figure 4.9 (h). The cellulase activity ranged from 0.04 - 1.21U/ml in which the highest activity was recorded at enzyme concentration of 5% ^{v}/v while the least was recorded at enzyme concentration of 10% ^{v}/v.

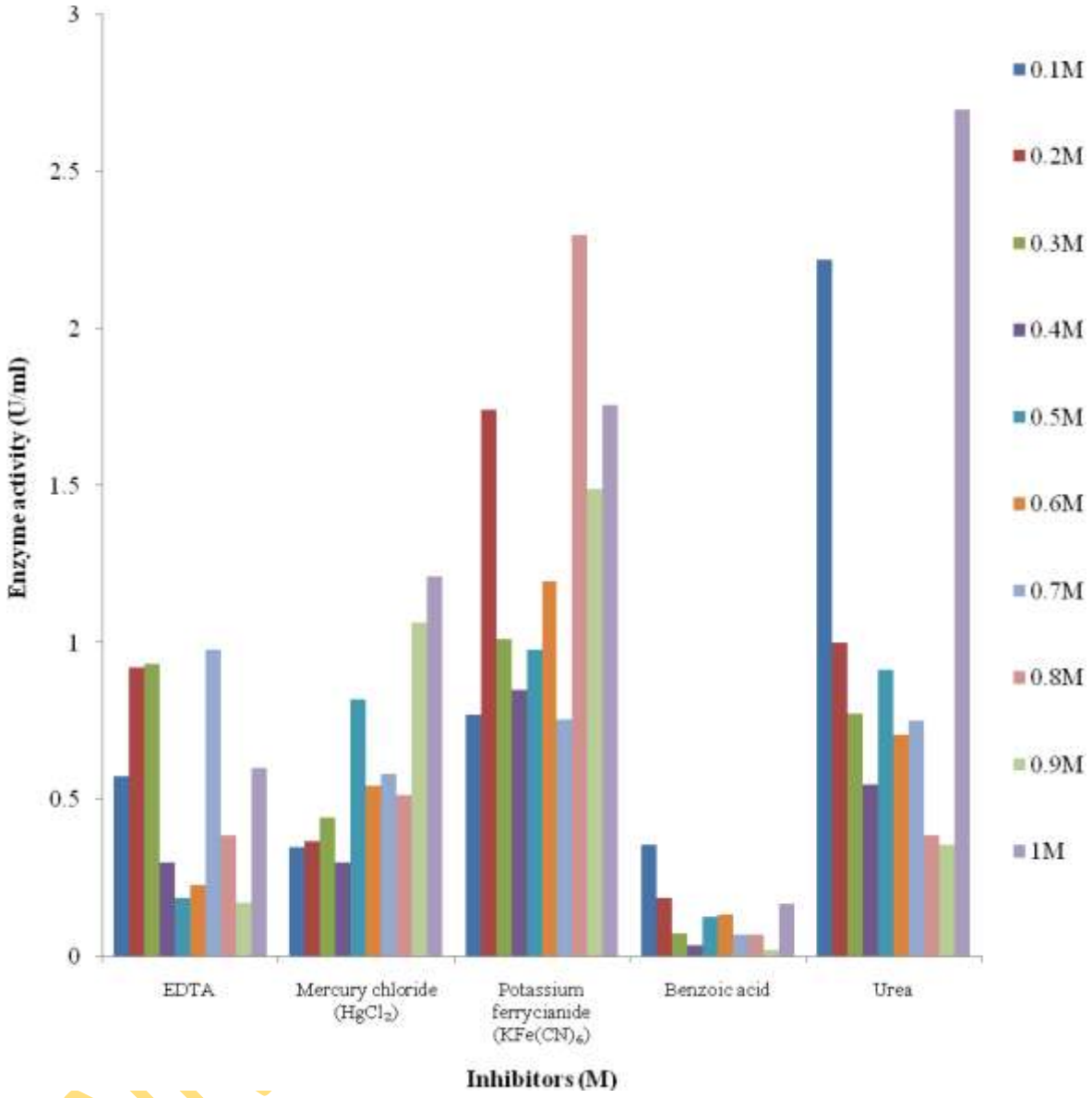


Fig. 4.9(e): Effect of different Inhibitors on the activity of *R. oryzae* Cellulase

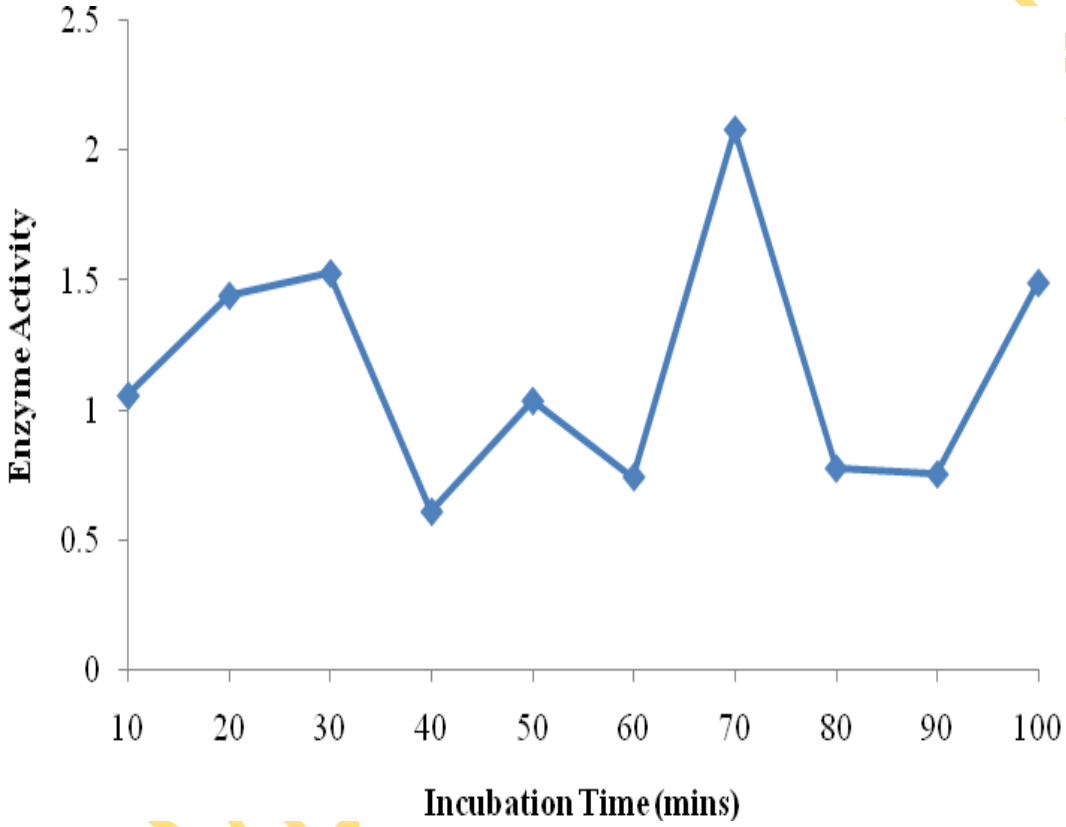


Fig. 4.9 (f): Effect of different Incubation times (mins) on activity of *R. oryzae* Cellulase.

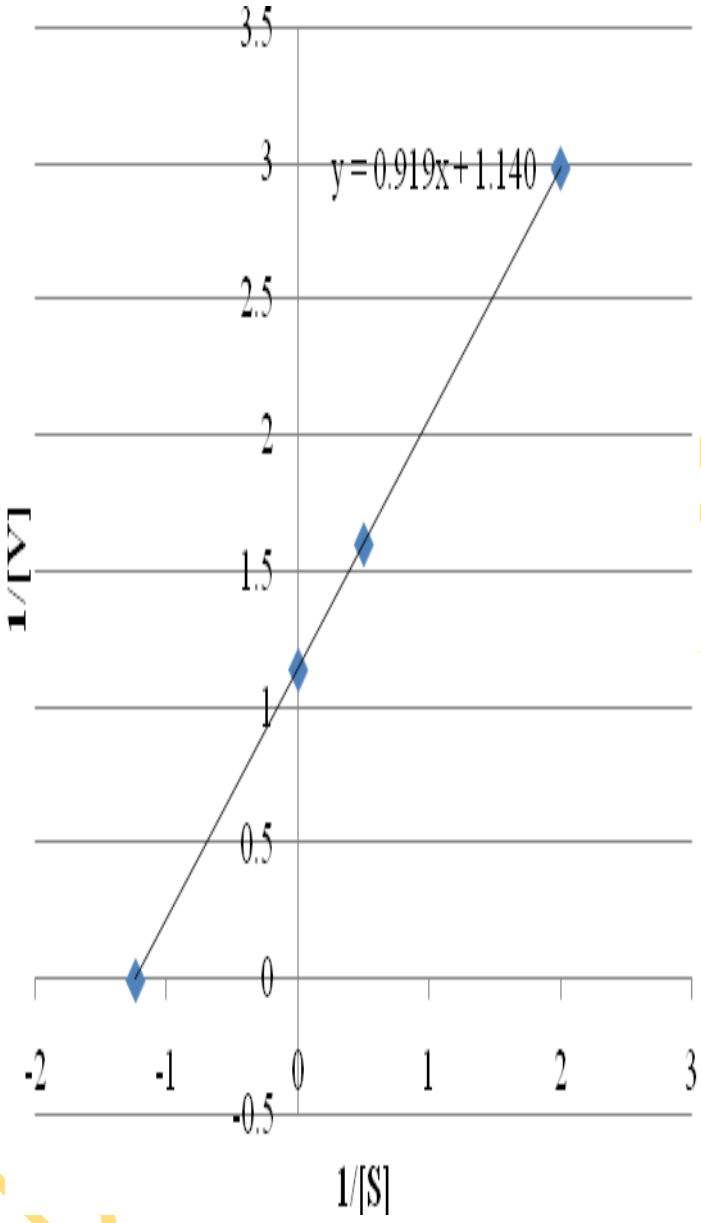


Fig. 4.9 (g): Characterization of Cellulase activities in different concentration of the substrates

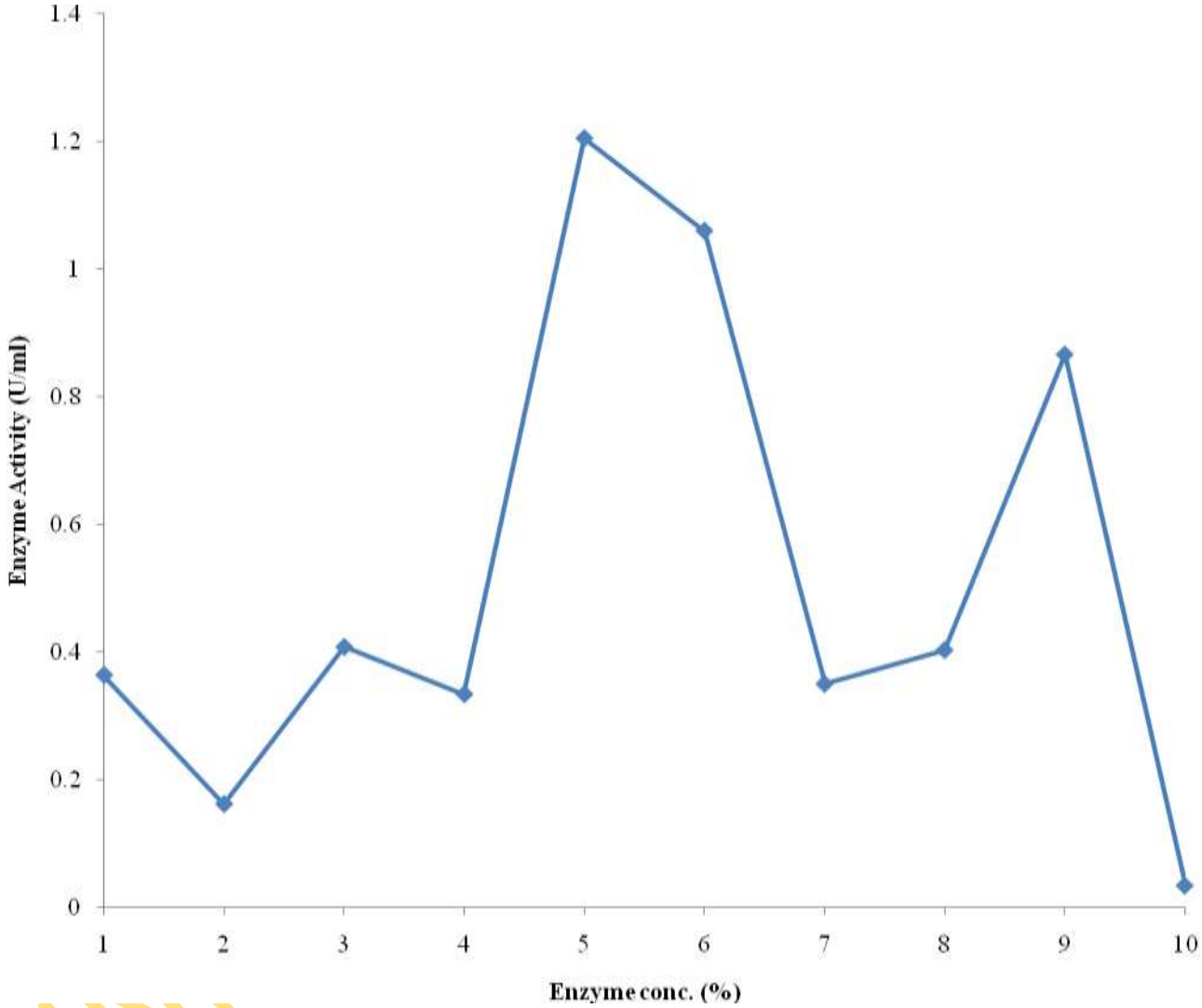


Fig. 4.9 (h): Characterization of Cellulase activities at its different concentrations of cellulase.

4.3. APPLICATION OF THE LIGNOCELLULOSIC SUBSTRATES IN FISH FEEDING PERFORMANCE CHARACTERISTICS

Table 4.7 shows the performance characteristics of local and Dutch *Clarias gariepinus* fed for 90 days with 7 different feed samples (Unfermented and Fermented Rice Bran (UFRB, FRB), and Palm Kernel Cake (UFPKC, FPKC), mixture of Unfermented and Fermented Rice Bran and Palm Kernel Cake (UFRB+PKC, FRB+PKC) and Fish Feed (FF) as the control feed).

The Mean Weight Gain (MWG) ranged from 0.08 – 1.80g in which fish fed with FF had the highest while the fish fed with UFRB had the least. The Feed Fed ranged from 0.08 – 0.18g. Fish fed with FF consumed 0.18g of feed, the highest, while the fish fed with FRB ate 0.08g, the lowest quatity of feed eaten. The Specific Growth Rate (SGR) ranged from 0.12 – 0.92 in which fish fed with FF had the highest of 0.92 while the fish fed with UFRB had 0.12, the least value of SGR. The Feed Conversion Ratio (FCR) ranged from 0.17 – 1.13 in which fish fed with UFRB had the highest while the fish fed UFPKC had the least. The Nitrogen metabolism ranged from 2.11 – 47.35 in which fish fed with FF had the highest while the fish fed with UFRB had the least. The Survival rate ranged from 23 – 67%. The fish fed with UFRB + PKC had the highest while the fish fed with FF had the least. The mortality rate ranged from 33 – 77%. Fish fed with FF had the highest, 77% while the fish fed with UFRB + PKC had the least mortality rate of 33%.

The performance characteristics of Dutch *Clarias gariepinus* fed for 90 days with the same sets of fish feed used to feed the local fish is also shown in Table 4.7 (a). The MWG ranged from 0.32 – 13.15g. Fish fed with FF had the highest with 13.15g while UFRB had the lowest of 0.32g. The amount of Feed Fed ranged from 8.04 – 23.92g in which fish fed with FF ate 23.92g, the highest while the fish fed with FPKC consumed 8.04g, the least amount of feed. The SGR ranged from 0.03 – 0.68 in which the fish fed with FF had the highest while the fish fed with UFRB had the least. The FCR ranged from 1.82 – 49.19 in which the fish fed with UFRB had the highest while the fish fed with FF had the least. Nitrogen metabolism ranged from 8.43 – 350.05 in which fish fed with FF had the highest while the fish fed with UFRB had the least. The Survival rate ranged from 6.67 – 33.3%. The fish fed with UFPKC had the highest survival rate of 33.3% while the fish fed with FRB and the fish fed with FF had the least.The Mortality rate ranged from 66.67 – 93.33% in which fish fed with FRB and fish fed with FF have the highest while the fish fed with UFPKC had the least.

Table 4.7 (a): Performance characteristics of Local and Dutch *Clarias gariepinus* (BURCHEL, 1822) fed with seven (7) different feed samples for 90 days

Parameters	Performance characteristics						
	RB		PKC		RB + PKC		FF
	UF	F	UF	F	UF	F	
Local*Claias gariepinus*							
Initial Weight (g)	0.39^{a*}	0.32^{b}	0.39a	0.28^{bc}	0.28^{bc}	0.28^{bc}	0.27^{bc}
Final Weight (g)	0.47^{e}	0.49e	0.85^{c}	0.96^{b}	0.77^{d}	0.70^{d}	2.07^{a}
Mean Weight Gain (g)	0.08f	0.17e	0.53^{c}	0.68^{b}	0.49^{c}	0.42^{d}	1.80^{a}
Feed fed (g)	0.09^{cd}	0.08^{d}	0.09^{bcd}	0.12^{b}	0.11^{bc}	0.09^{bcd}	0.18^{a}
Specific Growth Rate	0.12^{g}	0.25^{f}	0.32^{e}	0.61^{b}	0.48^{c}	0.45^{d}	0.92^{a}
Feed Conversion Ratio	1.13^{a}	0.47^{a}	0.17^{c}	0.18^{c}	0.23^{c}	0.19^{c}	1.10^{a}
Nitrogen Metabolism	2.11^{g}	4.48^{f}	24.06^{b}	18.01^{c}	12.91^{d}	11.07^{e}	47.35^{a}
Survival Rate	60^{b}	57^{c}	33^{e}	57^{c}	67^{a}	47^{d}	23^{f}
Mortality Rate	40^{e}	43^{d}	67^{b}	43^{d}	33^{f}	53^{c}	77^{a}
Dutch *Claias gariepinus*							
Initial Weight (g)	3.15^{a*}	1.86^{e}	1.76^{g}	1.88^{d}	1.82^{f}	2.24^{b}	1.98^{c}
Final Weight (g)	3.47^{d}	2.57^{e}	3.44^{d}	2.40^{f}	3.97^{c}	5.47^{b}	14.57^{a}
Mean Weight Gain (g)	0.32^{g}	0.71^{e}	1.68^{d}	0.52^{f}	2.15^{c}	3.21^{b}	13.15^{a}
Feed fed (g)	15.73^{c}	8.91^{f}	12.83^{e}	8.04^{g}	13.16^{d}	16.35^{b}	23.92^{a}
Specific Growth Rate	0.03^{e}	0.22^{d}	0.29^{c}	0.19^{d}	0.34^{c}	0.40^{b}	0.68^{a}
Feed Conversion Ratio	49.14^{a}	12.55^{c}	7.63^{d}	15.45^{b}	6.12^{e}	5.10^{f}	1.82^{g}
Nitrogen Metabolism	8.43^{g}	35.15^{e}	44.90^{d}	30.62^{e}	57.25^{c}	85.56^{b}	350.05^{a}
Survival Rate	16.67^{c}	6.67^{e}	33.33^{a}	23.33^{b}	13.33^{d}	13.33^{d}	6.67^{e}
Mortality Rate	83.33^{c}	93.33^{a}	66.67^{e}	76.67^{d}	86.67^{b}	86.67^{b}	93.33^{a}

*Valeus with the same superscripts are not significant different using Duncan's Multiple Range Test

Table 4.7 (b) shows the performance characteristics of Dutch *Clarias gariepinus* fed for 90 days with Unfermented and Fermented Rice Bran (UFRB and FRB) and Palm Kernel Cake (UFPKC and FPKC) substituted in the fish feed. Fish Feed (FF) was used as the control feed. It also shows the performance characteristics of Dutch *Claris gariepinus* fed with feed substituted with starter fermented and unfermented substrates for 90 days.

The MWG ranged from 15.28 – 31.46g. The fish fed with FF had 31.46g, the highest while the fish fed with FRB had 15.28g, the least. The Feed Fed ranged from 27.37 – 54.35g in which the fish fed with UFPKC consumed 54.35g, the highest while the fish fed with FRB ate 27.37g, the least. The SGR ranged from 0.67 – 1.21 in which the fish fed with FF had the highest while the fish fed with UFRB had the least. Nitrogen metabolism ranged from 411.14 – 964.04. The fish fed with UFPKC had the highest value while the fish fed with FRB had the least. The Survival Rate (%) ranged from 23.33 – 70%. The fish fed with FF had the highest survival rate of 70% while the fish fed with UFRB had the least with 23.33%. The Mortality Rate ranged from 30 – 76.67% in which UFRB had the highest while FF had the least.

The performance characteristics of Dutch *Clarias gariepinus* fed for 90 days with fish feed substituted with RB and PKC fermented with starter cultures is also shown in Table 4.7 (c). The MWG ranged from 12.84 - 43.30g. The fish fed with 10%PKC had 43.30g, the highest while 12.84g, the least was in the fish fed with 10%RB. The Feed Fed ranged from 29.70 - 59.90g in which the fish fed with 10%PKC had 59.90g, the highest while 29.70g, the least was in the fish fed with 20%PKC. The SGR ranged from 0.79 - 1.33 in which the fish fed with 10%PKC ranked highest with1.33 while 0.79, the least was in the fish fed with 10%RB. The Feed Convertion Ratio (FCR) ranged from 1.38 - 3.18 in which the fish fed with 10%RB had 3.18, the highest while 1.38, the least was in the fish fed with 10%PKC. Nitrogen metabolism ranged from 331.18 - 1117.14. The fish fed with 10% PKC had 1117.14, the highest while the fish fed with 10%RB had 331.18, the least. Survival Rate ranged from 19.09 - 30.00% in which the fish fed with 20%PKC had 30%, the highest while the fish fed with FF had 19.09%, the least. Mortality Rate ranged from 70 - 80.91% in which FF had the highest while 20%PKC had the least.

Table 4.7 (b): Performance characteristics of Dutch *Clarias gariepinus* fed for 90 days with feed substituted with unfermented and spontaneously fermented substrates.

Parameters	Performance characteristics				
	RB		PKC		FF
	UF	F	UF	F	
Initial Weight (g)	2.77^{c*}	3.01^{b}	2.59^{d}	3.14^{a}	2.15^{f}
Final Weight (g)	21.15^{d}	16.75^{e}	38.28^{a}	28.36^{c}	33.60^{b}
Mean Weight Gain (g)	18.93^{d}	15.28^{e}	35.84^{a}	25.21^{c}	31.46^{b}
Feed fed (g)	36.61^{d}	27.37^{e}	54.35^{a}	47.64^{c}	51.18^{b}
Specific Growth Rate	0.67^{c}	0.72^{c}	1.17^{a}	0.80^{b}	1.21^{a}
Feed Conversion Ratio	1.93^{a}	1.79^{c}	1.52^{e}	1.89^{b}	1.63^{d}
Nitrogen Metabolism	509.33^{d}	411.14^{e}	964.04^{a}	678.26^{c}	846.22^{b}
Survival Rate	23.33^{d}	43.33^{b}	43.33^{e}	33.33^{c}	70^{a}
Mortality Rate	76.67^{a}	56.67^{c}	56.66^{e}	66.67^{b}	30^{d}

* Values with same superscripts are not significantly different using Duncan's Multiple Range Test.

Table 4.7 (c): Dutch *Clarias gariepinus* fed for 90days with feed substituted with fungal fermented substrates.

	RB		PKC		FF
	10%	20%	10%	20%	
Initial Weight (g)	2.74[a*]	2.46[b]	2.37[c]	1.96[e]	1.98[d]
Final Weight (g)	15.58[e]	20.89[c]	45.67[a]	18.95[d]	24.58[b]
Mean Weight Gain (g)	12.84[e]	18.42[c]	43.30[a]	16.99[d]	22.60[b]
Feed fed (g)	40.85[c]	36.79[d]	59.90[a]	29.70[e]	44.21[b]
Specific Growth Rate	0.79[d]	1.00[c]	1.33[a]	1.01[c]	1.19[b]
Feed Conversion Ratio	3.18[a]	2.00[b]	1.38[e]	1.75[d]	1.95[c]
Nitrogen Metabolism	331.18[e]	475.17[c]	1117.14[a]	438.39[d]	583.15[b]
Survival Rate %	25[b]	25[b]	25[b]	30[a]	19.09[c]
Mortality Rate %	75[b]	75[b]	75[b]	70[c]	80.91[a]

* Values with same superscripts are not significantly different using Duncan's Multiple Range Test.

Table 4.8 shows the levels of Anti nutrient factors (percentage) and the levels of Vitamins and Sugars in the different feed samples.

The Alkaloid contents ranged from 18.60 - 36.50% in which PKC fermented with Starter had the highest while UFPKC had the least. Oxalate contents ranged from 18.24 - 32.10% in which UFPKC had the highest while FPKC had the least. Phytate ranged from 0.17 - 0.22% in which UFPKC had the highest while PKC fermented with Starter had the least. Tannin contents ranged from 0.14 - 0.45% in which FPKC had the highest while the least was detected in PKC fermented with Starter. Phytic acid ranged from 0.18 - 0.45% in which UFPKC had the highest while the least was detected in RB fermented with Starter. Trypsin Inhibitor ranged from 8.18 - 10.30% in which UFRB had the highest while UPKC had the least. Cyanogenic glycoside (mg/g) ranged from 8.18 - 10.12 in which FPKC had the highest while UFPKC had the least. Hemaglutinin Hg (unit/mg) ranged from 0.026 - 0.117 in which FRB had the highest while RB fermented with Starter had the least.

Vitamin C ranged from 0.0010 - 0.004 µmol/l in which UFPKC ranked highest while the least was FPKC. Thiamine ranged from 14.40 - 18.02 in which RBS fermented with starter produced the highest while the least was detected in FPKC. Riboflavin contents ranged from 0.221 - 0.412 in which RB fermented with Starter had the highest while FPKC had the least. Total sugar ranged from 24.20 - 66.29 g/l in which RB fermented with Starter had the highest with 66.29 while UFPKC had the least. The Non Reducing Sugar ranged from 23.32 - 65.68mg/ml in which RB fermented with Starter had the highest while UPKC had the lowest. The Reducing Sugar ranged from 0.32 - 0.88 mg/ml in which UPKC had the highest while URB had least.

Table 4.9 shows the toxin level in the fungal isolates. Mycotoxin was not detected in any of the isolates except in *A. tamari* in trace. The percentage (%) Aflatoxin ranged from 0.010 - 0.24 in which *A. versicolor* produced the greatest while *A. flavus* and *A. terreus* produced the least. Fumosin (%) ranged from 0.12 - 0.22 in which *Rhizopus* sp. and *A. tamarii* contained the highest while the least was detected in *R. oryzae*. The level of Alkaloid (%) ranged from 0.32 - 0.46 in which *A. terreus and Trichoderma sp.* produced the highest while the least was produced in *A. niger*. The Oxalate (%) level ranged from 16.12 - 22.16 in which *A. clavus* had the highest while *A. tamarii* had the least.

Table 4.8: Anti-nutrition factors, Vitamins and Sugar contents in the different feed sampls fed to *Clarias gariepinus* in the course of the experiment.

Parameters	RB			PKC		
	UF	F	+ Starter	UF	F	+ Starter
Antinutrient factors						
%Alkaloid	22.40^{b*}	32.00^{a}	31.50^{a}	18.60^{b}	28.80^{a}	36.50^{q}
% Oxalate	24.12^{a}	22.16^{b}	20.32^{b}	32.10^{a}	18.24^{b}	21.14^{b}
% Phytate	0.20^{e}	0.21^{f}	0.20^{e}	0.22^{f}	0.18^{f}	0.17^{e}
% Tannin	0.22^{e}	0.30^{e}	0.23e	0.15^{f}	0.45^{e}	0.14^{ef}
% Phytic acid	0.24^{e}	0.26^{f}	0.18e	0.45^{e}	0.20^{f}	0.20^{e}
% Trypsin Inhibitor	10.30^{c}	9.84^{d}	10.20^{c}	10.10^{c}	8.90^{d}	9.60^{d}
Cyanogenic glycoside (mg/g)	10.14^{d}	10.16^{c}	8.40^{d}	8.18^{d}	10.20^{c}	10.12^{c}
Hemaglutinin U/mg	0.104^{f}	0.117^{g}	0.026^{f}	0.065^{g}	0.052^{g}	0.065^{f}
Vitamins and Sugar						
Vitamin C (µmol/l)	0.0032^{b*}	0.0012^{d}	0.0024^{c}	0.0041^{a}	0.0010^{d}	0.0034^{b}
Thianin (µg/l)	17.89^{b}	16.32^{d}	18.02^{a}	14.42^{e}	14.40^{e}	16.58^{c}
Riboflavin (µg/l)	0.339^{c}	0.344^{b}	0.412^{a}	0.242^{d}	0.221^{f}	0.232^{e}
Total Sugar (g/l)	36.80^{d}	34.50^{e}	66.20^{a}	24.20^{f}	48.20^{b}	38.60^{c}
Non-ReducingSugar (mg/ml)	36.48^{d}	33.88^{e}	65.68^{a}	23.32^{f}	47.72^{b}	38.11^{c}
ReducingSugar(mg/ml)	0.32^{e}	0.62^{b}	0.56^{c}	0.88^{a}	0.48^{d}	0.49^{d}

* Values with same superscripts are not significantly different using Duncan's Multiple Range Test.

Table 4:9: Toxin concentration in the different fungal isolates from different lignocellulosic samples.

Parameters	Toxin level				
	Mycotoxin	Aflatoxin (%)	Fumosin (%)	Alkaloid (%)	Oxalate (%)
Aspergillus.*niger*	0.00	0.014^{d*}	0.17^{b}	0.32^{f}	22.12^{b}
Aspergillus flavus	0.00	0.010^{f}	0.16^{bc}	0.38^{c}	20.14^{f}
Aspergillus terreus	0.00	0.010^{f}	0.14^{cd}	0.46^{a}	21.16^{e}
Aspergillus clavatus	0.00	0.012^{e}	0.17^{b}	0.36^{d}	22.16^{a}
Aspergillus versicolor	0.00	0.024^{a}	0.13^{d}	0.38^{c}	22.04^{d}
Rhizopus oryzae	0.00	0.018^{b}	0.12^{d}	0.42^{b}	18.24^{h}
Rhizopus stolonifer	0.00	0.016^{c}	0.13^{d}	0.34^{e}	22.08^{c}
Rhizopus sp.	0.00	0.012^{e}	0.22^{a}	0.42^{b}	18.42^{g}
Aspergillus tamarii	Trace	0.012^{e}	0.22a	0.43^{b}	16.12^{i}
Trichoderma sp	0.00	0.014^{d}	0.18b	0.46^{a}	18.24^{h}

* Values with same superscripts are not significantly different using Duncan's Multiple Range Test.

Table 4.10 shows the quantitative analysis of minerals and metals (µg/g) in the feed samples. The Magnesium (mg) contents of the feed samples are not significantly different. The Calcium (Ca) contents ranged from 3.00 – 29.00µg/g in which the control feed (FF) had the highest while FRB had the least. Zinc (Zn) values ranged from 470.00 - 520.60µg/g in which the mixture of fermented RB+PKC had the highest while UFRB had the least. The Iron (Fe) contents ranged from 78.00 - 140.00µg/g in which UFRB had the highest while UFPKC had the least. The Potassium (K) contents ranged from 6.00 – 35.00µg/g in which the control feed had the highest while FRB had the least. The Copper (Cu) contents ranged from 20.00 - 170.00µg/g in which UFPKC had the highest while FPKC had the least. Heavy metals (Cadmum, Lead and Cobolt) were not detected in the feed samples

4.3.2. WATER QUALITY PARAMETERS

Tables 4:11 (a) to 4.11 (c): show the water quality parameters of the medium (water) in which the local and the Dutch *Clarias gariepinus* were reared and fed with the different types of feed sample (Unfermented and Fermented Rice Bran (UFRB, FRB), Palm Kernel Cake (UFPKC, FPKC), the mixture of the two substrats (UFRB+PKC, FRB+PKC), feed substituted with the substrates fermented with starters i.e, RB was fermented with *A. clavatus* and PKC was fermented with *A. tamarii*).

The pH ranged from 7.33 – 7.86 in which the highest was obtained with the fish fed with UFRB+PKC and FRB+PKC while the least was obtained with the fish fed with the control fish feed on tables 4.15b and 4.15d respectively. The temperature ranged from 24.42±0.75 - 46 .04±0.94^{o}C in which FRB+PKC had the highest while UFRB had the least (Tables 4.15b and 4.15a). The dissolved oxygen ranged from 2.33± 0.69 – 8.75± 1.79 mg/l in which 10%RB had the highest while the control feed had the least (Tables 4.15d and 4.15b). Conductivity ranged from 187.50±4.79 – 430.00±62.07µS/cm in which the feed control (Table4.15c) had the highest while the feed control (Table 4.15d) had the least.

Table 4:10: Quantitative analysis of minerals and metals (µg/g) in the feed samples

Feed Samples	Metals and Minerals Composition (µg/g)								
	Mg	Ca	Zn	Fe	K	Cu	Cd	Pb	Co
UF RB	8.80^{a*}	4.90^{d}	470.00^{b}	140.00^{a}	7.00^{d}	40.00^{c}	0.00	0.00	0.00
FRB	8.90^{a}	3.00^{e}	520.00^{a}	108.00^{a}	6.00^{e}	22.00^{cd}	0.00	0.00	0.00
UFPKC	8.60^{a}	11.50^{b}	510.00^{a}	78.00^{c}	14.00^{b}	170.00^{a}	0.00	0.00	0.00
FPKC	8.80^{a}	11.80^{b}	520.50^{a}	94.00^{b}	13.00^{bc}	20.00^{cd}	0.00	0.00	0.00
Fish Feed	8.58^{a}	29.00^{a}	520.30^{a}	127.00^{a}	35.00^{a}	74.00^{b}	0.00	0.00	0.00
UFRB + PKC	8.80^{a}	8.00^{c}	520.10^{a}	100.00^{a}	11.00^{cd}	128.00^{ab}	0.00	0.00	0.00
FRB + PKC	8.90^{a}	8.50^{c}	520.60^{a}	86.00^{bc}	12.00^{c}	102.00^{ab}	0.00	0.00	0.00

*Values with the same superscripts are not significantly different using Duncan's Multiple Range Test

Key: UF = Unfermented, F = Fermented, RB = Rice Bran, PKC = Palm Kernel Cake, 0.00 = Absent, Mg=Magnesium, Ca = Calcium, Zn = Zinc, Fe = Iron, K = Potassium, Cu = Copper, Cd = Cadmium, Pb = Lead and Co = Cobolt.

Table 4:11 (a):- Water quality parameters of the Local and Ductch *Clarias gariepinus* fed with seven (7) different feed samples

	Water quality							
	RB		PKC		RB + PKC		FF	Water
Parameters	U	F	U	F	U	F		
Local *Clarias gariepinus*								
pH	7.74^{a*}±0.15	7.79^{a}±0.13	7.54^{a}±0.20	7.70^{a}±0.14	7.75^{a}±0.13	7.75^{a}±0.15	7.66^{a}±0.12	6.96^{b}±0.05
Water Temperature ^{o}C)	25.17^{a}±0.93	25.25^{a}±0.93	25.25^{a}±0.93	25.24^{a}±0.93	25.32^{a}±0.92	46.04^{a}±0.94	24.85^{a}±0.90	23.20^{a}±0.74
Dissolved Oxygen (mg/l)	2.94^{a}±0.51	3.20^{a}±0.49	3.29^{a}±0.55	3.99^{a}±0.64	3.36^{a}±0.56	3.38^{a}±0.67	2.83^{a}±0.95	2.76^{a}±0.63
Conductivity (µS/cm)	218.00^{b}±25.55	226.00^{b}±24.60	222.00^{b}±18.00	317.00^{a}±31.20	265^{b}±23.54	288.90^{ab}±31.61	377.00^{a}±52.87	262.00^{b}±20.10
Ductch *Clariasgariepinus*								
pH	7.71^{a}±0.03	7.81^{a}±0.04	7.63^{a}±0.13	7.83^{a}±0.09	7.86^{a}±0.08	7.86^{a}±0.08	7.21$^{b\pm}$±0.13	6.96^{c}±0.05
Water Temperature (^{o}C)	24.42^{a}±1.07	25.00^{a}±1.18	25.17^{a}±1.12	24.71^{a}±0.75	25.14^{a}±0.77	25.86^{a}±0.51	25.71^{a}±0.61	25.20^{a}±1.16
Dissolved Oxygen (mg/l)	3.95^{a}±1.12	4.07^{a}±0.79	3.31^{a}±0.68	3.26^{a}±0.559	3.90^{a}±0.70	2.80^{a}±0.70	2.33^{a}±0.69	1.58^{a}±0.63
Conductivity (µS/cm)	370.00^{a}±68.70	244.29^{b}±26.71	270.00^{ab}±50.29	197.14^{c}±23.64	294.29^{ab}±42.25	285.71^{ab}±9.48	205.71b±28.52	228.00^{b}±20.59

The values with the same letters are not significantly different

Table 4.11 (b): Water quality parameters of Dutch *Clarias gariepinus* fed with fish feed substituted with fermented and unfermented RB and PKC.

Parameters	Water quality					
	RB		PKC			
	U	F	U	F	FF	Water
pH	$7.37^{a}\pm0.08$	$7.40^{a}\pm0.08$	$7.43^{a}\pm0.06$	$7.40^{a}\pm0.06$	$7.53^{a}\pm0.10$	$7.14^{b}\pm0.11$
Water Temperature (^{o}C)	$26.60^{a}\pm1.02$	$26.54^{a}\pm1.02$	$26.47^{a}\pm1.03$	$26.26^{a}\pm0.71$	$26.40^{a}\pm1.04$	$23.58^{a}\pm0.66$
Dissolved Oxygen (mg/l)	$5.55^{a}\pm1.07$	$4.83^{a}\pm0.69$	$4.97^{a}\pm0.55$	$4.77^{a}\pm0.65$	$2.84^{a}\pm1.00$	$5.50^{a}\pm1.14$
Conductivity (µS/cm)	$300.00^{ab}\pm32.66$	$322.86^{ab}\pm56.30$	$298.57^{ab}\pm28.99$	$344.29^{ab}\pm35.04$	$430.00^{a}\pm62.07$	$206.00^{b}\pm32.80$

*Values with same superscripts are not significantly different using Duncan's Multiple Range Test.

Table 4.11 (c): Water quality parameters and that of Dutch *C.gariepinus* fed with fish feed substituted with RB and PKC fermented with starter cultures.

	RB		PKC			
	10%	20%	10%	20%	FF	Water
pH	7.35^{a}±0.05	7.43^{a}±0.03	7.40^{a}±0.08	7.40^{a}±0.08	7.33^{ab}±0.05	7.15^{b}±0.07
Water Temperature (^{o}C)	26.08^{a}±0.74	25.93^{a}±0.78	27.75^{a}±0.48	27.00^{a}±0.41	26.85^{a}±0.64	26.25^{a}±0.85
Dissolved Oxygen (mg/l)	8.75^{a}±1.79	6.98^{a}±0.33	7.84^{a}±0.67	7.53^{a}±0.21	7.56^{a}±0.02	6.18^{a}±1.02
Conductivity (µS/cm)	220.00^{ab}±10.80	223.75^{ab}±8.51	217.50^{ab}±13.15	207.50^{b}±12.50	187.50^{b}±4.79	260.00^{a}±30.28

*Values with same superscripts are not significantly different using Duncan's Multiple Range Test.

Table 4.12: shows the proximate composition of all the different feeds used in the feeding trials. The Dry matter (%) ranged from 68.15 – 95.90 in which the UFRB+PKC had the highest while the RB fermented with a starter culture had the least. The Moisture content ranged from 4.10 – 31.85% in which the RB fermented with a starter culture had the highest while UFRB+PKC had the least. The Ash content ranged from 3.30 – 16.80% in which FRB had the highest while the PKC fermented with a starter culture had the least. The crude fat ranged from 4.50 – 23.85% in which the RB fermented with a starter culture had the highest while the UFRB had the least. Protein values ranged from 6.5 -52.27% in which the control feed (FF) had the highest while RB fermented with a starter culture had the least. The crude fibre ranged from 3.00 – 24.70% in which the UFRB had the highest FF had the least. The Nitrogen Free Extract (NFE) ranged from4.04 – 49.98% in which UFPKC had the highest while the RB fermented with a starter culture had the least. The carbohydrate content ranged from 25.85 – 64.84% in which UFRB had the highest while the RB fermented with a starter culture had the least.

Table 4.13 (a) shows the proximate composition of the local and Dutch *Clarias gariepinus* fed with 7 different feed samples. In the local fish, the Dry Matter ranged from 16.40 - 29.20% in which UFPKC had the highest while FPKC the least. The moisture contents ranged from 70.80 - 83.60% in which FPKC had the highest while UFPKC had the least. The Ash content ranged from 1.33 - 3.00% in which FRB had the highest while UFPKC had the least. The Crude fat ranged from 3.88 - 7.20% in which FRB had the highest while UFRB had the lowest. The crude Protein ranged from 8.68 - 20.83% in which UFPKC had the highest while FPKC had the lowest. The crude Fiber ranged from 0.30 - 0.90% in which UFRB+PKC had the highest followed by UFPKC while FF and FRB have the lowest. The Nitrogen Free Extract (NFE) ranged from 0.01 to 0.09% in which FF had the highest followed by UFPKC while UFRB and FRB+PKC were the lowest. The Carbohydrate content ranged from 0.33 - 0.92% in which UFRB+PKC had the highest, followed by UFPKC while UFRB had the lowest.

In the Dutch *Clarias gariepinus* fed with 7 different feed samples. The Dry Matter ranged from 17.52 - 24.10% in which FF had the highest followed by UFRB while the least was recorded in UFPKC. The Moisture content ranged from 75.90 - 82.48% in which UFPKC had the while the least was recorded in FF. The Ash contents ranged from 0.60 - 1.35% in which UFRB+PKC had the highest followed by FRB+PKC while FF had the least. Crude Fat ranged from 3.42 - 4.65% in which FF had the highest followed by UFPKC while the least was

recorded in FRB. The crude Protein ranged from 5.58 - 17.0%7 in which FF had the highest while FPKC had the least. The crude Fibre contents were not significant different from each other. NFE ranged from 0.88 - 3.10 in which UFPKC had the highest while UFRB had the least. The Carbohydrate contents ranged from 0.60 - 4.00 in which UFPKC had the highest while UFRB had the lowest.

Table 4:12: Proximate Composition (%) of the different feed used in the feeding trias

Parameters	Proximate Composition (%)								
	RB			PKC			RB + PKC		FF
	UF	F	Starter	UF	F	Starter	UF	F	
Dry Matter	95.00^{e*}	95.45^{cd}	68.15^{g}	95.60^{b}	95.35^{d}	70.70^{f}	95.90^{a}	95.85^{a}	95.50^{bc}
Moisture Content	5.00^{c}	4.55^{de}	31.85^{a}	4.40^{e}	4.65^{d}	29.30^{b}	4.10^{f}	4.15^{f}	4.50^{de}
Ash Content	16.10^{b}	16.80^{a}	11.95^{c}	4.60^{g}	4.40^{h}	3.30^{i}	10.00^{e}	11.15^{d}	8.05^{f}
Crude Fat	4.50^{i}	10.00^{f}	23.85^{a}	14.30^{d}	8.05^{g}	20.84^{b}	15.60^{c}	7.40^{h}	12.15^{e}
Crude Protein	9.56^{h}	12.13^{g}	6.50^{i}	19.12^{e}	26.10^{b}	17.86^{f}	19.85^{d}	20.22^{c}	42.27a
Crude Fibre	24.70^{a}	23.70^{b}	21.80^{c}	7.60^{h}	7.90^{g}	11.50^{f}	16.80^{e}	19.50^{d}	3.00^{i}
NFE	40.14^{c}	32.82^{f}	4.05^{i}	49.98^{a}	48.90^{b}	17.26^{h}	33.65^{e}	38.58^{d}	30.03^{g}
Carbohydrate	64.84^{a}	56.52^{e}	25.85^{i}	57.58^{c}	56.80^{d}	28.76^{h}	50.45^{f}	58.08^{b}	33.03^{g}

*Values with the same superscripts are not significantly different using Duncan's Multiple Range Test.

Table 4:13 (a): Proximate Composition of Local and Dutch *Clarias gariepinus* (Burtchell, 1822) fed with different seven (7) feed samples.

Parameters	Proximate Composition (%)						
	RB		PKC		RB + PKC		FF
	UF	F	UF	F	UF	F	
Local *Clarias gariepinus*							
Dry Matter	20.60^{e*}	28.58^{b}	29.20^{a}	16.40^{g}	20.28^{f}	26.01^{c}	25.20^{d}
Moisture Content	79.40^{c}	71.42^{f}	70.80^{g}	83.60^{a}	79.72^{b}	73.99^{e}	74.80^{d}
Ash Content	2.09^{d}	3.00^{a}	1.33^{f}	2.14^{c}	2.17^{b}	2.99^{a}	1.49^{e}
Crude Fat	4.91^{e}	7.20^{a}	6.38^{b}	5.03^{d}	3.88^{f}	5.97^{c}	6.02^{c}
Crude Protein	13.19^{e}	18.05^{b}	20.83^{a}	8.68^{f}	13.31^{d}	17.29^{c}	17.30^{c}
Crude Fibre	0.40^{cd}	0.30^{d}	0.60^{b}	0.50^{bc}	0.90^{a}	0.51^{bc}	0.30^{d}
NFE	0.01^{d}	0.03^{c}	0.06^{b}	0.05^{b}	0.02^{cd}	0.01^{d}	0.09^{a}
Carbohydrate	0.41^{e}	0.33^{g}	0.66^{b}	0.55^{c}	0.92^{a}	0.52^{d}	0.39^{f}
Dutch *Clarias gariepinus*							
Dry Mater	23.08^{b*}	20.97^{d}	17.52^{g}	21.78^{c}	18.01^{f}	20.03^{e}	24.10^{a}
Moisture Content	76.92^{f}	79.03^{d}	82.48^{a}	78.22^{e}	81.99^{b}	79.97^{c}	75.90^{g}
Ash Content	0.90^{b}	0.90^{b}	0.95^{b}	0.85^{b}	1.35^{a}	1.20^{a}	0.60^{c}
Crude Fat	3.85^{e}	3.42^{g}	4.41^{b}	3.60^{f}	4.12^{c}	3.98^{d}	4.65^{a}
Crude Protein	6.70^{e}	13.36^{b}	8.16^{d}	5.58^{f}	8.16^{d}	11.50^{c}	17.07^{a}
Crude Fibre	0.75^{d}	0.95^{bc}	0.90^{bc}	0.65^{e}	1.10^{a}	1.00^{b}	0.80^{cd}
NFE	0.88^{e}	2.34^{b}	3.10^{a}	1.05^{c}	2.35^{b}	2.35^{b}	0.98^{d}
Carbohydrate	0.60^{e}	3.29^{c}	4.00^{a}	1.70^{d}	3.45^{b}	3.35^{c}	1.78^{d}

*Values with the same supersripts are not significantly different using Duncan's Multiple Range Test.

Table 4.13 (b): shows the proximate composition of Dutch *Clarias gariepinus* fed with fish feed substituted with spontaneously fermented substrated and that of Dutch fish fed with feed substituted with the substrates fermented with starter cultures. In the fish fed with feed substituted with unfermented and spontaneously fermented substrates, the Dry Matter ranged from 24.24 - 27.10 in which UFRB had the highest while UFPKC had the least. Moisture contents ranged from 72.90 - 75.80 in which UFPKC had the highest while UFRB had the least. The Ash content ranged from 0.45 - 0.90 in which UFPKC had the highest while FRB and FF had the least. Crude Fat content ranged from 2.20 - 3.40 % in which FF had the highest while UFRB had the least. Crude Protein content ranged from 18.64 - 22.44 % in which UFRB had the highest while FF the least. The NFE ranged from 0.30 - 1.23 % in which FRB had the highest while FPKC had the least. The Carbohydrate contents ranged from 1.33 - 2.11 in which FF had the highest while FRB had the least.

In the Dutch *Clarias gariepinus* fed with feed substituted with the substrate fermented with stater cultures, its Dry Matter ranged from 29.19 – 30.95 in which the fish fed with feed substituted with 20% PKC had the highest while the fish fed with 10% PKC had the least. The Moisture content ranged from 69.05 – 70.80 in which the fish fed with 10% PKC had the highest while the fish fed with 20% PKC had the least. The Ash content ranged from 1.06 – 1.08 in which the fish fed with 10% PKC had the highest while the fish fed with the FF had the least. Crude fat ranged from 4.48 – 5.32 in which the fish fed with 10%RB had the highest while the fish fed with 20% PKC had the least. The crude protein ranged from 22.17 – 24. 49 in which the fish fed with feed substituted with 20%PKC had the highest while the fihe fish fed with feed substituted with 10%PKC had the least. The crude fibre ranged from 0.35 – 0.84 in which the fish fed with FF had the highest while the fish fed with the feed substituted with 10% PKC had the least. The NFE ranged from 0.20 – 0.70 in which the fish fed with the feed substituted with 20% PKC had the highest while the fish fed with the feed substituted with 10%RB had the least. The carbohydrate ranged from 0.70 – 1.15 in which the fish fed wiyh the feed substituted with 20% PKC had the highest while the fish fed with the feed substituted with 10% RB had the least.

Table 4:13 (b): Proximate Composition (%) Dutch *Clarias gariepinus* (Burtchell, 1822) fed with feed substituted with the unfermented and spontaneously fermented substrates

Parameters	Proximate composition (%)				
	RB		PKC		FF
	UF	F	UF	F	FF
Dry Matter	27.10^{a*}	25.50^{b}	24.24^{e}	24.40^{d}	24.60^{c}
Moisture Content	72.90^{e}	74.50^{d}	75.80^{a}	75.60^{b}	75.40^{c}
Ash Content	0.85^{b}	0.45^{d}	0.90^{a}	0.75^{c}	0.45^{d}
Crude Fat	2.20^{d}	2.80^{b}	2.35^{d}	2.65^{c}	3.40^{a}
Crude Protein	22.44^{a}	20.92^{b}	19.02^{d}	19.40^{c}	18.64^{e}
Crude Fibre	1.00^{c}	1.10^{bc}	1.20^{ab}	1.30^{a}	1.20^{ab}
NFE	0.61^{d}	1.23^{a}	0.73^{c}	0.30^{e}	0.91^{b}
Carbohydrate	1.61^{c}	1.33^{d}	1.93^{b}	1.60^{c}	2.11^{a}

*Values with the same supersripts are not significantly different using Duncan's Multiple Range Test.

Table 13 (c) shows the proximate composition (%) of all the fish samples fed in all the feeding trials. The Dry Matter ranged from 16.40 – 30.95 in whicxh the fish fed with 20% PKC fermented with a starter culture had the highest while the fish fed with FPKC had the least. The Moisture content ranged from 69.05 – 83 60 in which the fish fed with FPKC had the highest while the fish fed with 20%PKC fermented with a starter culture had the least. Ash content ranged from 0. 45 – 3.00 in which fish fed with FRB had the highest while the fish fed with feed substituted with FRB had the least.The crude fat values ranged from 2.20 – 7.20 in which the local fish fed with FRB had the highest while the fish fed with feed substituted with URB had the least. The crude protein ranged from 8.16 – 24.49 in which the fish fed with feed substituted with 20% PKC fermented with a starter culture had the highest while the Dutch fed with UFPKC as well as the Dutch fed with UFRB+PKC had the least. The fibre content ranged from 0.30 – 1.3 in which the fish fed with feed substituted with RPKC had the highest while the local fish fed with FRB and FF had the least. The NFE ranged from 0.01 – 3.10 in which the Dutch fish fed with UFPKC had the highest while the local fish fed with UFRB, UFRB+PKC and FRB+PKC had the least. The carbohydrate content ranged from 0.33 – 4.00 in which the Dutch fed with UFPKC had the highest while the local fish fed with FB had the least.

Table 4:13 (c): Proximate Composition (%) of Dutch *Clarias gariepinus* (Burtchell, 1822) fed with the substrates fermented with Starter Cultures (RB fermented with *A. clavatus*) and PKC (fermented with *A. tamarii* respectively)

	RB		PKC		FF
	10%	20%	10%	20%	
Dry matter	29.94^{c*}	30.00^{c}	29.19^{d}	30.95^{a}	30.50^{b}
Moisture Content	70.06^{b}	70.00^{b}	70.81^{a}	69.05^{d}	69.50^{c}
Ash Content	1.08^{a}	1.07^{ab}	1.07^{ab}	1.07^{ab}	1.06^{c}
Crude Fat	5.32^{a}	4.90^{c}	5.23^{b}	4.48^{e}	4.85^{d}
Crude Protein	22.84^{c}	22.51^{d}	22.17^{e}	24.49^{a}	23.50^{b}
Crude Fibre	0.50^{b}	0.45^{c}	0.35^{d}	0.38^{cd}	0.84^{a}
NFE	0.20^{d}	0.70^{a}	0.37^{c}	0.53^{b}	0.25^{d}
Carbohydrate	0.70^{c}	1.15^{a}	0.72^{c}	0.91^{b}	1.09^{a}

*Values with the same superscripts are not significantly different using Duncan's Multiple Range Test.

Table 4:13 (d): Proximate Composition (%) of the fish samples fed in the four (4) feeding trials

	PROXIMATE COMPOSITION (%)																							
	RB								PKC								RB +PKC				FF			
	UF		F		Substituted		+Starter		UF		F		Substituted		+Starter		UF		F		1	2	3	4
	1	2	1	2	UF	F	10%	20%	1	2	1	2	UF	F	10%	20%	1	2	1	2				
Dry Matter	20.60^{p*}	23.08^{m}	28.58^{e}	20.97^{o}	27.10^{f}	25.50^{h}	29.94^{c}	30.00^{c}	29.20^{d}	17.52^{t}	16.40^{u}	21.78^{n}	24.20^{i}	24.40^{k}	29.19^{d}	30.95^{c}	20.28^{q}	18.01^{s}	26.01^{g}	20.03^{r}	25.20^{i}	24.10^{l}	24.60 l	30.50 b
Moisture Content	79.40^{f}	76.92^{i}	71.42^{q}	79.03^{g}	72.90^{p}	74.50^{n}	70.06^{s}	70.00^{s}	70.80^{r}	82.48^{b}	83.60^{a}	78.22^{h}	75.80^{j}	75.60^{k}	70.81^{r}	69.05^{u}	79.72e	81.99^{c}	73.99^{o}	79.97^{d}	74.80 m	75.90^{j}	75.40 l	69.50 t
Ash Content	2.09^{c}	0.90^{gh}	3.00^{a}	0.90^{gh}	0.85^{ghi}	0.45^{j}	1.08^{efg}	1.07efg	1.33^{de}	0.95^{gh}	2.14c	0.85^{gh}	0.90^{gh}	0.75^{hi}	1.07^{efg}	1.07^{efg}	2.99^{b}	1.35^{d}	2.99^{a}	1.20^{ef}	1.49^{d}	0.60^{ij}	0.45^{j}	1.06^{fg}
Crude Fat	4.91^{gh}	3.85^{m}	7.20^{a}	3.42^{o}	2.20^{s}	2.80^{p}	5.32^{e}	4.90^{fg}	6.38b	4.41^{j}	5.03^{f}	3.60^{n}	2.35^{r}	2.65q	5.23^{e}	4.48^{j}	5.17^{e}	4.12^{k}	5.17^{e}	3.98^{l}	6.02^{c}	4.65^{i}	3.40^{o}	4.85^{h}
Crude Protein	13.19^{q}	16.70^{p}	18.05^{k}	13.36^{p}	22.44^{d}	20.92^{f}	22.84^{c}	22.51^{d}	20.83^{g}	8.16^{t}	8.68^{s}	15.58^{o}	19.02^{i}	19.40^{h}	22.17^{e}	24.49^{a}	17.29^{t5}	8.16^{t}	17.29^{l}	11.50^{r}	17.30^{l}	17.07 m	18.64 j	23.50 b
Crude Fibre	0.40^{hij}	0.75^{ef}	0.30^{j}	0.95^{cd}	1.00^{c}	1.10^{b}	0.50^{g}	0.45^{hij}	0.60^{fg}	0.90^{cd}	0.50^{gh}	0.65^{fg}	1.20^{ab}	1.30^{a}	0.35^{j}	0.38^{ij}	0.51^{hi}	1.10^{c}	0.51^{hi}	1.00^{c}	0.30^{j}	0.80^{de}	1.20^{a} b	0.84^{de}
NFE	0.01^{l}	0.88^{e}	0.03^{kl}	2.34^{b}	0.61^{g}	0.23^{j}	0.20^{j}	0.70^{f}	0.06^{kl}	3.10^{a}	0.05^{kl}	1.05^{c}	0.73^{f}	0.30^{i}	0.37^{i}	0.53^{h}	0.01^{l}	2.35^{b}	0.01^{l}	2.35^{b}	0.09^{k}	0.98^{cd}	0.91^{d} e	0.25^{j}
Carbohydrate	0.41^{n}	1.63^{g}	0.33^{o}	3.29^{c}	1.61^{g}	1.33^{h}	0.70^{kl}	1.15^{i}	0.66^{l}	4.00^{a}	0.55^{m}	1.70^{f}	1.93^{e}	1.60^{g}	0.72^{kl}	0.91^{j}	0.52^{m}	3.45^{b}	0.52^{m}	3.35^{c}	0.39^{no}	1.78^{f}	2.11^{d}	1.09^{i}

*Values with the same superscripts are not significantly different using Duncan's Multiple Range Test

CHAPTER 5

5.0 DISCUSSION

Rice Bran (RB), Palm Kernel Cake (PKC) as well as other agricultural by-products also known as Agro-Industrial by-products (AIBs) are abundantly produced in areas the particular farm products are planted and processed in Nigeria. Wudiri (1992) attested to the fact that Nigeria is able to produce 200,000 metric tons of rice bran from the 500, 000 metric tons of rice she produces annually. Porla (2000) and Gere (2004) ascertained the abundance of oil palm, the source of PKC in Asia and Africa especially the West African sub-region where Nigeria is located.

Rice Bran (RB**)** is the dry, outer cover of rice grain which is always removed during the milling of rice. It accounts for 5-8% of the rough rice weight (FAO, 1964; Houstgon, 1972). As in other agro-industrial by-products, rice bran is of no direct nutritional value to man. In most mills it is often discarded and allowed to rot away. In some areas, it may be collected and used as litter material or fire making. World rice production is greater than the production of any other single crop (Carter *et al*., 1979).

Palm Kernel Cake (PKC), which is a by-product of the African Palm Oil industry, is a possible optimal feed ingredient for animals. Palm Kernel Cake is the shaft obtained after the extraction of oil from palm kernel. The global production of palm kernel cake (PKC), a by-product of oil extraction from palm kernel, is ever increasing due to the tremendous growth of the oil palm industry in many parts of Asia and Africa (Porla, 2000).

RB, PKC and its different forms like Deoiled Cake (DOC) from the industries, Palm Kernel Cake Sludge in its wet and dried forms ($PKCS_W$ and PKC_D) was collected in abundance from rice mills in New-Bussa, Niger State, North central part of Nigeria and also from Ibadan, Oyo State, western part of Nigeria. This is in agreement with the report of Fetuga and Tewe, (1985), Omole and Tewe, (1989) who reported that AIBs are abundantly produced and processed in Nigeria

The fungi isolated from the spontaneously fermenting lignocellulosic material include *Aspergillus* (*Aspergillus niger, A. flavus, A. terreus, A. clavus, A. versicolor, A. tamarii*), *Rhizopus oryzae, Rhizopus stolonifer* and *Trichoderma* sp. The most predominant fungal strain isolated was *Aspergillus flavus* (32.25%) while *Trichoderma* sp. (2.42%) was the least. Mistra *et al*. (1994) also reported that agro-industrial residues are generally considered

the best substrates for the Solid State Fermentation (SSF) processes. During the SSF of the substrates the temperature ranged from 31 - 38^{o}C. The highest temperature recorded at day 5 during SSF of the samples may be as a result of the metabolic activity of the isolates which can be term exothermic in nature.

There was a significant difference in pH changes during the period of spontaneous fermentation of the substrate which ranged from 4.18- 6.46. The lowest pH was recorded at day 5 during spontaneous fermentation of the samples**.** The pH reduction at the last day may be due to the acidic by-product such as lactic acid which is similar to the observation of Onilude (1999). The irregularity in the temperature and pH readings may be explained on the fact that SSF has some limitations such as a poor pool of microorganism capable of growth under restricted conditions and the controlling and monitoring of parameters such as temperature, pH, humidity and air flow (Nahara *et al*., 1982).

Generally the digested substrates of some samples had higher values of metabolizable energy than the undigested forms. One of the reasons is the increased percentage fat contents of the fermented samples. The energy in feeds is not available until the complex molecules are broken down to simpler ones by fermentation. Secondly, the enzyme produced by the microorganisms during the fermentation must have contributed to the increase in the ME. This is in line with the work of Cowan *et al.* (1996), who concluded that addition of enzymes to feed resulted in improved energy availability.

Proximate compositions of the unfermented and spontaneously fermented ligno-cellulosic substrates were determined. The spontaneous fermentation resulted in decreased percentage (%) of dry matter, fibre and Nitrogen Free Extract (NFE); increased ash contents of most samples, moisture, fat and protein though not high. The decrease in the dry matter and increased in the moisture content might be due to the addition of the sterilized distilled water in the ratio of 1:1 volume/weight ratio to induce the natural fermentation.

The Fibre content of the unfermented and fermented samples ranged from 6.10 - 33.90% and 5.00 - 18.00%. The highest was recorded in UFIndPKC2 while the FLPKC1 had the least. The reduction in the fibre (%) and NFE contents of the fermented samples could be due to the degrading activities of enzymes produced by the fungal growth on the non- starch polysaccharides. The different sugar moieties and crystalline build-up of different samples explains the higher fibre (%) contents recorded in the unfermented samples than in the fermented samples. Theodorou *et al*. (1989) reported on different microbes utilizing

different sugar moieties at different rates. Any factor that affects the crude fibre will definitely affect the value of the NFE. The increase in percentage ash contents of the fermented forms of the samples with the exception of rice bran samples is in agreement with the observation of Ng *et al*. (2002) that the ash content of fermented palm kernel cake was markedly higher than the raw palm kernel cake.

The protein content ranged from 5.15 - 18.92% and 5.68% - 22.53%. The highest was recorded in FLPKC1 while UFRB1 had the least. This increase in the percentage protein contents could be due partly to the protein content of the mycelia of the fungi present during the natural fermentation. Smith *et al*. (1996) obtained increase in crude protein value in biodegraded wheat offal and sweet potato residues with *Trichoderma resei*. Cheah *et al*. (1998) also reported that the protein content of the palm kernel cake substrates increased to over 20% when fermented with various fungal strains.

The cellulose contents of most of these samples were higher in their digested forms than the cellulose contents of their digested forms. Cellulose content is also based on the proximate composition of the samples. The fermentation of cellulosic substrates requires rigorous control of hydrogen ion concentration, temperature, aeration, agitation and size of inoculums for excellent microbial growth. This implies that the conversion of cellulose to single cell protein is more efficient under aerobic than anaerobic conditions which are more available under submerged fermentation than under solid state fermentation in which this work was carried out. The general observation about the degraded forms of all the lignocellulosic samples is not too far from the observation made by Belewu (1998) that biodegraded feeds contain more free sugars and protein, less cellulose and lignin with an increase content of ash when compared with the un-degraded forms of the samples. The nutritive value of RB and PKC can be enhanced by subjecting them to fungal degradation (solid state fermentation) to digest the Non-Starch Polysaccharides (NSP) so as to improve the bioavailability of their nutrients and increase their protein contents.

Fermentation of samples had profound influence on amino acid content of the samples. There was a significant difference in amino acid content of the fermented and unfermented samples. Generally, the undigested lignocellulosic samples have higher amino acid contents than the digested samples. In the 17 Amino acid (Lysine, Histidine, Arginine, Aspartic acid, Theonine, Serine, Glutamic acid, Proline, Glycine, Alanine, Cystine, Valine, Methionine, Isoleocine, Leucine, Tyrosine, and Phenylaline) analyzed it was discovered that most of the unfermented samples have higher contents than their fermented samples. General reduction

in amino acid during fermentation of the substrates may be due to the fact that the available amino acid in the unfermented substrates were used as a precursor metabolites for the synthesis of proteins and other essential building molecules such as ATP and enzymes (Prescott *et al,* 1999). Reduction in Amino acid content may be due to fungal growth on these substrates especially PKC, a process which might have used up part of the little Amino acid available in them. Ng (2004) recommended the need for Amino acid supplementation of plant-based diet for fish. Fungal proteins had also been reported to be deficient in Amino acid (Ng *et al*., 2002).

Screening of the fungal isolates for Amylase production

Amylase production by the screened isolates ranged from 10.24 - 63.17UI/ml in which *Aspergillus niger* had the highest while *Aspergillus flavus* had the least. About 50% of the isolates (*Aspergillus niger, A. terreus, R.* sp., *R. oryzae* and *A. tamarii)* are good producer of amylase while 20% (*A. versicolor* and *Rhizopus stolonifer*) produced amylase moderately and about 30% (*Aspergillus flavus, A. clavatus* and *Trichoderma* sp.) are poor amylase producer.

Out of all the 10 fungi screened for amylase production, *A. niger* produced the highest value with 63.17U/ml and so was selected for further physiological work. Amylolytic enzymes are commonly produced by filamentous fungi and the preferred strains belong to the species of *Aspergillus* and *Rhizopus* (Pothiraj *et al*., 2006). Singh *et al*. (2012) noted that filamentous fungi especially those belonging to the *Aspergillus* have been most commonly employed for the production of α- amylase. Studies on fungal amylases especially in developing countries have concentrated mainly on *A. niger*, probably because of their ubiquitous nature and non-fastidious nutritional requirements (Abu *et al*., 2005). Ikenebomeh and Chikundu (1997), Yigitogler (1992) found that the high amylolytic activity of *A. niger* made it superior to other species of *Aspergillus* and strains of fungi in biomass yield from agricultueal waste.

Physico-chemical parameters

The effects of physico-chemical parameters (carbon sources, nitrogen sources, pH, Inoculums load, Temperature and Incubation Time) on amylase production by the selected *Aspergillus niger* was determined. Galactose, xylose and starch supported the highest production amylase respectively. Generally, most of the simple carbon sources supported maximum amylase production while complex carbon sources repressed maximum amylase production by *Aspergillus niger*. Sucrose, galactose and mannitol supported the highest

mycelial growth at different days of incubation while sugar cane baggase supported the least growth by *Aspergillus niger*. Various physical and chemical factors have been known to affect the production of α- amylase such as temperature, pH, period of incubation, carbon sourses acting as inducers, surfactants; nitrogen sources phosphate, different metal ions, moisture and agitation with regards to solid state fermentation and submerged fermentation respectively. The interactions of these parameters are reported to have a significant influence on the production of the enzyme (Singh *et al*., 2012).

Corn steep liquor and Urea repressed the production of amylase while NH_4NO_3 supported the highest growth of *Aspergillus niger*. Suganthi *et al*. (2011) noted that nitrogen source increased the yield of amylase produced. Ampama and Ravindra, (2001) observed remarkable increase in amylase production with Ammonium nitrate and Ammonium chloride as well as increase in biomass cropped. The mycelia dry weight obtained in this work was highest by day 3 with Ammonium nitrate which is in agreement with the report of Ampama and Ravindra (2001). Growth of mycelium is crucial for extracellular enzymes like α-amylase (Carlsen, 1996). Previous findings have shown that peptone, $NaNo_3$ and casein hydrolysate are good nitrogen supplements for amylase production in *A. fumigatus, A. niger* and *A. oryzae* (Pandey *et al*., 1994; Got *et al*., 1998; Pederson and Neilson, 2000).

The effect of pH on amylase production by *A. niger* showed amylase production range between 1.83 - 173.033UI/ml while pH 6 and 7 supported the highest amylase production. This is higher than the recommended pH range for hydrolysis of starch with commercially available fungal amylases between 4.0 and 4.5 (Boyce, 1986).

4ml and1ml inoculum size supported the highest amylase production by *A.niger*. 45^{o}C, 35^{o}C and 20^{o}C and induced the highest amylase production while 35^{O}C and 30^{O}C supported the highest growth of *Aspergillus niger* on day 3, 5 and 7 of fermentation.

Ability of *Aspergillus niger* to produce the highest amylase at 20^{o}C is in contrast with the report of Moreira *et al* (1999) who recorded 30^{o}C for *A. tamarii*. Rao et al (2005) reported that the temperature on the activity of α amylase from the pericarp of *Borassus indica* increases up to 37^{0}C before a slight decrease in activity.

During this study *Aspergillus niger* produced the highest amylase at day 7 and 3 and the least enzyme production and growth of *Aspergillus niger* was recorded at day 21. Moreira *et*

al (1999) on the contrary recorded maximum activities and biomass production on day 4 for the amylase from *A. tamarii*.

Optimization of Amylase production by *Aspergillus niger*

Optimization of various parameters and manipulation of media are one of the most important techniques used for the over production of enzymes in large quantities to meet industrial dmands (Tanyildizi and Elibol, 2005). Production of α- amylase in fungi is known to depend on both morphological and metabolic state of the culture (Carlsen, 1996).

It was observed that 6 and 7g of xylose and 7g of Oat meal chaft induced optimum production of amylase by *A. niger* while 8g/l concentration of xylose and 8g/l of oatmeal chaff supported the optimum growth. Optimum amylase production by *A. niger* was recorded in 12% and 0.5g Corn steep liquor and yeast extract.

Amylase production and mycelia dry weight of *Aspergillus niger* under different pH studied shows optimum production at pH 6. This is in line with the observation made by Moreira *et al.*, (1999) for *A. tamarii* with optimal pH of 6. On the contrary, Omemu *et al*, (2005) observed optimum pH 4 for amylase of *A. niger* hydrolysing tuber starches.

During this research work, it was observed that amylase production and mycelia dry weight of *Aspergillus niger* under different inoculums load ranged from 18.167 – 28.91 UI/ml. Optimum production was supported by 7ml. 25^{o}C induced optimum production of amylase by *A. niger* this result contradicts the findings of Morcira *et al*. (1999) who reported that 50 - 55^{o}C for *A. tamarii*. Amylase production and mycelia dry weight of *Aspegillus niger* under different optimized Incubation time ranged from 7.33 - 24.65UI/ml and optimum production was at day 7. Mycelia dry weight of *Aspergillus niger* ranged from 0.05 – 0.29g in which Day 12 supported optimum growth of *Aspergillus niger*.

The highest activity and stability of *Aspergillus niger* was recorded at 50^{o}C and it ranged from 3.04 – 12.32UI/ml and 2.68 – 14.35 UI/ml. This amylase demonstrated optimal activity and stability at 50^{o}C. This is in agreement with the report of Obineme *et al*. (2003) when the amylase from *A. oryzae* strain isolated from the soil was characterized demonstrated optimal activity and stability at 50^{o}C. This observation is also in agreement with the temperature range of 40 - 60^{o}C recorded for most amylases from the genus *Aspergillus* (Fogarty and Kelly, 1990; Okolo *et al*., 2000) whereas the amylase produced by *A. tamarii* was stable at 55^{o}C.

pH 12 and 3 supported the highest *Aspergillus niger* amylase activity and stability. The activity and stability ranged from 2.27 - 16.85UI/ml and 1.96 – 18.04UI/ml. The optimum activity of the enzyme was recorded at pH6. Activity and stability of the enzyme at pH 12 and pH 3 is in contrast to the report of Suganthi *et a*l. (2011) and Varalakshmi *et al.* (2009) who reported maximum enzyme activity at pH 9.5, Hermandez *et al.* (2006) reported acidic pH optima for amylase for *A. niger*.The stability of the amylase from *A. tamarii* reported to be between pH 4 – 7 did not agree with the finding in this report either.

Enzyme activity can be affected by other molecules which could either be inhibitors that decrease enzyme activity or activators which increase the enzyme activity (Groves, 1997). The amylase produced by A. niger in this study was characterized by inclusion of 0.1 – 1M of cations; anions and inhibitors (EDTA, HgCl, FeKCN, Benzoic acid and Urea). KNO_3 (1M), 1M of $FeSO_4$ and 0.7M of FeKCN induced the production of amylase while KNO_3 (0.3M) as cation, 0.1M of $CaCO_3$ as anion and 0.3M of HgCl suppressed the production of amylase. Most amylases are known to be metal ion-dependent enzymes, namely divalent ions like Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Fe^{2+} etc (Okolo *et al*., 2000; Goyal *et al*., 2005). Amylases appear to vary considerably in their specific requirement for divalent cations (Okolo *et al*., 2000). Some ions found stimulatory in some reports may be found non-stimulatory in some others (Obineme *et al*., 2003). The cations are generally believed to function by protecting the enzyme protein against heat denaturation, thus contributing to thermostability (Fogarty and Kelly, 1980). Amylase from *Pseudomonas* sp. was activated by most metals including Calcium (Varalakshmi *et al*., 2012) which inhibited the activity of amylase in this report.

Enzyme activity is also affected by temperature, chemical environment (e.g. pH) and the concentration of the substrates (Groves, 1997). Amylase production increased as the substrate (starch) increased. At 2.5% concentration, the highest amylase was produced. Oboh (2005) and Omemu *et al.* (2005) observed a similar report of increase in enzyme activity with increase in starch concentration from 1.0 to 3.0%. Alli *et al.* (1998) indicated that increase in substrate concentration from 1 to 3% led to progressive increase in amylase activity by some fungi.

At 90 mins, the highest activity of 20.70U/ml was observed. On the contrary, the enzyme activity increased exponentially with the time of incubation up to 30 mins (Rao *et al*. 2005) for amylase from *A. tamarii*.

5.2.1.3.2. Cellulase

Cellulase is a complex of enzymes containing chiefly endo- and exo- β glucanases plus cellobiase. Cellulases are a group of hydrolytic enzymes capable of degrading cellulose to smaller sugar components like glucose units (Onsori *et al*., 2005). There are three different kinds of enzymes believed to be involved in crystalline cellulose decomposition; endo- β-1, 4-glucanase, exo-β-1, 4-glucanase and β-glucosidase (Enert *et al*., 1974).

Cellulolytic enzymes are synthesized by a number of microorganisms. Fungi and bacteria are the main natural agents of cellulose degradation (Lederberg, 1992); the cellulose-utilizing population includes filamentous fungi (Alexander, 1961). Cellulose is the world's most abundant organic substance (Rottloff, 1987) and comprises a major storage from photosynthesized glucose. It is the major components of biomass energy (Scott *et al*., 1987). Since a large proportion of vegetation added to the soil is cellulose, therefore, decomposition of cellulose has a special significant in the biological cycle of carbon (Lederberg, 1992). In the industry, cellulases have found novel application in production and processing of chemicals, food and manufactured goods such as paper, rayon etc., and extraction of valuable components from plants and improvement of nutritional values of animal feed (Wiseman, 1995).

However, fungi are well known agents of decomposition of organic matter, in general, and of cellulosic substrate in particular (Lynd *et al.*, 2002). Among the 10 isolated fungi, a few strain of Tricodermal species (2%) were isolated which may be due to the possibility that the growth of the Trichoderma was suppressed by rapidly growing fungi like *Aspergillus* and *Rhizopus.*

The fungal isolates obtained from lignocellulosic materials were screened for their ability to produce cellulase and mycelia growth using submerged fermentation. The best cellulase producer was selected, subjected to different production condition and optimization and the enzyme produced was characterized. The fungal isolates was screened for cellulase production and it ranged from 2.82 to 3.13 UI/ml in which *R. oryzae* had the highest while *Rhizopus stolonifer* had the least. About 40% of the isolates (*R. oryzae, A. versicolor, R. sp* and *A. terreus)* are good producer of cellulase and 60% (*A. flavus, A. niger, A. clavertus, R. stolonifer, A.tamarii and Trichoderma sp.*) produced cellulase moderately.

According to Nwagu *et al*. (2012), *Rhizopus* species had a higher cellulase activity than *Aspergillus* specie, in that the cellulase activity of *Aspergillus* specie was surpassed by the activity of *Rhizopus* specie. Pothiraj *et al*. (2006) also reported that *Rhizopus stolonifer* cultivated on cassava waste gave a higher cellulase activity than *Aspergillus niger* cultivated in the same cassava waste. Similar observation has been expressed by Vipan *et al* (1994).

The higher cellulase activity of *Rhizopus oryzae* might be due to better growth of its mycelial biomass in rice bran and palm kernel cake which led to higher enzyme activities. It could also result from their pH tolerance. On the contrary, Sadaf Jahangeer *et al*. (2005) found that cellulase activity of *Aspergillus* species were relatively towards the higher side and *Rhizopus* moderate range cellulase activity. Ray *et al*, (1993) made a contrary conclusion that *A. niger* has been mostly used for production of cellulose.

The properties of cellulolytic enzyme as proteins are modified by prevailing physical condition such as temperature and pH. Enzyme exhibits its catalytic activity within these ranges of physical conditions**.** Concentration, composition and quality of substrate as well as enzyme concentration and reaction time are also important factors that determine the rate of hydrolysis and the final yield of the product (Godfrey, 1996; Philippidis, 1994).

The effects of physico-chemical parameters on cellulase production by the selected *R. oryzae* were determined. The effect of different carbon sources on cellulase production and mycelia dry weight of *R. oryzae* at different incubation time was determined.

Xylose, a simple sugar and Rice bran, a complex sugar induced the highest production of cellulace of 14.10 and 5.5.92U/ml respectively by *R. oryzae* in this study. This is contrary to the report of Gautam *et al*. (2010) and Rai *et al*. (2012) that glucose was the best carbon source for *Trichoderma viride* and *Candida* sp. to yield high cellulase respectively. When *A. niger* was grown on cellulose as the sole carbon source the production of cellulase was induced while a very low rate of enzyme production was observed when glucose was the sole carbon source (Jahangeer *et al*., 2005). Residual enzyme activity was noted when cellulolytic fungi were grown in the presence of glucose and many fold increase in enzyme yield were reported in the presence of cellulosic substrate (Lederberg, 1992; Lynd *et al*., 2002). The production of cellulase for the utilization of cellulose is induced only in the presence of specific substrate but suppressed when easily utilizable sugars such as glucose are available (Lynd *et al*., 2002). Although cellulases are inducible, but there is a low level of constitutive production of these enzymes suggesting that there may be isozymes, some of

them remain repressed in the absence of inducer and, the presence of inducer greatly affect the enzyme yield (Yalpani, 1987). Mukesh kumar *et al* (2012) reported lactose as the best carbon source for *Bacillus subtilis* toproduce the highest cellulase. Baig (2005) used banana biomass as source of carbon for *Trichoderma lignorum* to produce the highest activity of cellulase. Cellulase production is, however, influenced by several other factors such as carbon, nitrogen, phosphorus sources , the ratio of carbon to nitrogen provided, trace elements, pH and aeration rate (Philippidis, 1994).

Raffinose, Xylose and sugarcane bagasse supports the highest growth of the *R. oryzae* in this study. It was observed that KNO_3, followed by CSL and casein as organic nitrogen sources supported the highest production of cellulase (11.28, 10.15 and 1.0U/ml) by *R. oryzae*. Urea is an inorganic nitrogen source that gave the highest cellulase production. Rajoka (2004) reported ammonium (inorganic) compounds as the most favourable nitrogen source for protein and enzyme synthesis. Peptone was the best nitrogen source for *B. subtilis* as reported by Mukesh Kumar *et al.* (2012). Malt extract and NH_4SO_4 (organic and inorganic) compound were recorded by Rai *et al.* (2012) as the best nitrogen source for cellulase production by *Candida* sp. Peptone supported the highest growth of *R. oryzae.*

The effect of inoculum size (1ml - 4ml) on cellulase production and mycelia dry weight of *R. oryzae* was determined**.** The highest cellulase (5.018U/ml) produced was at pH 4. The highest growth of 0.33g of R. oryzae was obtained with 3ml. It was discovered that 4ml and 2ml inoculum size supported the highest while 3ml, 3ml and 4ml supported the lowest. 3ml inoculum size supported the highest while 2ml, 2ml and 1ml supported the lowest

R. oryzae produced the highest cellulase (3.278U/ml) at 20^{o}C. This contrasts the reports of Sone *et al.* 1999 and Jahangeer *et al.* 2005 who reported maximum cellulase production at 37^{o}C by *Aspergillus* species Ability of *R. oryzae* to produce the highest cellulase at 20^{o}C may be due to the fact that the enzyme is not thermostable. Philippidis (1994) however reported higher enzyme yield at 30^{o}C by some *Aspergillus* strains. Akinyosoye *et al*. (1995) recorded as high as 45^{o}C for the highest activity of the culture filtrates.

The highest growth of *R. oryzae* (0.31g) was at 45^{o}C which is in agreement with the report of Akinyosoye *et al.,* (1995) who observed maximum mycelia growth at 45^{o}C *Aspergillus niger*.

During this study the effect of different incubation time (days) on cellulase production and mycelia dry weight of *R. oryzae* was determined. The highest Cellulase production was recorded at day 3 while the highest mycelia dry weight of *R. oryzae* was at day 14 (0.10g). Optimal cellulase production was supported by 8g/l of xylose and 7% of Corn steep liquor. Rai *et al*. (2012) recorded optimum production cellulase on 1% malt extract for *Candida* sp. The best optimum growth of *R oryzae* was recorded on 5g/l of xylose (0.08g) and 7% of CSL (0.91g).

Optimum production of cellulase was obtained on 3g/l of KNO_3 and 5g/l of urea. Optimum growth of *R. oryzae* was obtained on 7g/l of KNO_3 (0.09g) and urea (0.11g). Cellulase has an optimum pH within which its activity is optimum and at higher or lower pH values, its activity decreases (Lehninger *et al*, 1993). Most enzymes function between pH 6 and 8 except pepsin that works best at a pH of 2 in the stomach (Schnell *et al*., 2006). Opimization of cellulase production and mycelia dry weight of *R. oryzae* under different pH yielded high production of cellulase as from pH 3 – 6; and the optimum production was recorded at pH 4 in this study. This is in agreement with the findings of Lee *et al*. (2002) and Omojasola *et al*. (2008) who reported CMCase activities exhibited a pH optimum of approximately 4 by *A. niger*; Akinyosoye *et al*. (1995) observed a pH of 6 by *A. niger*. The optimum pH for fungi cellulases varies from species to species (Garg and Neelakantan, 1981). Optimum pH 5.5 was recorded for *Candida* sp. by Rai *et al*, (2012), pH 7, neutral pH for *B subtilis* by Mukesh Kumar *et al*. (2012), Rathman et al. (2013) and Shaikh *et al*, (2013). Maleekka Begum *et al*. (2012) recorded optimum pH 5.6 – 5.8 for cellulase production by *T. lignorum* on banana agro-waste. Wang *et al*. (2003) also reported pH 5.0 for *T. reesei* on corn staw. The best growth of 0.12g of *R. oryzae* was at pH 5 and 6. Optimal production of cellulase and mycelia growth was obtained with 2ml and 2, 5 and 6ml of the inoculums load.

During this study, optimum cellulase production and mycelia dry weight of *R. oryzae* under different temperatures (10 - 60^{o}C**)** was determined. Optimum cellulase production was recorded at 35^{o}C and 55^{o}C. This agrees with the work of Okeke and Obi (1993) that cellulases show highest activity at temperature range of 30 – 35^{o}C. Above 35^{o}C there was a drastic decrease in cellulase activity which indicates the sensitivity of enzymes to high temperature which could result to denaturation or destuction. On the contrary, Wang *et al*. (2012) recorded 28^{o}C as the optimum temperature for *T. reesei*; and 45^{o}C for *T. lignorum* on banana agro-waste by Jha *et al*. (1995). Akinyosoye *et al*. (1995) reported the optimum

cellulase activity as 45^{o}C for *A. niger*. Optimum production of cellulase was however different in the reports of Rai *et al*. (2012) and Shaikh (2013) as 50^{o}C for *Candida* sp and *Bacillus* respectivly, 30^{o}C and 40^{o}C for *Bacillus subtilis* by Mukesh Kumar *et al* (2012) and Rathman *et al.* (2013) respectively.

Optimum growth of *R. oryzae* (0.23g) obtained at 55^{o}C during this work contrast the report of Akinyosoye *et al*. (1995) who reported 45^{o}C as the opimum temperature for maximum mycelial growth of *A. niger* and reported that 55^{o}C supported minimum mycelial growth whereas Ogundero (1982) reported an optimum mycelial production for both *A. niger* and *A. flavus* as 30^{o}C. Akinyosoye *et al*. (1995) attributed the difference in the optimum temperature of growth to the differences in the prevailing environmental conditions from which the organisms were isolated which invariably might have affected their physiological activities.

Opimum cellulase production and mycelia dry weight of *R. oryzae* under different incubation time determined showed that the optimum cellulase production was recorded at day 3 and 21. *T. lignorum* produced maximum cellulase by day 8 of incubation on banana waste. Solomon *et al*. (1999) observed the highest activity of cellulase at about 12th hour by *Aspergillus flavus* on bagasse.

Under different incubation temperature, 10 and 50^{o}C supported the highest cellulase activity and stability respectively. Akinyosoye *et al*. (1995) observed the highest cellulase activity at 45^{o}C. It was observed that pH 8 supported the highest cellulase activity and stability this may be as a result of the fact that R. oryzae cellulase is alkalinogenic in nature. NaCl (0.7M), 0.5M$FeSO_4$ and 1M urea induced the highest cellulase activity of *R. oryzae* while 0.4M$CaCO_3$, 0.1M$CuSO_4$ and 0.9M benzoic acid suppresed the activity in this study.

Under different incubation time cellulase of *R. oryzae* when characterized 0.61 - 2.08U/ml in which the highest activity of 2.08U/ml was recorded after 70min of incubation. Activity of cellulase increased with the increase of the substrate (Carboxylmethyl Cellulose (CMC)). 2.0% substrate (CMC) concentration supported the highest cellulase activity of *R. oryzae.* Activity was high at 5 – 6% of the enzyme, cellulase while the highest activity was recoerded at 5%. The increase in the CMC had a positive effect on the cellulase activity. The linear increase obtained in this report was also reported in the enzyme activity of *Poronia punctata* (Robertson and Koehn, 1978). This increase in activity with increase in substrate concentrations may be due to the effective biding of the substrate to the active

sites. It may further be explained in terms of the enzyme-substrate affinity and the rate of breakdown of enzyme-substrate complex to form products (Akinyosoye *et al*.,1995).

Aquaculture is currently the fastest growing animal production sector in the world (Ng, 2004) which must be supported by a corresponding increase in the production of formulated diets for cultured aquatic animals. The world's dependence on its wild aquatic catch for food has become grossly inadequate. Food and Agriculture Organization (FAO) (1997), reported the rate of increase in the total food supply which is generally less than the rate of increase in human population. This suggests that fish food is under-produced, thus necessitating an aggressive, intensive aquaculture (Akintomide *et al*., 2005). Ng (2004) reported an increase in the cost of imported feed ingredients used in commercial aqua feeds in many developing countries due to increased global demand and fluctuation in foreign currency exchange value. El-Sayed (1999) stated that an escalating cost of fish meal used as a major source of dietary protein in commercial aqua feeds have stimulated much research into the use of alternative plant protein sources.

As a result of rising cost of conventional feed ingredients, an alternative source of fish feed such as rice bran (RB) and Palm kernel cake (PKC) has been in use. This helps in avoiding competition of stuff with human beings, reduction of the environment impact from the disposal of these lignocellulosic by-products. Longe (1985) and Babatunde (1989) reported that incorporation of agro-industrial by-products (AIBs) in animal feeds holds tremendous potentials in alleviating the existing critical situation of high cost and inadequate supply of feeds.

Fish, like other animals are fed on adequate quantity diets to improve their growth rate and general production performance. Standard quality feeds found to be expensive have been developed to meet the nutritional needs of some fish species. Healthy state of animal like fish is a manifestation brought about by the combination of a set of organic elements known as nutrients brought together in different proportions at feeding (Falayi, 2009a). The currently recognized feed nutrients are carbohydrates, proteins, lipids, vitamins, minerals and water.

The performance characteristics of the *Clarias gariepinus* fingerling in this work showed that the fingerling fed with feed substituted with 10%PKC had the best Mean Weight Gain (MWG), Specific Growth Rate (SGR) Nitrogen Metabolism (NM) and feed fed. The feed conversion Ratio (FCR) of 0.17 recorded in fish fed with feed substituted with UPKC was

the best of all. Adikwu (2003) concluded that a lower FCR value implies efficient feed utilization by fish. The FCR of the feed with 10% PKC was 1.38. These performance were better than those of the fish fed with the control feed samples. The feed with the 10% PKC must have been a feed with a better quality that was well accepted, digested and utilised better by the fish. The starter culture (*Aspergillus tamarii*) must have digested or fermented the PKC adequately to make the locked up nutrients in it to be available to support the growth of the fish.

The survival rate of local *Clarias gariepinus* fed with U (RB+PKC) 67% and URB 60% were next to that of Dutch *Clarias gariepinus* fed with the control feed. The survival rate of fish especially Dutch fed with fermentented substrates were generally low. It could be that the local species is naturally resistant to the effect of the presence of the fungi, their secretions and other conditions within their rearing system. Asiwaju *et al* (2011) observed that the rearing of catfish is still faced with several problems as the fingerlings exhibit strong cannibalism, which results in low survival rate and overall growth performance. They feed better under dark condition (Hossain *et al*, 1998) which is not obtainable in the glass aquaria in which this feeding trial was carried out.

Although PKC supplies both protein and energy, it is looked upon more as a source of protein (Chin, 2001). Fish eat primarily to satisfy energy requirements. If there is too little energy compared with protein, part of the dietary protein will be used for energy (Carmen and Geoff, 2007). The energy in feed is not available until the complex molecules are broken down to simpler molecules by digestion. The products of digestion are then absorbed into the body of the animal where oxidation processes occur which release the energy. According to Cowan *et al.* (1996), addition of enzymes to feed resulted in improved energy availability.The metabolizable energy profiles of the samples utilized in this work were between 1440.9893 – 6177.8593kcal/kg.

The protein contents of the different feeds ranged from 6.5 – 42%. The general requirement of *C. gariepinus* is 35 to 40% protein and 13 to 17kcal/kg of energy (Van Weerd, 1995). The protein content UPKC was between 12.57% and 18.92% while that of the FPKC wasbetween 13.35% to 22.53%. Ng and Chen (2002) conducted a feeding trial with hybrid catfish the result of which showed that up to 20% raw PKC could be incorporated in catfish diets without any negative effects on the growth performance.

The proximate composition of all the feed samples used in the feeding trials includes moisture, protein, lipid (fat) and carbohydrate are referred to as macronutrients as considered by Carmen and Geoff (2007) as the major components of feeds. The dry matter ranged from 68.15 – 95.90 in which the URB+PKC had the highest while the RB fermented with a starter culture (*A. clavatus)* (SFRB) had the least. Moisture content ranged from 4.10 – 31.85 in which SFRB had the highest while the URB+PKC had the least. The low dry matter and high moisture content in starter fermented substrates may be due to production of higher water molecules in one of the products of the fermentation.

The ash contents ranged 3.30 – 16.80%, Crude fat ranged from 4.5 – 23.85%, Protein ranged from 6.5 – 52.27%, Fibre ranged from 3.00 – 24.70%, NFE ranged from 4.04 – 49.98% and Carbohydrate ranged from 25.85 – 64.84%.

The starter fermented RB had the least ash content, NFE, carbohydrate but higher crude fat contents. The starter cultures (*Aspergillus clavatus* and *Aspergillus tamarii*) activities must have made the fat contents to be more available in the course of digesting the substrates. These different fungi isolates and the feed samples were subjected to different analysis like toxin, anti-nutrient factors, vitamins, sugar and metals to investigate certain factors that could probably affect their performance. Aflatoxins, fumosin, Alkaloid and oxalate were detected in all the 10 fungi isolated from the lignocellulosic substrates when they were analysed for toxin production. It was the trace of mycotoxin that was detected in *A. tamarii* alone. These must have been secreated into the lignocellulosic substrates that constituted to their anti-ntrient contents.

The presence and levels of anti-nutrient analysed in the different fish feeds showed that the percentage alkaloid in PKC fermented with a Starter (*A. tamarii*) was the highest (18.60 – 36.50). The alkaloid might have been secreted in higher quantity by the starter culture. The content of alkaloid dected in *A. tamarii* was as high as 43%. The percentage oxalate (18.24 – 32.10), phytic acid (0.18 – 0.45%), cyanogenic glycoside (8.18 – 10.30mg/g) and hemagglutinin (0.026 – 0.117u/mg) in the SFRB and the percentage phytate and tanning in SFPKC were less than their values in other fish feds. This must be as a result of the effect of fermentation by the inoculated fungi. However the alkaloid and tannin contents of the URB and the alkaloid and cyanogenic glycoside of UPKC were less than their contents in the other feeds. Apart from the production of different enzymes, the fungi fermenting the substrates must have secreted these anti-nutrients. Strong (1974) concluded that these

substances are an intrinsic part of the plant or animal food material as it was formed in nature. Many chemical components of natural food products have been identified as toxicants; some of which include cyanogenic glycoside, hemaglutinin, saponin, gossypol, goistrogen, trypsin inhibitor, oxalate, phytates and antivitamins (Onwuka, 2005).

Vitamins are complex organic substances, usually of comparatively small molecular size (molecular weight usually less than 1000). They are distributed in feedstuff in small quantities and form a distinct entity from other major and minor food components (Cho *et al.*, 1985). Vitamins are needed for normal growth, maintenance, and reproduction of animals. Deficiency have been found to cause depressed appetite and reduced growth rate in channel catfish. Other symptoms include discoloration, lack of coordination, nervousness, hemorrhages, lesions, fatty liver and increased susceptibility to bacterial infection (Dupree and Huner, 1984). *Clarias* fingerling requires vitamin B_1 (thiamin), B_2 (riboflavin), B_6 (pyridoxine), pantothenic acid, folic acid, niacin and vitamin C (ascorbic acid) for proper growth (Omitoyin, 1997).

UPKC had the highest value of vitamin C which ranged from 0.0010 – 0.0041µmol/l while FPKC had the least. Thiamine ranged from 14.40 – 18.02mg/100g, Riboflavin ranged from 0.221 – 0.412mg/100g and total sugars ranged from 24.20 – 66. 29g/l. As for the vitamins detected, the high contents of thymine, riboflavin, total sugar and the non-reducing sugar in SFRB and the thyamine in SFPKC must have been made available by the fermentative actions of the fungi on the substrates. The higher contents of vitamin C in URB and vitamin C, riboflavin and the reducing sugars of the UPKC must have been used up or exhausted by the growing fungi biomass during fermentation.

Minerals are required for maintenance of salt and water tissue balance for the metabolism of other nutrients and for major structural elements on the tissue of fish. Most studies of mineral requirement by Nose and Arai (1979); Dupree and Huner (1984) have involved calcium and phosphorous. Calcium can be absorbed in sufficient quantity from the water and dietary phosphorous has been reported for fish.

The magnesium ranged from 8.50 – 8.90µg/g, Calcium ranged from 3.00 – 29.00µg/g, Zinc ranged from 470.00 – 520.60µg/g. The Iron contents ranged from 78.0 – 140.00µg/g, Potassium ranged from 6.00 – 35.00µg/g, Copper ranged from 20. – 170.00µg/g while Cadmium, Lead and Cobolt were not detected. Metals (minerals) like Magnesium and Zinc detected in FRB and magnesium, calcium, zinc and iron in FPKC were higher in contents

probably due to the ability of the fungi to make them more available at fermentation. However, the calcium, iron, pottasium and cupper in URB and Potassium and Copper in UPKC were higher. They are probably essential for the growth of the fungi and have been used up by them. All the samples free of cadmium lead and cobalt as they were not detected in any of the substrates. Minerals and vitamins are referred to as micronutrients and are considered by Carmen and Geoff (2007) as part of the major components of feeds.

Water quality includes several interacting physico-chemical and biological factors, which must be in optimal levels for fish rearing activities to yield maximum result. These parameters majorly depend on the source of water (Ovie and Ovie, 2010). The water quality parameters become more critical in culture systems where fish is raised in artificial enclosures with reduced self-purification capabilities as compared to natural systems (Ovie and Adeniji, 1990). Among the physico - chemical water quality variables is temperature, pH and dissolved oxygen (DO) which have immense effects on the activities of the fish.

Each fish species have its natural thermal tolerance outside which it cannot live. The optimum temperature for maximum growth rate and feed conversion as observed by Varreth and Bieman (1987) is 27.5%. The rate of metabolism in fish is also temperature dependent. As temperature increases basal metabolism increases. Nutrient requirements of fish are temperature dependent and that near certain upper thresholds, feed digestibility, metabolism and nutrient utilization are enhanced. Temperature also has effect on the reproduction of *C. gariepinus* (Delince *et al*., 1985).

The temperature of fish varies with the water temperature (poikilothermy) and so each fish species has specific ranges of temperature within which all activities, especially growth is optimal (Ovie and 2010). The temperature range for *Clarias* species is 25 – 35^{o}C (Chakroff, 1976), recommended for growing catfish is 22 – 28^{o}C (Akinwole and Faturoti, 2007).

pH is a measure of the hydrogen ion (H^{+}) concentration and indicates the level of acidity or alkalinity of the water. Fish grow best in water at pH 6.5 - 9.0 and prefer slightly alkaline water close to neutral pH. The pH of water is greatly influenced by the concentration of carbon dioxide; however the optimal acceptable pH level varies with species. The acid and alkaline death points for pond fish are approximately pH 4 and pH11 respectively

The measure of the hydrogen ion concentration in water is its pH. A pH between 6.5 and 9 is good for fish culture (Ovie, 2010). Boyd and Lichtkoppler (1979) recommended a pH range of 6.7 – 8.6, and a pH range of 6.0 – 8.0 (Akinwole and Faturoti, 2007).

The atmosphere is 21% oxygen by volume, but this oxygen is not directly available to fish. For fish to utilize oxygen it must be dissolved in water. The exact concentration of Dissolved Oxygen (DO) required for maintaining fish in healthy condition is variable; however, a DO of 4mg/l or more will prevent the detrimental effects of low oxygen (Schwedler, 1983). Adequate DO is necessary not only to prevent massive fish kill but also to keep fish healthy and make them grow well. Low DO concentrations adversely affect fish even at levels which do not cause mortality, making them more susceptible to parasites and diseases (Plumb *et al.*, 1976). DO concentration as low as 1mg/l can be tolerated by healthy catfish for a short period, however prolonged exposure to 1.5mg/l DO can cause histological damage to catfish (Scott and Rogers, 1981). In addition fish do not feed or grow when concentration remain continuously below 4 or 5mg/l. More oxygen is needed at feeding because much of biological and chemical activities are taking place, and there is the need to supplement normal oxygen composition of ponds by external or additional aeration (Falayi, 2009a). There is a direct relationship between feeding rates, dissolved oxygen and other water quality characteristics (Dupree and Huner, 1984). Dissolved oxygen (DO) is the amount of oxygen that is available in water and can be influenced by the water temperature, weather conditions. It has been observed that a minimum constant value of 5mg/l is satisfactory for most species and stages of culture fish (EIFAC/19). The recorded water temperature range (24.42 – 27.75^{o}C), pH range (7.21 – 7.86) and the range of DO (2.33 – 8.75mg/l) in this work are within the range of those recommended above. They are also in line with the findings of Obasa *et al* (2009) that recorded 26.25 – 28^{o}C for temperature, 6.0 – 6.8 for pH and 5.53 – 6.2mg/l for DO.

6.0 CONCLUSION

Agriculture Industrial by-products, especially like rice bran and palm kernel cake otherwise known as ligno-cellulosic materials are not to be considered as waste products but sources of wealth. The previous negative attitude in which wastes were viewed as valueless and even offensive and for disposal only has been replaced in large part by a positive view in which wastes are recognised as raw materials of potential value (Pranner, 1979). They are good substrates for fungi cultivation as 10 fungi of *Aspergillus, Rhizopus* and *Trichoderma* were successfully isolated from the unfermented and naturally fermented RB and PKC. They can be converted to useful materials by fungi fermentation (either by solid state or in the submerged) as animal feed and to produce valuable enzymes for food, medicinal, paper and some other industries.

Aspergillus tamarii could be used as starter culture to inoculate and ferment lignocellulosic substrates especially palm kernel cake to feed animal generally, particularly fish and specifically *Clarias gariepinus.* At 10% inclusion, *Clarias gariepinus* performed better than other fish feed including the control feed used in this work. Apart from increasing the source of income to farmers, as it will lead to more profit margin, it will also improve the health status of the consumers which makes the adage health is wealth true. Fish (especially *C. gariepinus*), a source of animal protein will become cheap and more available for the increasing population of Nigeria. Job opportunities will be created for more Nigerian youth, retirees and other interested Nigerians will be encouraged to venture into fish especially catfish farming to make gain or as hobbies. Our environment will be kept clean as the disposal of these AIBs will be taken care of. However further research is necessary to improve the survival rate of the animals especially fish fed with feed made with these materials.

Conclusively, Fungi are potential producer of amylases and cellulases. Fungi-degraded palm kernel cake and rice bran supported the growth of *Clarias gariepinus* better than the control feed and could be good sources of fish feed components.

The result demonstrated that diversity of fungi in fermented and unfermented lignocellulosic substrates as potential amylase and cellulase producers. Combination of cultural condition and appropriate fermentation strategies increased enzyme yield which will contribute to fermentation of lignocellulosic substrates as a feed substitute which will solve the problem of feed shortage and reduce the cost of fish feeds. More oil seeds, legumes and nuts that

have little value as nutrient sources for human population should be investigated to see if they will have good replacement values in the diets for fish. It is adviced that there is need for more research to guarante that these feed that contain fungi will not be injurious to human beings that consum the fish that feed on them.

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APPENDIX

Starch Yeast Extract [SYE] medium components

Soluble starch [BDH] - 5g, Yeast Extract [Difco] - 2g, K_2HPO_4 - 1g, $MgSO_4.7H_2O$ - 0.5g,

Distilled Water - 1 litre

Starch Yeast Extract Agar [SYEA] components

Starch Yeast Extract [SYE] medium [above] + Agar - 2%.

Amylase Assay medium:-

1% soluble starch in 0.02M phosphate buffer, pH 6.9.

Dinitrosalicylic acid reagent

3, 5- Dinitrosalicylic acid - 1.0g, 2N NaoH - 20ml, Potassium Sodium tartrate - 20g.

Distilled water - 100ml.

Oatmeal chaff preparation:-

Oatmeal chaff - 20g. Yeast extract - 5g, Distilled water - 1 litre.pH - 6.8.

Soluble starch - 2.0, $MgSO_4.7H_2O$ - 0.025, KH_2PO_4 - 0.05, $CaCo_3$ - 0.5, Yeast Extract - 0.1.

Olive oil - 1.0 and Agar - 1.0.

MOISTURE CONTENT (AOAC, 1980)

Principle:- When samples are dried at 100- 102^0c for 15hours in an air oven, the loss in weight is reported as moisture. (Compounds other than moisture may be lost). The dry matter is left behind.

ASH CONTENT:-

Principle

When foods and food products are heated to temperature of 500^0c and above water and other volatile constituents are evolved as vapours, organic constituents also burn to carbon dioxide and water. The mineral constituents remain in the residue as oxides, sulfates,

phosphates and so on depending on conditions of incineration and the composition of the food ignited. This inorganic residue constitutes the ash in the food products. (AOAC, 1980)

CRUDE PROTEIN: AOAC, 1980 pgs 126 – 127

Principle

This method is used to measure the crude protein content in foods as it gives the amount of all the reduced nitrogen in the food in the form of amonos, ammonium compounds, urea, amino – acids and so on. The procedure involves digesting the material with concentrated H_2S0_4 and converting the nitrogen to ammonium hydrogen sulfate. The digestion is accelerated by addition of a catalyst (Potassium Sulfate, mercury, selenium or copper salt) to increase the boiling point. The mixture is made alkalin by adding NaoH solution. The NH_3 produced is distilled into boric acid. The exact amount of NH_3 is determined by titration with Hcl. Protein values are obtained by multiplying the total nitrogen by a factor of 6.25, 6.38, 5.30 or 5.55 depending on the food source.

CRUDE FAT (soxhlet method) (AOAC, 1980)

Principle

This method measures crude fat which is extracted by solvents such as petroleum ether or hexane. The extraction is based on the sparingly solubility of lipids in water and their considerable solubility in non polar organic solvents (that is hydrophonic nature). The solvent is evaporated off to get the fat. The measured fat consists of all the soluble materials present in the sample.

CRUDE FIBER DETERMINATION (Tricholoroacetic acid method) (AOAC, 1980)

Principle

The crude fiber content of any food (raw or processed) is indicative of the indigestible matter or roughage in the food. The method involves delating the food material. This is followed by hydrolyzing the protein and carbohydrate in the food using a mixture of acids.

NITROGEN FREE RXTRACT (NFE)

Principle

The calculation of nitrogen free extract (NFE) is made after the ash, crude fiber, ether extract and crude protein content were determined. The calculation is made by adding the percentage values on dry basis of these analyzed contents and subtracting them from 100%. (Crampton and Harris, 1969)

ACID - DETERGENT FIBER (ADF)

Principle

A quaternary/detergent/cetyl trimethyl ammonium bromide (CTAB) dissolves plant protein in acid media. The residue obtained by this reagent has been named acid – detergent fiber (ADF). Compositional studies show ADF to consist chiefly of lignin and polysaccharides (cellulose). At times some protein will be attached to the fiber cell walls. In this procedure, air dried sample is refluxed with one normal sulfuric acid containing 20% CTAB for one hour. Digestion of the samples is best accomplished using these conditions. The residue is filtered, washed with hot water to remove the acid – detergent solution, washed with acetone to remove the plant pigment, dried and weighed. (Van Soet, 1963, AOAC, 1980)

NEUTRAL DETERGENT FIBER (cell wall constituents) (NDF).

Principle

This is a rapid method for the total fiber in fibrous plant feeds stuffs. It appears to divide the dry matter of feeds near the point that separates the nutritively available and soluble constituents from those which are incompletely available or dependent on microbiology fermentation. This method can not be used for feeds with high protein and low fiber contents. (Goering and Van Soets, 1970; Van Soet et al, 1991).

LIGNIN DETERMINATION (ACID DETERGENT) (ADL):- (A.O.A.C., 1980)

Principles

The acid- detergent lignin (ADL) procedure utilizes the acid – detergent fiber as a preparatory step. The detergent removes the protein and other acid–soluble material which would interfere with the lignin determination. The principle of the procedure is that the acid

detergent fiber residue is primarily lignocellulose of which the cellulose is dissolved by the 72% H_2S0_4 solution. The remaining residue consists of lignin and acid – insoluble ash, however, with samples containing large amounts of cutin (seed coats), this is also measured as part of the lignin.

ALGEBRAIC EQUATIO METHOD USED FOR FISH FEED fFORMULATION

It is also known as quadratic or simultaneous equation.

To prepare 40% crude protein fish feed using Soya Bean Meal (x) having crude protein (c.p.) content 48.5% and (a) Unfermented Rice Bean (URB) (y) having c.p. content 9.56%, (b) Fermented Rice Bran (FRB) (y) having c.p. content 12.13%, (c) Unfermented Palm Kernel Cake (UPKC) (y) having c.p. content 19.12% and (d) Fermented Palm Kernel Cake (FPKC) (y) having c. p. Content 26.1%.

URB

Assuming x = kg of Soya Bean Meal (SBM) per 100kg of the fish feed

y = kg of URB per 100kg of the fish feed

x + y = 100 equation (i)

To get equation (ii), divide the c. p. of URB and SBM by 100

i.e, 0.485x + 0.0956y = 40% (c. p. of the fish feed) equation (ii)

Multiply equation (i) by 0.0965to get equation (iii)

i e, 0.0965x + 0.0965y = 9.65 equation (iii)

Subtract equation (iii) from (ii)

0.3894x = 30.44

x = 78.17kg of SBM

y = 100 – 78.17 = 21.83 kg of URB

FRB

x + y = 100 equation (i)

0.485x + 0.1213y = 40 equation (ii)

0.1213x + 0.1213y = 12.13 equation (iii)

0.3637x = 27.87

x = 76.63kg of SBM

y = 23.37kg of FRB

UPKC

x + y = 100 equation (i)

0.485x + 0.1912y = 40 equation (ii)

0.1912x + 0.1912y = 19.12 equation (iii)

0.2938x = 20.88

x = 71.07 kg of SBM

y = 28.93 kg of UPKC

FPKC

x + y = 100 equation (i)

0.485x + 0.261y = 40 equation (ii)

0.261x + 0.261y = 26.1 equation (iii)

0.224x = 13.9

x = 62.15kg of SBM

y = 37.85kg of FPKC

ENZYME

AMYLASE

Amylase production by 10 fungi isolates (Screening).

Fungal Isolate	Enzyme Production
1BK	63.170[a]
2GR	10.237[j]
3BR	53.047[b]
4BL	11.973[i]
5OR	31.355[g]
6GY	42.873[d]
7DW	39.756[f]
8WT	47.904[c]
10DB	41.018[e]
11PK	17.923[h]

Effects of Physico-Chemical Parameters on Amylase Production by (1BK) *Aspergillusniger*.

Simple Carbon Sources

	Enzyme Activity		
Carbon sources	INCUBATION (DAYS)		
Simple carbon sources	3	5	7
D – glucose	192.334f	102.453^{j}	30.030^{g}
Sucrose	234.443^{b}	143.755^{i}	15.728^{i}
Fructose	120.636^{h}	100.452^{k}	26.123^{h}
Maltose	148.059^{g}	181.181^{f}	110.986^{e}
Xylose	198.266^{d}	303.427^{a}	49.223^{f}
Manitol	4.355^{l}	15.387^{m}	-3.055^{k}
Galactose	235.068^{a}	186.912^{d}	136.636^{c}
Sorbitol	0.000^{m}	11.121^{n}	13.052^{i}
Lactose	194.012^{e}	216.405^{c}	130.432^{cd}
Starch	64.118^{i}	161.995^{g}	179.963^{a}
Saccharose	56.418^{j}	40.204^{l}	10.723^{ij}
Raffinose	190.850^{g}	226.630^{b}	158.940^{b}
Mllibiose	214.020^{c}	182.071^{e}	123.161^{d}
Trehalose	16.229^{k}	159.268^{h}	15.027^{i}
Complex carbon sources			
Rice bran	13.349^{a}	2.746^{c}	3.579^{c}
Palm Kernel Cake	13.513^{a}	0.126^{d}	1.628^{e}
Wheat bran	0.814^{a}	9.404^{b}	8.760^{a}
Oat Meal Chaff	13.942a^{a}	33.434^{a}	2.505^{d}
Sugar Cane Bagasse	14.605a^{a}	4.898^{d}	3.654^{b}

(C) Nitrogen Sources (Simple)

Nitrogen Source	Enzyme Activity		
	INCUBATION PERIOD (DAYS)		
Inorganic nitrogen Source	3	5	7
Ammonium nitrate	14.024[b]	49.292[a]	50.561[c]
Ammonium sulphate	33.002[a]	38.033[c]	51.255[b]
Potassium nitrate	5.018[d]	48.352[b]	42.261[d]
Corn steep liquor	0.303[e]	34.744[d]	57.207[a]
Soya meal	11.581[c]	-11.203[e]	2.935[e]
Complex nitrogen source			
Peptone	32.523[a]	5.497[c]	28.950[d]
Yeast Extract	31.917[b]	36.764[a]	39.680[a]
Urea	6.217[d]	0.088[d]	33.318[c]
Casein	2.733[c]	10.578b[b]	39.623[b]

(e)pH
(f) Inoculum load (Seed medium in ml)

pH	Enzyme Activity		
	INCUBATION TIME(DAYS)		
	3	5	7
4	80.464[b]	5.762[e]	88.776[e]
5	-1.931[f]	147.573[b]	176.277[b]
6	173.033[a]	229.217[a]	154.730[c]

	Enzyme Activity		
	INCUBATION TIME(DAYS)		
Inoculum Load (ml)	**3**	5	7

7	40.923[c]	0.372[d]	230.644[a]	1	0.00	222.319[a]	145.724[b]
8	-7.328[f]	1.830[e]	101.803[d]	2	0.00	0.00	75.857[d]
9	12.364[d]	3.112c	12.850[f]	3	0.00	64.730[b]	110.790[c]
10	6.002[e]	1.685e	14.699[f]	4	20.512[a]	16.353[c]	189.841[a]

(h) Time (day)

(g)Temperature (T^{o}c)

	Enzyme Activity		
	INCUBATION TIME (DAYS)		
Temperature (^{o}C)	3	5	7
20	11.045[e]	0.00	211.590[a]
25	4.727[f]	0.372[e]	121.122[b]
30	11.999[d]	41.510[c]	19.761[c]
35	15.122[b]	130.861[a]	11.771[d]
40	13.046[c]	63.960[b]	0.00
45	19.988[a]	14.302[d]	11.013[e]

Time (day)	Enzyme Activity
3	0.00
7	123.419[a]
14	46.218[b]
21	41.693[c]

AMYLASE

MYCELIAL GROWTH (WEIGHT)/BIOMASS MEATUREMENT)

Physico-Chemical Parameters on Amylase production by *Aspergillusniger*.

(a) Carbon Sources (Simple)

	Mycelia weight (g)		
Parameter	Day3	Day5	Day 7
D-glucose	0.13[d]	0.05d	0.03d
Sucrose	0.35[a]	0.05d	0.03d
Fructose	0.11[e]	0.09b	0.06b
Maltose	0.17[c]	0.05d	0.06b
Xylose	0.32[b]	0.05[d]	0.02[e]
Manitol	0.08[f]	0.07[c]	0.07[a]
Galactose	0.09[f]	0.12[a]	0.02[e]
Sorbitol	0.08[f]	0.03[f]	0.04[c]
Lactose	0.01[h]	0.02[g]	0.04[c]
Starch	0.08[f]	0.07[c]	0.01[f]
Saccharose	0.05[g]	0.01[h]	0.02[e]
Raffinose	0.04[g]	0.04[e]	0.04[c]
Melibiose	0.01[h]	0.02[g]	0.04[c]
Trehalose	0.02[h]	0.04[e]	0.04[c]

(b)Carbon Sources (complex)

	Mycelia weight (g)		
	INCUBATION TIME(DAYS)		
Parameter	Day 3	Day 5	Day 7
Rice bran	0.02[b]	0.08[a]	0.02[c]
Palm Kernel Cake	0.06[a]	0.03[c]	0.04[b]
Wheat bran	0.06[a]	0.04[b]	0.02[c]
Oat meal chaff	0.02[b]	0.03[c]	0.02[c]
Sugar cane bagasse	0.01[c]	0.02[d]	0.02[c]
Corn steep liquor	0.06[a]	0.04[b]	0.05[a]

(b) Nitrogen Source (Simple)

	Mycelia weight (g)		
Parameter	Day 3	Day 5	Day 7
Ammonium nitrate	0.08[a]	0.02[b]	0.05[a]
Ammonium sulphate	0.04[c]	0.02[b]	0.04[b]
Potassium nitrate	0.01[d]	0.04[a]	0.03[c]
Corn steep liquor	0.06[b]	0.04[a]	0.05[a]
Soy meal	0.04[c]	0.04[a]	0.05[a]

Nitrogen Source (Complex)

	Mycelia weight (g)		
Parameter	Day 3	Day 5	Day 7
Peptone	0.02d	0.04a	0.03b
Yeast Extract	0.07a	0.02b	0.02c
Urea	0.06b	0.01c	0.04a
Casein	0.03c	0.01c	0.04a

(c) pH

Parameter	Mycelia weight (g)		
Ph	Day 3	Day 5	Day 7
4	0.03f	0.11a	0.04b
5	0.15c	0.10b	0.03c
6	0.08e	0.05d	0.04b
7	0.21b	0.05d	0.04b
8	0.02f	0.02e	0.02d
9	0.12d	0.09c	0.21a
10	0.41a	0.05d	0.04b

Temperature (^{o}C)

(d) T

e	Mycelia weight (g)		
m Temperature (^{o}C)	Day 3	Day 5	Day 7
p 20	0.01[c]	0.04[b]	0.01[d]
e 25	0.02[b]	0.O2[d]	0.02[c]
r 30	0.02[b]	0.03[c]	0.05[a]
a 35	0.03[a]	0.06[a]	0.03[b]
t 40	0.01[c]	0.06[a]	0.05[a]
u 45	0.01[c]	0.02[d]	0.01[d]
r			
e			
(			

(e) Time (day)

Time (day)	Mycelia weight (g)
3	0.19[a]
7	0.04[b]
14	0.03[c]
21	0.01[d]

AMYLASE

Optimization of the Physico-Chemical Parameters on amylase production by *Aspergillusniger*.

Carbon Source (Simple) – Xylose (5g/litre) ; (Complex) – Oat Meal Chaff (5g/litre)

Parameter	Day 3	Day 5	Day 7
Xylose (g/litre)	Enzyme Activity	Enzyme Activity	Enzyme Activity
2	31.166f	21.244g	16.763g
3	263.282e	39.036f	72.770f
4	286.204c	243.462d	267.395c
5	284.600d	74.910e	267.035d
6	378.684a	267.938c	276.988b
7	378.684a	285.219b	286.418a
8	328.818b	288.456a	82.503e

Parameter	Day 3	Day5	Day 7
OMC(g/l)	Enzyme Activity	Enzyme Activity	Enzyme Activity
2	35.003b	17.407d	18.808g
3	30.831d	17.849c	24.684e
4	27.322g	15.703g	23.062f
5	30.219e	15.848f	29.657c
6	28.748^{f}	24.539^{a}	29.474^{d}
7	36.991^{a}	16.479^{e}	37.307^{a}
8	33.842^{c}	21.863^{b}	34.385^{b}

Nitrogen Source – Corn steep liquor (10% v/v)

	Day 3	Day 5	Day 7
C S Liquor (% v/v)	Enzyme Activity	Enzyme Activity	Enzyme Activity
7	44.773^{f}	42.179^{c}	378.684^{a}
8	98.843^{c}	22.797^{d}	21.011^{e}
9	91.061^{d}	86.214^{a}	28.111^{d}
10	76.368^{e}	13.759^{g}	378.684^{a}
11	91.061^{d}	71.956^{b}	32.295^{c}
12	124.966^{a}	13.771^{f}	378.684^{a}
13	109.844^{b}	16.776^{e}	43.984^{b}

(a) Nitrogen Source (Complex) – Yeast Extract 2g/litre

Y.Extract (g/litre)	Enzyme Activity	Enzyme Activity	Enzyme Activity
0.5	58.557a	14.106e	18.196g
1.0	34.520d	12.187f	27.808c
1.5	44.937b	12.187f	51.066a
2.0	33.198e	19.199c	23.914e
2.5	31.936f	18.877d	37.679b
3.0	35.672c	28.603a	26.615d
3.5	25.561g	22.311b	22.544f

pH

pH	Enzyme Activity	Enzyme Activity	Enzyme Activity
5.5	15.267g	52.101b	11.613f
6.0	16.858f	52.183a	8.571g
6.5	17.445e	9.448g	12.755e
7.0	23.188b	10.243f	16.485d
7.5	22.891c	25.069d	19.704c
8.0	20.998d	25.302c	21.415b
8,5	23.201a	13.973e	21.661a

(b) Temperature (^{o}C)

Temperature (^{o}C)	Enzyme Activity	Enzyme Activity	Enzyme Activity
10	25.467c	20.582d	15.955g
20	20.481f	15.577f	18.896c
25	45.726a	14.731i	9.461j
30	23.056d	19.439e	16.814e
35	22.450e	9.945j	35.943a
40	16.902j	22.645b	15.715h
45	17.699h	15.267h	15.337i
50	18.480g	15.406g	16.037f
55	17.255i	22.967a	25.706b
60	33.640b	22.570c	17.849d

Inoculum size (ml)

ml	Enzyme Activity	Enzyme Activity	Enzyme Activity
1	22.904d	7.081f	12.421f
2	18.158g	11.203e	10.976g
3	20.701f	4.860g	13.235d
4	22.084e	13. 765b	12.433e
5	25.820c	13.216c	20.405c
6	27.335b	12.011d	28.187b
7	28.913a	36.884a	36.859a

Time (Day)

Time (day)	Enzyme Activity
2	19.016b
3	13.999c
5	12.547d
7	24.646a
9	
10	8.413f
11	7.328g
12	8.508e

AMYLASE

Mycelia Weight

Optimization of the Physico – Chemical Parameters on mycelia dry weight of *Aspergillus niger*.

Carbon Sources (Simple) – Xylose

Parameter	Mycelia Weight (g)		
Xylose (g/litre)	Day 3	Day 5	Day 7
2	0.11a	0.09c	0.12d
3	0.05d	0.13a	0.12d
4	0.09b	0.11b	0.14b
5	0.07c	0.11b	0.13c
6	0.06cd	0.08c	0.13c
7	0.05d	0.09c	0.12d
8	0.10ab	0.12ab	0.15a

Conplex – Oat Meal Chaff

Parameter	Mycelia Weight (g)		
O M C (g/l)	Day 3	Day 5	Day 7
2	O.09c	0.10d	0.15b
3	0.10b	0.11cd	0.13d
4	0.09c	0.13b	0.13d
5	0.08d	0.12bc	0.14c
6	0.09c	0.14a	0.15b
7	0.11a	0.14a	0.13d
8	0.08d	0.12bc	0.17a

Nitrogen Source (Simple) – Corn Steep Liquor;

	Mycelia Weight (g)		
C.S.Liquor (%v/v)	Day 3	Day 5	Day 7
7	0.11ab	0.11b	0.13b
8	0.08c	0.12a	0.13b
9	0.10b	0.10c	0.11d
10	0.12a	0.10c	0.14a
11	0.07d	0.11b	0.12c
12	0.08c	0.10c	0.13b
13	0.10b	0.09d	0.10e

Complex –Yeast Extract

Parameter	Mycelia Weight (g)		
Yeast Extract (g/litre)	Day 3	Day 5	Day 7
0.5	0.09c	0.11e	0.14a
1.0	012a	0.12d	0.13b
1.5	0.08d	0.12d	0.13b
2.0	0.10b	0.16a	0.14a
2.5	0.12a	0.13c	0.14a
3.0	0.09c	0.14b	0.13b
3.5	0.10b	0.11e	0.13b

pH

Parameter	Mycelia Weight (g)		
pH	Day 3	Day 5	Day 7
5.5	0.08d	0.09e	0.08b
6.0	0.07e	0.11c	0.04e
6.5	0.12a	0.13a	0.07c
7.0	0.10b	0.13a	0.07c
7.5	0.08d	0.09e	0.07c
8.0	0.01f	0.12b	0.05d
8.5	0.09c	0.10d	0.09a

Inoculum size (ml)

Parameter	Mycelia weight (g)		
Inoculum size (ml)	Day 3	Day 5	Day 7
1	0.07c	0.09b	0.14a
2	0.10b	0.09b	0.03e
3	0.12a	0.09b	0.05d
4	0.07c	0.11a	0.12c
5	0.10b	0.09b	0.12c
6	0.12a	0.09b	0.13b
7	0.08c	0.06c	0.13b

Temperature (^{o}C)

Parameter	Mycelia Weight (g)		
Temperature (^{o}C)	Day 3	Day 5	Day 7
10	0.14c	0.07h	0.12f
20	0.12d	0.14a	0.18a
25	0.09f	0.09f	0.09g
30	0.i	0.11d	0.06h
35	0.08g	0.08g	0.14d
40	0.06h	0.13b	0.13e
45	0.11e	0.12c	0.18a
50	0.18a	0.12c	0.16c
55	0.11e	0.14a	0.17b
60	0.15b	0.10e	0.14d

Time (days)

Time (days)	Mycelia Weight (g)
2	0.08e
3	0.07f
5	0.11d
7	0.05h
9	0.06g
10	0.13c
11	0.18b
12	0.29a

CHARACTERIZATION OF AMYLASE ACTIVITIE

Temperature (°C)

	Enzyme Activity
10	4.323h
20	3.042i
30	5.895f
40	8.173c
50	12.320b
60	7.309d
70	5.472g
80	6.009e
90	14.125a
100	1.452j

Temperature (°C) Stability pH pH Stability

Temperature (°C) Stability	Enzyme Activity	pH	Enzyme Activity	pH	Enzyme Activity
10	7.403d	3	4.570h	3	18.038a
20	2.676j	4	8.962d	4	10.275e
30	6.356e	5	8.931e	5	4.412i
40	5.074i	6	14.617b	6	12.099d
50	14.352b	7	4.191i	7	15.999b
60	5.535h	8	7.833g	8	4.967h
70	5.642g	9	2.278j	9	5.535g
80	8.243c	10	11.190c	10	1.963j
90	5.882f	11	8.262f	11	12.124c
100	28.692a	12	16.851a	12	6.154f

Magnesium sulphate ($MgSO_4$)

Magnesium sulphate ($MgSO_4$)	Enzyme Activity
0.1M	1.925f
0.2M	2.651c
0.3M	1.092j
0.4M	1.843g
0.5M	2.215d
0.6M	1.742h
0.7M	1.502i
0.8M	3.919b
0.9M	7.378a
1M	1.994e

Calcium chloride (CaCl)	Enzyme Activity
0.1M	2.045e
0.2M	1.849f
0.3M	4.254b
0,4M	3.175c
0.5M	1.237h
0.6M	0.966j
0.7M	6.248a
0.8M	1.105i
0.9M	1.313g
1M	2.569d

Cations –

1. Calcium chloride (CaCl)

Manganese sulphate ($MnSO_4$) Potassium nitrate (KNO_3) Sodium chloride ($NaCl_2$)

Manganese ($MnSO_4$)	Enzyme Activity
0.1M	1.105j
0.2M	1.218i
0.3M	6.210c
0.4M	3.175e
0.5M	3.642d
0.6M	8.268a
0.7M	6.431b
0.8M	1.407h
0.9M	1.698g
1M	2.884f

Potassium nitrate (KNO_3)	Enzyme Activity	Sodium chloride ($NaCl_2$)	Enzyme Activity
0.1M	1.199h	0.1M	7.214a
0.2M	1.893g	0.2M	3.440g
0.3M	0.278j	0.3M	3.743f
0.4M	5.396d	0.4M	5.775c
0.5M	5.958c	0.5M	1.363j
0.6M	5.276e	0.6M	4.484e
0.7M	4.607f	0.7M	3.206h
0.8M	0.682i	0.8M	2.335i
0.9M	9.953b	0.9M	5.131d
1M	10.193a	1M	6.375b

Anions

$NaNO_3$) $FeSO_4$) $FeCl_2$ $CuSO_4$ $CaCO_3$

Sodium nitrate ($NaNO_3$)	Enzyme Activity
0.1M	4.721g
0.2M	7.157c
0.3M	6.652d
0.4M	3.219h
0.5M	6.217e
0.6M	1.887i
0.7M	1.755j
0.8M	14.043a
0.9M	8.053b
1M	5.933f

Iron sulphate ($FeSO_4$)	Enzyme Activity
0.1M	6.785f
0.2M	8.401c
0.3M	10.338b
0.4M	6.980d
0.5M	4.639h
0.6M	4.860g
0.7M	4.418i
0.8M	3.919j
0.9M	6.949e
1M	18.795a

Iron chloride ($FeCl_2$)	Enzyme Activity	Copper sulphate ($CuSO_4$)	Enzyme Activity	Calcium carbonate ($CaCO_3$)	Enzyme Activity
0.1M	10.054b	0.1M	8.098c	0.1M	0.202j
0.2M	5.750d	0.2M	9.208b	0.2M	4.670c
0.3M	3.579f	0.3M	9.776a	0.3M	3.503f
0.4M	10.515a	0.4M	4.412h	0.4M	4.159e
0.5M	1.824i	0.5M	5.106f	0.5M	4.734b
0.6M	3.030g	0.6M	4.102i	0.6M	3.181h
0.7M	5.650e	0.7M	3.314j	0.7M	1.174i
0.8M	3.017h	0.8M	5.447e	0.8M	3.383g
0.9M	1.098j	0.9M	6.116d	0.9M	5.257a
1M	6.709c	1M	4.866g	1M	4.551d

Inhibitors

EDTA HgCl FeKCN Benzoic acid Urea

EDTA	Enzyme Activity	Mercury chloride (HgCl)	Enzyme Activity	Iron potassium ferricyanide (FeKCN)	Enzyme Activity	Benzoic acid	Enzyme Activity	Urea	Enzyme Activity
0.1M	1.590i	0.1M	4.373f	0.1M	1.931j	0.1M	5.144h	0.1M	1.168j
0.2M	4.159d	0.2M	7.889b	0.2M	3.263i	0.2M	9.827b	0.2M	7.889c
0.3M	3.724e	0.3M	0.707j	0.3M	5.794f	0.3M	2.449j	0.3M	4.210g
0.4M	2.165h	0.4M	1.603h	0.4M	5.908e	0.4M	10.704a	0.4M	4.386f
0.5M	5.144c	0.5M	1.477i	0.5M	6.501b	0.5M	6.311f	0.5M	9.253b
0.6M	6.589b	0.6M	5.466e	0.6M	6.097d	0.6M	7.397c	0.6M	4.134h
0,7M	7.069a	0.7M	6.778d	0.7M	13.311a	0.7M	5.655g	0.7M	6.987e
0.8M	2.834g	0.8M	9.492a	0.8	4.588g	0.8M	6.709e	0.8M	9.492a
0.9M	3.332f	0.9M	1.919g	0.9M	3.629h	0.9M	6.867d	0.9M	1.919i
1M	1.521j	1M	7.069c	1M	6.185c	1M	4.185i	1M	7.069d

Time (minutes) Substrate (starch) concentration Enzyme concentration

Time (minutes)	Enzyme Activity	Substrate (starch) concentration (%w/v)	Enzyme Activity	Enzyme concentration (%v/v)	Enzyme Activity
10	10.269d	0.5	1.086g	1	1.401h
20	8.520e	1.0	4.008b	2	1.092i
30	8.167h	1.5	0.940j	3	2.127g
40	5.775j	2.0	0.966i	4	3.269c
50	11.646c	2.5	6.564a	5	2.177e
60	8.079h	3.0	2.979c	6	5.106b
70	19.016b	3.5	2.853d	7	0.871j
80	8.363f	4.0	0.997h	8	7.296a
90	20.695a	4.5	1.212f	9	2.272d
100	6.053i	5.0	2.809e	10	2.165f

CELLULASE

Screening of 10 fungi isolates for Cellulase production.

Fungi isolate	Enzyme Activity
1BK	2.85fg
2GR	2.86f
3BR	2.99d
4BL	2.83gh
5OR	3.09b
6GY	3.12a
7DW	2.82h
8WT	3.02c
10DB	2.83gh
11PK	2.87e

Physico – chemical parameters effect on cellulase production.

Carbon Source: (simple)

Parameter	Enzyme activity	Enzyme activity	Enzyme activity
D-glucose	8.313e	0.608e	0.385f
Sucrose	10.402d	0.008m	0.349g
Fructose	4.567h	0.511f	2.370e
Maltose	5.391f	3.812b	0.029l
Xylose	14.101a	5.180a	0.301i
Manitol	1.117n	0.352j	4.268a
Galactose	12.899c	2.283c	0.309h
Sorbitol	1.445k	0.477g	2.658d
Lactose	1.706j	0.419h	3.963c
Starch	1.361m	0.257l	0.002m
Saccharose	1.416l	0.509f	4.060b
Raffinose	5.320g	0.400i	0.037k
Mellibiose	12.992b	0.298k	0.037k
Trehalose	1.940i	0.672d	0.285j

Carbon Source (complex)

Parameter	Enzyme Activity	Enzyme Activity	Enzyme Activity
Rice bran	1,783b	0.377b	5.918a
Palm kernel cake	0.474e	0.323d	4.782c
Wheat bran	1.615c	0.358c	4.957b
Oat meal chaff	1.477d	0.409a	2.576e
Sugarcane bagasse	2.055a	0.311e	4.540d

(a) Nitrogen sources (Simple) (Complex)

Parameter	Enzyme Activity	Enzyme Activity	Enzyme Activity				
Ammonium nitrate	6.735c	0.059e	11.172b	Parameter	Enzyme Activity	Enzyme Activity	Enzyme Activity
Ammonium sulphate	2.901d	0.227b	5.573d	Peptone	2.503c	0.463b	4.843d
Potassium nitrate	8.155b	0.072d	11.283a	Yeast extract	2.060d	0.032d	6.927c
Corn steep liquor	10.151a	0.311a	6.153c	Urea	9.768a	0.081c	8.775a
Soy bean meal	5.372d	0.105c	2.079e	Casein	5.937b	1.002a	8.252b

pH Inoculum size (ml)

pH	Enzyme Activity	Enzyme Activity	EnzymeActivity				
4	3.718a	0.040e	0.115d	Inoculum load (drop)	Enzyme Activity	Enzyme Activity	Enzyme Activity
5	3.140b	0.025f	0.058f	1	2.372c	1.473b	0.286b
6	3.032c	0.205b	0.814a	2	2.931b	2.200a	0.332a
7	2.455f	4.051a	0.429b	3	1.522d	0.053d	0.097c
8	2.882d	0.105c	0.091e	4	5.018a	0.727c	0.076d
9	2.794e	0.063d	0.043g				
10	2.365g	0.027f	0.391c				

Temperature (°C) Time (Days)

Parameter	Day 3	Day 5	Day 7
Temperature (°C)	Enzyme Activity	Enzyme Activity	Enzyme Activity
20	3.278a	0.295d	0.206e
25	0.460d	0.837a	0.142f
30	1.301b	0.133f	0.432d
35	0.075e	0.626b	0.561c
40	0.051f	0.325c	1.551b
45	0.665c	0.178e	1.708a

Time (days)	Enzyme Activity
3	3.465a
7	1.107c
14	1.205b

CELLULASE

MYCELIA WEIGHT – Effects of physic chemical parameters on the mycelia dry weight of *Rhizopus oryzae*.

(a) Carbon sources (simple) (Complex)

	Mycelia weight		
Parameter	Day 3	Day 5	Day 7
D – glucose	0.07g	0.06f	0.14b
Sucrose	0.08f	0.26b	0.07g
Fructose	0.11d	0.09e	0.09e
Maltose	0.06h	0.24c	0.08f
Xylose	0.11d	0.30a	0.15a
Manitol	0.08f	0.06f	0.11c
Galactose	0.18b	0.23c	0.14b
Sorbitol	0.07g	0.09e	0.11c
Lactose	0.10e	0.09e	0.08f
Starch	0.06h	0,12d	0.09e
Saccharose	0.10e	0.06f	0.06h
Raffinose	0.19a	0.10e	0.09e
Mellibiose	0.12c	0.09e	0.10d
Trehalose	0.12c	0;02g	0.10d

	Mycelia weight		
Parameter	Day 3	Day 5	Day 7
Rice bran	0.07b	0.07c	0.11c
Palm kernel cake	0,07b	0.08b	0.08d
Wheat bran	0.09a	0.09a	0.08d
Oat meal chaff	0.07b	0.06d	0.12b
Sugarcane bagasse	0.07b	0.06d	0.18a

Nitrogen sources (simple or organic)

	Mycelia weight						
Parameter	Day 3	Day 5	Day 7		Mycelia weight		
Ammonium nitrate	0.07d	0.10b	0.11a	Parameter	Day 3	Day 5	Day 7
Ammonium sulphate	0.11b	0.06c	0.08c	Peptone	0.13a	0.15a	0.14a
Potassium nitrate	0.08c	0.11a	0.11a	Yeast extract	0.10c	0.08b	0.06c
Corn steep liquor	0.12a	0.10b	0.09b	Urea	0.10c	0.07c	0.12b
Soy bean meal				Casein	0.11b	0.06d	0.12b

pH

	Mycelia weight		
pH	Day 3	Day 5	Day 7
4	0.06e	0.08c	0.1f
5	0.08d	0.07d	0.13c
6	0.10b	0.13b	0.11e
7	0.11a	0.17a	0.12d
8	0.06e	0.06e	0.09g
9	0.09c	0.04f	0.16a
10	0.07e	0.03g	0.14b

	Mycelia weight (g)		
Inoculum size(ml)	Day 3	Day 5	Day 7
1	0.26b	0.13b	0.12c
2	0.03d	0.09d	0.13b
3	0.33a	0.25a	0.15a
4	0.17c	0.10c	

(b) Inoculum load (seed medium in drops) Time (Days)
Temperature (^{o}C)

Parameter	Mycelia weight (g)				
Temperature (^{o}C)	Day 3	Day 5	Day 7	Time (days)	Mycelia weight (g)
20	0.09d	0.12a	0.12e	3	0.09b
25	0.15b	0.04d	0.13d	7	
30	0.07e	0.01e	0.15b	14	0.10a
35	0.12c	0.11b	0.18a		
40	0.09d	0.06c	0.09f		
45	0.31a	0.12a	0.14c		

CELLULASE

OPTIMIZATION OF THE PHYSICO – CHEMICAL PARAMETERS ON CELLULASE PRODUCTION BY *Rhyzopusoryzae*.

(a) Carbon sources (simple) Xylose (complex) Corn Steep Liquor

Xylose (g)	Enzyme Activity	Enzyme Activity	Enzyme Activity	Corn steep liquor (% v/v)	Enzyme Activity	Enzyme Activity	Enzyme Activity
2	11.647g	*1*.682d	0.883e	7	8.486a	2.356c	0.245g
3	14.966f	1.716c	1.991b	8	7.601d	2.450b	1.370b
4	15.277e	1.919a	0.221g	9	6.295g	1.815g	1.316d
5	16.814d	1.891b	1.556d	10	8.104b	2.802a	1.335c
6	20.575c	1.168g	0.864f	11	7.479e	2.271d	1.138e
7	22.373b	1.373e	2.073a	12	7.713c	1.837f	1.481a
8	24.827a	1.212f	1.565c	13	6.537f	1.929e	0.994f

.

Nitrogen source (organic) Potassium nitrate

(b) Nitrogen source (inorganic) Urea

Potassium nitrate (g/litre)	Enzyme Activity	Enzyme Activity	Enzyme Activity
2	12.316f	1.264c	1.765e
3	16.443a	1.333b	1.837d
4	11.059g	1.202d	1.855c
5	12.400e	1.059e	1.956b
6	14.343c	0.138g	2.082a
7	15.317b	0.816f	0.981f
8	13.227d	1.358a	1.855c

Urea (g/litre)	Enzyme Activity	Enzyme Activity	Enzyme Activity
2	6.050e	1.478a	0.343f
3	1.227g	0.986f	1.594c
4	7.154d	1.461b	1.614b
5	13.231a	1.408c	0.409e
6	11.214c	1.344e	0.556d
7	12.163b	1.366d	0.207g
8	1.297f	0.962g	9.866a

pH

	Day 3	Day 5	Day 7
pH	Enzyme Activity	Enzyme Activity	Enzyme Activity
3.0	10.773f	3.901c	0.490g
3.5	12.445e	4.648b	1.405e
4.0	15.268a	1.497g	1.783b
4.5	14.017b	3.523d	1.567c
5.0	13.517c	7.509a	1.550d
5.5	12.567d	3.491e	1.373f
6.0	10.292g	2.924f	1.936a

Inoculum (ml)

Parameter	Day 3	Day 5	Day 7
Inoculum size (ml)	Enzyme Activity	Enzyme Activity	Enzyme Activity
1	11.841b	3.221d	0.223g
2	13.243a	3.138e	0.961f
3	9.415g	3.264c	1.400c
4	11.534c	5.403a	1.640b
5	11.367d	1.775g	1.214e
6	10.841f	5.226b	1.788a
7	10.945e	2.001f	1.347d

Temperature (^{o}C)

	Day 3	Day 5	Day 7
Temperature (^{o}C)	Enzyme Activity	Enzyme Activity	Enzyme Activity
10	0.107i	0.825g	0.418h
20	1.310f	1.567d	1.247b
25	11.367b	3.810b	1.067e
30		2.542c	0.909f
35	13.441a	5.880a	1.474a
40	1.659d	0.354i	0.616g
45	1.116g	0.419h	0.193j
50	1.774c	1.264e	1.213c
55	1.357e	1.065f	1.187d
60	0.300h	0.043j	0.207i

Time (Days)

Time (days)	Enzyme Activity
2	2.282d
3	11.168a
5	3.467c
7	1.365f
9	1.793e
10	1.002h
11	1.324g
12	0.984i
21	8.932b

CELLULASE

MYCELIA WEIGHT – Optimization of the physico chemical parameters on Cellulase production by *Rhizopus oryzae*.

Carbon source (simple)– Xylose (complex) Corn Steep Liquor

	Mycelia weight (g)						
Xylose (g/l)	Day 3	Day 5	Day 7		Mycelia weight (g)		
2	0.05b		0.05b	Corn steep liquor (% v/v)	Day 3	Day 5	Day 7
3	0.07a	0.05a	0.04c	7	0.08d	0.04d	0.91a
4	0.01d	0.02c	0.01e	8	0.09c	0.05c	0.09b
5	0.05c	0.01d	0.08a	9	0.09c	0.10a	0.03d
6	0.01d	0.04b	0.02d	10	0.04e	0.06b	0.05c
7	0.06b	0.04b	0.04c	11	0.09c	0.05c	0.05c
8	0.06b	0.02c	0.01e	12	0.11a		0.04cd
				13	0.10b	0.04d	0.01e

(a) Nitrogen source – Potassium nitrate

	Mycelia weight (g)		
Potassium nitrate (g/litre)	Day 3	Day 5	Day 7
2	0;06c	0.04b	0.08b
3	0.06c	0.07a	0.09a
4	0.07b	0.01	0.06c
5	0.07b	0.02c	0.08b
6	0.06c	0.01d	0.04d
7	0.09a	0.02c	0.04d
8	0.06c	0.07a	0.09a

Nitrogen source (Complex) – Urea pH Inoculum size (ml)

	Mycelia weight (g)	Mycelia weight (g)							
Urea (g/litre)	Day 3	pH	Day 3	Day 5	Day 7		Mycelia weight (g)		
2	0.01d	3.0	0.08d	0.08c	0.09b	Inoculum size (ml)	Day 3	Day 5	Day 7
3	0.07b	3.5	0.12a	0.08c	0.09b	1	0.07d	0.09d	0.08a
4	0.07b	4.0	0.12a	0.10b	0.01d	2	0.13a	0.08e	0.06c
5	0.06c	4.5	0.09c	0.06d	0.08c	3	0.10b	0.08e	0.08a
6	0.06c	4.0	0.08d	0.08c	0.10a	4	0.08c	0.10c	0.06c
7	0.11a	5,5	0.06e	0.06d	0.09b	5	0.13a	0.08e	0.08a
8	0.06c	6.0	0.11b	0.11a	0.08c	6	0.08c	0.13a	0.07b
						7	0.10b	0.12b	0.08a

(b) Temperature (^{o}C) Time (Days)

	Mycelia weight (g)				
Temperature (^{o}C)	Day 3	Day 5	Day 7	Time (days)	Mycelia weight
10	0.03f	0.07d	0.09	2	0.04g
20	0.07d	0.09c	0.10e	3	0.12c
25	0.07d	0.09c	0.10e	5	0.08e
30	0.07d	0.09c	0.17d	7	0.12c
35	0.05e	0.07d	0.10e	9	0.07f
40	0.10a	0.11b	0.20b	10	0.16b
45	0.09b	0.14a	0.20b	11	0.10d
50	0.10a	0.14a	0.17d	12	0.10d
55	0.08c	0.09c	0.23a	21	0.20a
60	0.05e	0.07d	0.19c		

CELLULASE

CHARACTERIZATION OF CELLULASE ACTIVITY

(a) Temperature (°C) Temperature (°C) Stability

Temperature (°C)	Enzyme Activity	Temperature (°C) Stability	Enzyme Activity
10	1.257a	10	0.825f
20	0.805c	20	0.689i
30	0.417f	30	0.208j
40	0.637e	40	0.723h
50	0.345g	50	1.173c
60	0.825b	60	0.727g
70	0.417f	70	1.007d
80	0.825b	80	1.530a
90	0.641d	90	1.187b
100	0.300h	100	0.851e

pH pH Stability

pH	Enzyme Activity	pH	Enzyme Activity
3	2.087h	3	0.997d
4	1.683j	4	0.947f
5	2.142g	5	1.034d
6	2.615e	6	0.450i
7	1.875i	7	0.383j
8	4.415a	8	1.296a
9	3.714b	9	0.771h
10	3.086c	10	0.869g
11	2.359f	11	0.981e
12	2.927d	12	1.280b

Cations

$CaCl_2$ $MgSO_4$ $MnSO_4$ KNO_3) $NaCl_2$)

Calcium chloride ($CaCl_2$)	Enzyme Activity	Magnesium sulphate ($MgSO_4$)	Enzyme Activity	Manganese sulphate ($MnSO_4$)	Enzyme Activity	Potassium nitrate (KNO_3)	Enzyme Activity	Sodium chloride ($NaCl_2$)	Enzyme Activity
0.1M	0.616c	0.1M	0.945e	0.1M	0.113j	0.1M	0.628h	0.1M	1.347e
0.2M	0.369g	0.2M	1.258c	0.2M	1.864h	0.2M	0.948d	0.2M	1.095g
0.3M	0.686b	0.3M	0.728i	0.3M	1.974g	0.3M	0.894e	0.3M	0.608i
0.4M	0.198i	0.4M	2.746a	0.4M	1.575i	0.4M	1.996a	0.4M	2.368c
0,5M	0.403e	0.5M	1.164d	0.5M	2.053f	0.5M	1.854b	0.5M	0.687h
0.6M	0.895a	0.6M	0.930f	0.6M	3.266d	0.6M	0.691g	0.6M	4.110b
0.7M	0.388f	0.7M	1.164d	0.7M	2.894e	0.7M	0.701f	0.7M	6.845a
0.8M	0.324h	0.8M	1.560b	0.8M	3.477c	0.8M	0.325j	0.8M	1.126f
0.9M	0.189j	0.9M	0.763g	0.9M	4.028b	0.9M	1.026c	0.9M	1.385d
1M	0.415d	1M	0.740h	1M	4.159a	1M	0.337i	1M	0.545j

(b) Anions

1. $NaNO_3$ $FeSO_4$ $FeCl_2$ $CuSO_4$ $CaCO_3$)

Sodium nitrate ($NaNO_3$)	Enzyme Activity	Iron sulphate ($feso_4$)	Enzyme Activity	Iron chloride ($FeCl_2$)	Enzyme Activity	Copper sulphate ($CuSO_4$)	Enzyme Activity	Calcium carbonate ($CaCO_3$)	Enzyme Activity
0.1M	1.673d	0.1M	0.924j	0.1M	0.032i	0.1M	0.004j	0.1M	0.070i
0.2M	0.927g	0.2M	1.880b	0.2M	0.153h	0.2M	0.133f	0.2M	0.118h
0.3M	2.016c	0.3M	1.416e	0.3M	0.160g	0.3M	0.236c	0.3M	0.060j
0.4M	2.724a	0.4M	1.257g	0.4M	0.582b	0.4M	0.086h	0.4M	0.139g
0.5M	2.672b	0.5M	3.435a	0.5M	0.267e	0.5M	0.282b	0.5M	1.908a
0.6M	1.057e	0.6M	1.577d	0.6M	0.290d	0.6M	0.516a	0.6M	0.546c
0.7M	1.011f	0.7M	1.052i	0.7M	0.860a	0.7M	0.149e	0.7M	0.700b
0.8M	0.296j	0.8M	1.263f	0.8M	0.546c	0.8M	0.069i	0.8M	0.283e
0.09M	0.860h	0.9M	1.866c	0.9M	0.180f	0.9M	0.210d	0.9M	0.487d
1M	0.802i	1M	1.159h	1M		1M	0.091g	1M	0.231f

INHIBITORS

E.D.T.A HgCl $KFe(CN)_6$ Benzoic acid Urea

(EDTA)	Enzyme Activity	Mercury chloride (HgCl)	Enzyme Activity	Potassium ferrycianide ($KFe(CN)_6$)	Enzyme Activity	Benzoic acid	Enzyme Activity	Urea	Enzyme Activity
0.1M	0.577e	0.1M	0.350i	0.1M	0.770i	0.1M	0.358a	0.1M	2.222b
0.2M	0.924c	0.2M	0.369h	0.2M	1.743c	0.2M	0.187b	0.2M	1.002c
0.3M	0.932b	0.3M	0.444g	0.3M	1.013f	0.3M	0.077f	0.3M	0.777e
0.4M	0.300g	0.4M	0.302j	0.4M	0.852h	0.4M	0.039h	0.4M	0.551h
0.5M	0.190i	0.5M	0.821c	0.5M	0.980g	0.5M	0.128e	0.5M	0.915d
0.6M	0.231h	0.6M	0.545e	0.6M	1.196e	0.6M	0.137d	0.6M	0.708g
0.7M	0.978a	0.7M	0.583d	0.7M	0.756j	0.7M	0.070g	0.7M	0.752f
0.8M	0.389f	0.8M	0.515f	0.8M	2.300a	0.8M	0.070g	0.8M	0.386i
0.9M	0.174j	0.9M	1.064b	0.9M	1.490d	0.9M	0.022i	0.9M	0.358j
1M	0.602d	1M	1.213a	1M	1.757b	1M	0.171c	1M	2.697a

Time (minutes) Substrate (CMC) Concentration Enzyme concentration (%v/v)

Time (minutes)	Enzyme Activity	Substrate (CMC) concentration (%g/v)	Enzyme Activity	Enzyme concentration %(v/v)	Enzyme Activity
10	1.056e	0.5	0.337e	1	0.365f
20	1.441d	1.0	0.562b	2	0.163i
30	1.527b	1.5	0.520c	3	0.409d
40	0.610j	2.0	0.608a	4	0.335h
50	1.036f	2.5	0.179h	5	1.206a
60	0.743i	3.0	0.365d	6	1.061b
70	2.080a	3.5	0.184g	7	0.351g
80	0.777g	4.0	0.277f	8	0.404e
90	0.754h	4.5	0.157i	9	0.867c
100	1.490c	5.0	0.119j	10	0.035j

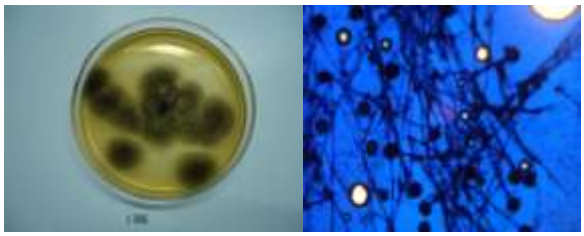

Plate 4.1- *Aspergillus niger*

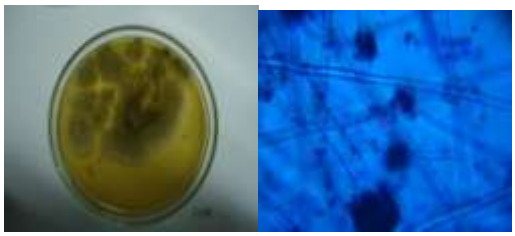

Plate 4.2- *Aspergillus flavus*

Plate 4.3- Aspergillus *terreus*

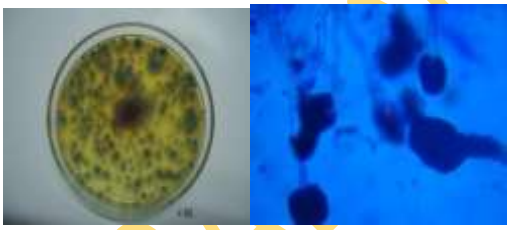

Plate 4.4.-*Aspergillusclavatus*

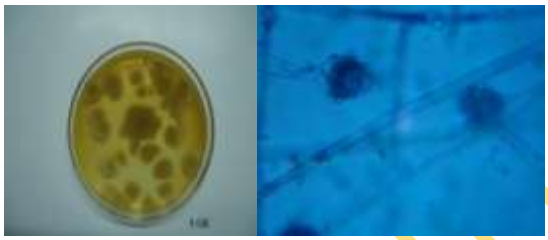

Plate 4:5:- *Aspergillus versicolor*

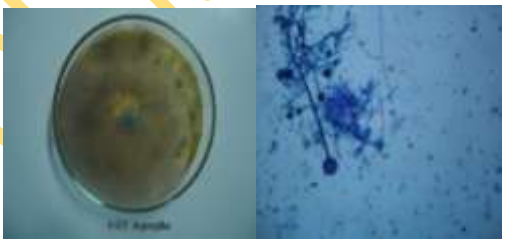

Plate 4:6:- *Rhizopus oryzae*Plate

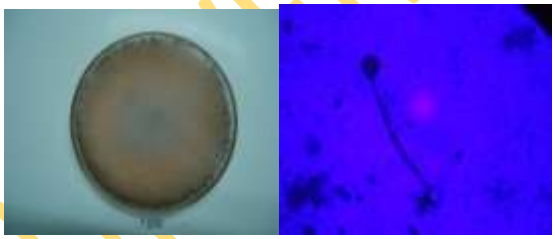

Plate 4:7:- Rhizopus *stolonifer*

Plate 4:8:- Rhizopus *sp.*

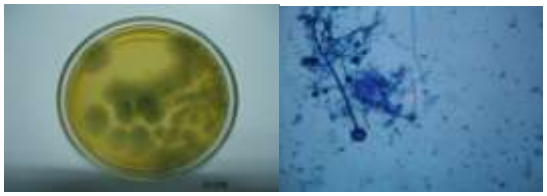

Plate 4:9:- *Aspergillus tamarii*

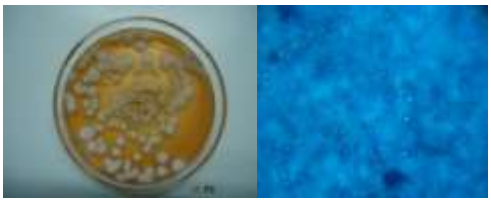

Plate 4:10:- *Trichoderma sp.*