**CHAPTER ONE**

**INTRODUCTION**

* 1. **Introduction:**

Biotechnology offers an increasing potential for the production of goods to meet various human needs. In enzyme technology- a subfield of biotechnology- new processes have been and are being developed to manufacture both bulk and high value added products utilizing enzymes as biocatalysts in other to meet needs such as food (bread, cheese,beer, vinegar) fine chemicals (e.g amino acids, vitamins) and pharmaceuticals. Enzymes are also used to provide services, as in washing and environmental processes or for analytical and diagnostic purposes.

The driving force in the development of enzyme technology, both in academia and in industry, has been and will continue to be development of new and better products, processes and services to meet these needs or the improvement of processes to produce existing products from new raw materials such as biomass. The goal of these approaches is to design innovative products and processes that not only are competitive but also meet criteria of sustainability. The concept of sustainability was introduced by the World Commission on Environment and Development, WCED, 1987 with the aim to promote a necessary “… development that meets the needs of the present without compromising the ability of future generations to meet their own needs.” This definition is now part of the Cartagena protocol on biosafety to the convention on biological Diversity, an international treaty governing the movements of living modified organisms (LMOs) resulting from modern biotechnology from one country to another. It was adopted on January 29, 2000 as a supplementary agreement to the conventionon Biodiversity and entered into forceon September 11, 2003. It has now been ratified by 160 states.

Enzymes, the biological catalysts are considered as nature’s gifts. They function as protein robots inside the cells and speed up the biological processes without undergoing any change (Lanka and Latha, 2015). ). Enzymes are widely applied in the field of scientific research, cosmetic production, medical diagnostics, chemical analyses, therapeutic applications and industrial catalysis (Sharma *et al* 2001b).

Cellulase enzyme has been used for the bioconversion of lignocellulosic to these useful products. The production of bio-based products and bioenergy from less costly renewable lignocellulosic materials would bring benefits to the local economy, environment, and national energy security (Zhang, 2008). Many fungi produce enzymes that enable them to break down polysaccharides and proteins into sugars and amino acids that can be assimilated easily. These enzymes are important industry. Approximately 90% of all industrial enzymes are produced in submerged fermentation (SmF), frequently using specifically optimized, and genetically manipulated microorganisms. In this respect SmF processing offers an insurmountable advantage over Solid state fermentation (SSF). Interestingly, fungi, yeasts and bacteria that were tested in SSF in recent decades exhibited different metabolic strategies under conditions of solid state and submerged fermentation.

Cellulases are the enzymes that hydrolyze β -1, 4 linkages in cellulose chains. They are produced by fungi, bacteria, protozoans, plants, and animals. The catalytic modules of cellulases have been classified into numerous families based on their amino acid sequences and crystal structures (Henrissat, 1989). In nature, complete cellulose hydrolysis is mediated by a combination of three main types of cellulases: (1) endoglucanases (EC 3.2.1.4), (2) exoglucanases, including cellobiohydrolases (CBHs) (EC 3.2.1.91), and (3) β -glucosidase (BG) (EC 3.2.1.21). To hydrolyze and metabolize insoluble cellulose, the microorganisms must secrete the cellulases (possibly except BG) that are either free or cell-surface-bound.

Notwithstanding the fact that only 2% of the world’s microorganisms have been recognized as enzyme sources ( Jayesree *et al*., 2014), microbial enzymes have a number of advantages over enzymes of plant and animal origin including the fact that: they have a greater potential for catalytic diversity, higher productivity within a short period of time, ease of genetic manipulation/optimization, independent of seasonal fluctuations, rapid growth of the producing microorganisms on inexpensive media, greater functional stability (Wiseman, 1995)

**1.2 Aim and Research objectives**

The aim of this research is to produce cellulase enzyme from *Aspergillus* species using *Cyperus esculentus.* The objectives are to determine the,

* effect of temperature,
* effect of pH,
* metal ions,
* And buffer solutions during the assay of cellulose.

**CHAPTER TWO**

**LITERATURE REVIEW**

**2.1 *Cyperus esculentus***

*Cyperus esculentus* or tiger nut is an edible perennial grass like plant and lesser known vegetable that produces sweet nut like tubers known as earth almonds Coskuner *et al* (2002)

The principal component of tiger nut is carbohydrate. The oil content is about 24.5%. *Cyperus esculentus* has a very high content in fiber, starch carbohydrate with 8.91, 29.90. 43.30% respectively (*Vol.11, 2012. Comprehensive reviews in food science and food safety***)** making it a good source of substrate for the production of cellulase

**2.2 Cellulose**

Cellulose is an organic compound with the formular (C6H10O5)n it is a linear polysaccharide of glucose residues connected by β-1, 4 linkages. Figure 2.3 shows the schematic illustration of cellulose chain. Cellulose is an important structural component of the primary cell wall of green plants

**2.3 Substrate pre-treatment**

Some features of natural cellulosic materials are known to inhibit their degradation/bioconversion. These are degree of crystallinity and lignification and the capillary structure of cellulose. The crystallinity and lignification limit the accessibility and susceptibility of cellulose to cellulolytic enzymes and other hydrolytic agents (Fan *et al*., 1987). However, many physical, chemical and microbial pre-treatment methods for enhancing bioconversion of cellulosic materials have been reported (Kansoh *et al*., 1999). Pre-treatment of cellulose opens up the structure and removes secondary interaction between glucose chains (Fan *et al*., 1987).

For enzymatic processes to be effective, some kind of pre-treatment process is thus needed to break the crystalline structure of the lignocellulose and remove the lignin to expose the cellulose and hemicellulose molecules. Depending on the biomass material, either physical or chemical pretreatment methods may be used. Physical methods may use high temperature and pressure, milling, radiation, or freezing; all of which require high-energy consumption. The chemical method uses a solvent to break apart and dissolve the crystalline structure. The lignocellulosic material may be used as is or may be subjected to a pretreatment using conventional method known in the art. For example, physical pretreatment techniques can include various types of milling, irradiation, steaming/steam explosion, and hydrothermolysis; chemical pretreatment techniques can include dilute acid, alkaline, organic solvent, ammonia, sulfur dioxide, carbon dioxide, and pH-controlled hydro hermolysis; and biological pretreatment techniques can involve applying lignin-solubilizing microorganisms.

**2.4 Fermentation**

Fermentation is a chemical reaction in which sugars are broken down into smaller molecules that can be used in living systems. Alcoholic beverages, such as beer, wine, and whiskey, are made from the controlled use of fermentation. Fermentation is an anaerobic process.

Fermentation typically refers to the conversion of sugar to alcohol using yeast. The process is often used to produce wine and beer, but fermentation is also employed in production of cellulase enzyme. The science of fermentation is known as zymology. Fermentation (formerly called zymosis) is the anaerobic metabolic breakdown of a nutrient molecule, such as glucose, without net oxidation. Fermentation does not release all the available energy in a molecule because it merely allows glycolysis (a process that yields two ATP per glucose) to continue by replenishing reduced coenzymes. Fermentation yields lactate, acetic acid, ethanol, or other reduced metabolites. Fermentation is also used much more broadly to refer to the bulk growth of microorganisms on a growth medium. No distinction is made between aerobic and anaerobic metabolism when the word is used in this sense. Fermentation usually implies that the action of the microorganisms is desirable.

In alcoholic fermentation, such as occurs in brewer's yeast and some bacteria, the production of lactic acid is bypassed, and the glucose molecule is degraded to two molecules of the two-carbon alcohol, ethanol, and to two molecules of carbon dioxide. Many of the enzymes of lactic acid and alcoholic fermentation are identical to the enzymes that bring about the metabolic conversion known as glycolysis. Alcoholic fermentation is a process that was known to antiquity

**2.5 Cellulase enzyme**

Cellulase refers to a group of enzymes which, acting together, hydrolyze cellulose. (www.worthington-biochem.com.html). The cellulolytic enzyme may any enzyme involved in the degradation of lignocellulose to glucose, xylose, mannose, galactose, and arabinose. The cellulolytic enzyme may be a multicomponent enzyme preparation, e.g., cellulase, a monocomponent enzyme preparation, e.g., endoglucanase, cellobiohydrolase, glucohydrolase, beta- glucosidase, or a combination of multicomponent and monocomponent enzymes. The cellulolytic enzymes may have activity, i.e., hydrolyze cellulose, either in the acid, neutral, or alkaline pH-range.

*Trichoderma reesei* and *Aspergillus terreus* has an extensively studied cellulase enzyme complex. This complex converts crystalline, amorphous, and chemically derived celluloses quantitatively to glucose. (Henrissat *et al*, 1998)

**2.5.2 Physical and chemical properties**

Most cellulase studied has similar pH optima, solubility and amino acid composition. Thermal stability and exact substrate specificity may vary. However, it should be remembered that cellulase preparations generally contain other enzymatic activities besides cellulase, and these may also affect the properties of the preparations. The optimum pH for cellulase preparations is effective between pH 3 and 7 but the optimum pH generally lies between 4 and 5. Besides that, the optimum temperature is between 40 - 50 °C. (Henrissat *et al*, 1998)

**2.5.3 Stability and storage**

The activity of cellulase preparations has been found to be completely destroyed after 10-15 minutes at 80 °C. Solutions of cellulase at pH 5-7 are stable for 24 hours at 4 °C. These products should be stored at 4 °C, in a dry place in tightly closed containers. If stored in this manner, lyophilized preparation is stable for several months without significant loss of activity. (Henrissat *et al*, 1998)

**2.5.4 Applications**

Cellulase enzyme is used in production of ethanol. The greatest potential for ethanol production from biomass lies in enzymatic hydrolysis of cellulose using cellulase enzymes. Then the other application of cellulase enzyme is various industries such as in alcoholic beverages industries to produce wine. It’s also important in chemicals and food industries. . (Sukumaran *et al*, 2005)

2.6**Cellulase Producing Organisms**

Cellulolytic microorganisms mostly degrade carbohydrates and cannot utilize lipids and proteins as source ofenergy for metabolism and growth. Among them, most important microorganisms are bacteria, cytophaga, cellulomonas can degrade carbohydrates other than cellulose. Anaerobic microbial species have limitedcellulolytic activity restricted to cellulose and its hydrolytic products. *Trichoderma reesai*is the most widely studied fungus and has ability to convert desired as well as native celluloseto glucose. Among most widely studied organisms having notably high cellulolytic activity, include various fungal species like *Humicola*, *Trichoderma*, *Penicillium* and *Aspergillus*. Some bacterial species include; *Pseudomonas*, *Bacilli*, *Actinomycetes*, *streptomycetes*, *Cellumonas*, *Streptomyces* and *Actinomucor*. Because of the ability of fungi to consume cellulose for energy consumption, only certain species could be used practically for cellulose hydrolysis. Despite of *T. reesai*, other fungal species include *Aspergillus*, *Penicillium.* And *Humicola* have practical implementation to produce high yields of cellulases

Certain aerobic bacterial species such as *Cytophaga*, *Cellumonas* and *Cellovibrio* have ability to degrade cellulose in pure culture. The most accepted commercially applicable microbes are *A. niger recombinant*, *T. reesai*, *H. insolens*, *Thermomonas porafusa*, *Bacillus* species and some other organisms (Table 2)

 Table 1: Microorganisms having cellulolytic abilities

|  |  |
| --- | --- |
| Microorganism | Genus |
| Fungi | Soft rot fungi*Aspergillus niger; A. nidulans; A. oryzae; A. terreus; Fusarium solani; F. oxysporum; Humicola insolens; H.**grisea; Melano carpusalbomyces; Penicillium brasilianum; P. occitanis; P. decumbans; Trichoderm areesei; T.**longibrachiatum; T. harzianum; Chaetomium cellulyticum; C. thermophilum; Neurosporacrassa; P. fumigosum;**Thermoascusaurantiacus;Mucorcircinelloides; P. janthinellum; Paecilomycesinflatus; P. echinulatum; Trichoderma**atroviride*Brown rot fungi*Coniophoraputeana; Lanzitestrabeum; Poria placenta; Tyromycespalustris; Fomitopsis sp.*White rot fungi*Phanerochaetechrysosporium; Sporotrichum thermophile; Trametesversicolor;*  |
| Bacteria  | Aerobic bacteria*Acinetobacterjunii; A. amitratus; Acidothermus cellulolyticus; Anoxybacillus sp.; Bacillus subtilis; B. pumilus; B.**amyloliquefaciens; B. licheniformis; B. circulan; B. flexus; Bacteriodes sp.; Cellulomonas biazotea; Cellvibriogilvus;**Eubacterium cellulosolvens; Geobacillus sp.; Microbispora ; Paenibacillus curdlanolyticus*; *Pseudomonas**cellulosa; Salinivibrio sp.; Rhodothermus marinus*Anaerobic bacteria*Acetivibrio cellulolyticus;; Clostridium thermocellum; C. cellulolyticum; C. acetobutylium;* |
| Actinomycetes | *Cellulomonas fimi; C. bioazotea; C. uda; S. lividans; Thermomonos porafusca; T. curvata* |

**2.7 Enzyme assay**

Enzymatic activity is dramatically affected by pH, temperature and substrate concentration. Enzymes are generally active over a specific range of temperature and pH. Model substrates are often employed in place of native substrates in order to assay certain activities.

Enzymes are assayed by measuring the rate at which they produce reducing sugars from their respective substrates. Reducing sugar assays such as the Neslon-Somogyi method or the Dinitrosalicylic acid (DNS) method are used to assay for the product sugars. Reactions are carried out by mixing and incubating a dilution of the enzyme preparation with a known amount of substrate at a buffered pH and set temperature. Xylanase assays are similar to cellulase assays except that a solution of xylan (oat spelts or birch) is substituted for CMC or filter paper. The DNS assay is easier to use than the Nelson-Somogyi assay. The DNS assay is satisfactory for cellulase activities.

**2.8 *Aspergilus species:***

*Aspergilus* consists of a few hundred species and it is defined as a group of conidial fungi- that is fungi in an asexual state some of them are known to be telemorph (sexual state), members of the genus *Aspergilus* can tentatively be considered members of the Ascomycota, Bennett (2010). They have the ability to grow where a high osmotic pressure exists (high concentration of sugar, salt, etc. *Aspergilus* species are highly aerobic and are found in oxygen rich environments where they commonly grow as molds on the surface on the surface of a substrate, as a result of the high oxygen tension.

*Aspergilus* species are common contaminants of starchy food (bread, potatoes) and grow mostly on many plants and trees. (Geisa, 2009).

Table 2: Scientific classification of *Aspergilus* species

|  |  |
| --- | --- |
| Kingdom | Fungi  |
| Phylum | Ascomycota |
| Class  | Eurotiomycetes |
| Order  | Eurotiales |
| Family  | Trichocomaceae |
| Genus  | Aspergilus |

Micheli (1729)

**2.8.1: Importance of *Aspergilus***

Species of *Aspergilus* are both important both medically and commercially. Some causes infection both in animal and plants. For humans a range of diseases such as infection to the external ear, skin lesions and ulcers Thom and Church, (1929).

Other species are important in commercial microbial fermentation example beverages such as Japanese sake which are often made from rice and starchy ingredients (manioc) rather than from malted baileys and grapes

**CHAPTER THREE**

**MATERIALS AND METHODS**

**3.1 Collection of Samples**

Samples were collected fromdecaying wood within the vicinity of Godfrey Okoye University Enugu, Nigeria with sterile sample bottles and transported to microbiology laboratory. Samples were stored at 40C in refrigerator before use.

**3.2. Isolation and Identification of Fungal**

Mould isolation for cellulase was initiated by plating out serially diluted decaying woods in a Potato Dextrose Agar (PDA) using the pour plate technique. The serial dilution procedure and pour plating followed the procedures previously described (Orji *et al*., 2016). Incubation at room temperature was done for five (5) days. Sub-culturing was carried out until pure cultures of *Aspergillus* spp were obtained and the pure *Aspergillus* spp colony was used to produce cellulase. After 4 days of cultivation, a small portion of the mycelia growth was carefully picked with the aid of a sterile wire loop and placed on a drop of lacto phenol cotton blue on a microscope slide and covered with a cover slip. The slide was examined under the microscope with (40x) objective lens for morphological examination as described by Cheesbrough (2006). The isolates were characterized based on the colour of aerial, shape and kind of asexual spores, and the characteristics of spore head.

**Serial dilution of the samples (decayed food and decayed wood)**

* Eighteen (18) test tubes with 9mls of distilled H2O in each were sterilized using the autoclave at 121◦c for 15mins
* One (1) g of each of the sample was weighed using an electric balance
* The test tubes were placed on the rack, 9 test tubes for each sample and labeled101,102,103,104,105,106, 107, 108, 109.
* One ml of the sample was placed in the test tubes labeled 101
* The test tubes containing the decayed food and the other containing the decayed wood were shaken vigorously.
* One ml was pipetted out of the test tube labeled 101using a micropipette and placed in the test tube labeled 102.
* One ml was pipetted from test tube 102 and placed in test tube 103
* The process of dilution was repeated until the last test tube

**Preparation of a potato dextrose agar medium**

* To prepare 42g/L PDA, 6.3g of PDA was weighed using electrical weighing balance. It was solubilized in 150 mL of distilled water using hotplate for 15mins (magnetic stirrer hot plate), autoclaved at 121°c for 15minutes and allowed to solidify. It was then put in the oven at 45°c for 30minutes to dry.

**Pour plate inoculation method**

* Random selection was done and 1ml of 104 and 107from decayed food was pipetted into two petri dishes containing potato dextrose agar and allowed for the fungi to grow. One ml each from 102, 103, 104, 105, 107 dilutions of the sample from decayed wood was pipetted into five petri dishes containing potato dextrose agar and allowed for the fungi to grow.

**Incubation process**

The petri dishes containing PDA was placed in the incubator at 37°c for 5days

**3.3 Screening for Hyper Producing Strains of Fungi and Identification of Moulds**

The screening method previously described with some modification was adopted (Camila *et al*., 2012). Potato Dextrose Agar (Oxoid) was prepared according to the manufacturer’s instruction, and 2% Crystalline Cellulose (Merck) was incorporated into the Potato Dextrose Agar (PDA**).** Under a sterile condition provided by a Bunsen burner, a sterile cork borer was used to bore 6mm holes on the centre of the sterile PDA (containing 2% cellulose). Another sterile cork borer was used to bore a 6mm hole on PDA containing the test organism (*Aspergillus*), and with the help of an inoculating needle the 6 mm agar containing the *Aspergillus* was transferred into the 6mm hole of the sterile PDA (with 2% Cellulose). Incubation was done for 3days at room temperature, after which the surface of the agar was flooded with Grams Iodine for 10 minutes. The excess Gram’s iodine was drained off, and the zone of clearance of the cellulose was measured using meter rule.

**3.4 Enzyme Production Medium and Experimental Design**

Composition of mineral media for cellulase production followed previous studies with modifications (Oyeleke *et al*., 2012, Orji *et al*., 2016).Tigernut (350 grams) or 350mls of mineral medium containing KH2PO43.0 g/l, MgSO4.7H2O 1.0 g/l, CaCl2.H2O 0.5 g/l, ZnSO4.7H2O 1.6 g/L, FeSO4.7H2O0.05 g/l, CoCl2.H2O0.5 g/l. Each set-up had 1% Crystalline cellulose (Merck) used as inducer. The experiment was set-up in a rectangular fermentation tray of 6.6 cm x 95.5 cm dimension. The pH of the amended substrates was adjusted to pH 6.0 with freshly prepared neutral Phosphate buffer. The amended substrates were placed into different muslin bags and labeled according to the time of incubation. Autoclaving was done at 121°C, 15 psi, and a holding time of 15minutes. After autoclaving, the cooled amended substrates were transferred to fermentation pans previously sterilized using hot air oven. Solution of sterile Tween 80 (10 ml) solution was used to wash off spores of seven days old *Aspergillus* spp into the sterile substrates in trays. The fungal mycelia (5.6 x 105 spores/ml of tween 80) were mixed under laminar air flow. The trays were covered with sterile foil paper and incubation was done at room temperature for seven (7) days.

Table 3:Substrate concentration

|  |  |
| --- | --- |
| Substrate | Grams |
| Base 1 | 350 |
| Base 2 | 350 |
| Control | 100 |

* Base 1 was divided into 7 different muslin clothes (50g each)
* Base 2 was divided into 7 different muslin clothes (50g each)
* Base 3 was divided into 4 different muslin clothes (25g each)
* They were then sterilized in the autoclave
* The substrates were mixed with a sterilized mineral medium

Procedures for the preparation of a sterilized mineral medium

Table 4 Materials and reagents

|  |  |
| --- | --- |
| **MATERIALS/ REAGENTS** | **MASS(g)** |
| Cellulose powder | 30 |
| MgSO4.7H2O | 1.0 |
| KH2PO4 | 3 |
| CoCl2 | 0.5 |
| ZnSO4.7H2O | 1.6 |
| FeSO4.7H2O | 0.05 |
| KNO3 | 3.0 |

All the reagents listed in table 2.0 with the exception of cellulose powder were weighed accurately and dissolved in 800ml of distilled water. The cellulose powder was added after autoclaving to avoid denaturation by heat.

* The reagents were weighed using an electric weighing balance and put in a conical flask
* A volume of 250ml of distilled H2O was added into the conical flask containing the reagents and homogenized using a magnetic stirrer.
* After homogenization, 750ml of distilled H2O wasadded to make it up to 1000ml.
* It was then placed in the autoclave for sterilization
* After sterilization, the base 1 and the base 2 substrates which were divided into 50g in 8 places were mixed with 50ml of the mineral medium
* The control was divided into 8 places, 25g each and mixed with 25ml of the mineral medium
* The three samples were placed in the autoclave for 15mims at 121oC
* Fermentation pans were swabbed with ethanol, covered with aluminium foil and kept in the oven for 2h at 100°C for sterilization.
* Base 1 and base 2 which contained 50g each were placed in 8 fermentation trays while 25g each of the control were put in 8 different fermentation pans
* The sterilized distilled H2O was placed on the surface of the culture of *Aspergilus* spp*.* to remove the spores
* The watery spores were put back in the 200ml and used as the inoculum
* A volume of 15ml of the inoculum were put in the substrates weighing 50g (treatment) while 10ml of the inoculum were put in the substrates weighing 25g (control)
* Carboxyl methyl cellulose (CMC) was also added in the samples containing 50g (base 1 and base 2), 0.5g each.

Thus the 15ml of inoculum, 0.5g of CMC and 50g of the base 1 and base 2 each in 8 different fermentation bowels were meshed properly.

* CMC was added in the samples containing 25g (control), 0.25g each.

Thus the 15ml of inoculum, 0.25g of CMC and 25g of the control each in 8 different fermentation bowel were meshed properly.

* The fermentation pans were covered with foil properly to prevent contaminations
* The experiments were placed randomly and samples were collected on 0hrs, 24hrs, 48hrs, 72hrs, 96hrs and 120hrs

**3.5 Pre-treatment of substrates using NaOH**

The substrates were pre-treated with 0.1N NaOH to remove the lignin content in the tiger nut bagasse. The essence of pre-treatment was to loosen up the highly crystalline structure of cellulose, and extend the amorphous areas and also remove lignin. Lignin hinders the activity of microorganisms. 0.1N NaOH was prepared by dissolving 0.8g of NaOH in 200ml of distilled water.

**Procedure**:

The required amount of NaOH was weighed and dissolved in distilled water and the required amount of tiger nut bagasse (100 grams) was weighed into 2 separate trays. Twenty seven (27) mls of NaOH was added, meshed properly and tied in a muslin cloth. These were tagged treatments, and for the control, NaOH was not added. Autoclaving was done at 1210C, 15psi and a holding time of 30 minutes.

After pre-treatment of the tiger nut, it was kept in the oven to dry and measured again

Table 5: Measurement of substrate after pre-treatment

|  |  |
| --- | --- |
| Tiger nut before heating | Tiger nut after heating |
| Base 1 350g | 320.23g |
| Base 2 350g | 310.78g |
| Control 100 | 73.65g |

The base pre-treatment was adopted from Orji *et al (*2016).

Sodium acetate buffer was prepared to wash the substrate in other to stabilize its pH for the microorganism to function properly.

**Preparation:**

A volume of 6.7ml of acetic acid was measured and 1193.1ml of DH2O was added to make up to 1200ml and 2.04g of sodium acetate was measured and dissolved in 1500ml of DH2O into a conical flask and the pH was adjusted. 1600ml of the buffer was measured and used to wash each of the 16 substrates.

The buffer was drained out using funnel and placed in an incubator for 3-4days

**3.6 Enzyme Extraction and determination of cellulase activity**

The cellulose enzyme was extracted and assayed every 24hour for 7 days using sterile 0.05 M acetate buffer of pH 5.0. In order to extract the extracellular enzyme, 3000 ml of the 0.05 citrate buffer were sterilized, and poured into each fermentation tray containing 300 grams of fermented tiger nut substrate, and after gentle agitation for 30 minutes, the enzyme –buffer solution was filtered using sterile muslin cloth. The cellulase extract was preserved using 5grams of sodium meta-bisulphite under 4°C until required for assay. The activity was determined according to the method previously reported by Ogwuche *et al,* (2012) with modifications. The reducing sugar product was assayed by the dinitrosalicylic acid (DNSA) method (Bertrand *et al*., 2004), using glucose as the sugar standard. One unit of activity was defined as the amount of enzyme required to release 1μm of reducing sugar in glucose equivalent per-minute under the assay conditions.

**3.7Optimization of pH:**

The effect of pH on cellulase production was carried out by growing the *Fungi* species at different pH range (5.5-10). Cellulase activity was measured by assaying for glucose using the T80+ UV/VIS spectrophotometer at 540nm wavelength

**3.8 Effects of Inoculum size**

The inoculum size based on the number of spores was studied using spores concentration of 1 x 103, 104 and 105 cfu/ml. The study was run for 5days and for each 24 hours crude cellulase activity was determined using the method previously reported by Ogwuche *et al.* (2012)

**CELLULASE CHARACTERISATION**

**3.9Determination of the Optimum pH for Cellulase**:

This was achieved by determining activity of the crude cellulase enzymes at varying pH range (3.0- 9.0).The reaction mixture comprised 0.5ml of the crude enzyme with 0.5ml of 1% of CMC in 0.1M sodium acetate buffer. Incubation was carried out for 30minutes at 50°C according to the method previously described by Ogwuche *et al*. (2012).

**3.10Determinationof Optimum Temperature for Cellulase:**

The determination of the optimum temperature was carried out at the optimum pH. Cellulase activity at varying temperatures (25°C to 50°C) was determined by using CMC as the substrate for the assay. The reaction mixture comprised of 1% CMC in 0.1M sodium acetate buffer, 0.5ml of the crude enzyme and were allowed to react at 50°C for 30minutes.

**3.11 Effects of different buffer solutions**

Concentrations of 0.1M of various buffers (sodium citrate, sodium acetate, sodium phosphate and distilled water as the control) were used to study the optimum solution for the cellulase activity. The buffers were prepared by dissolving 0.105gram of citric acid in 50mls of distilled water, 0.147gram of sodium citrate in 50mls. A 31.5mls of citric acid was mixed with 18.5mls of sodium citrate diluted to 100mls and pH adjusted to 6.5.

* NaH2PO4.H2O (1.331grams) was dissolved in 100mls,
* K2HPO4.3H2O (2.2822grams) was dissolved in 100mls.
* NaH2PO4.H2O (70mls) was mixed with 30mls of K2HPO4.3H2O (pH 6.5).
* Acetic acid (0.23mls ) was dissolved in 39.77mls of distilled water,
* 0.49218grams were dissolved in 60mls.
* Acetic acid solution (40mls) was mixed with 60mls of sodium acetate and pH was adjusted to 6.5.

**3.12 Determination of Effect of Metal Ions**

The reaction of the enzyme and 1% CMC was allowed to proceed at 45°C with duplicate test tubes containing 10mM of Mg, MnSO4, ZnSO4, and FeSO4. Test tubes were labeled according to the above mentioned salts. 0.5 ml of the crude enzyme was pipetted into the labeled test tube already loaded with 0.5 m of CMC. Thereafter, 0.5 ml of 10 mM solution of each of the above mentioned salts (one salt to one test tube) was pipetted into a test tube. The incubation of their action mixture was done at 45°C, and the assay done using previously described DNSA Method (Miller G 1948; Orji *et al*. 2013). Tubes containing distilled water and substrates (without the enzyme) but treated in the same way as sample served as the blank.

**CHAPTER FOUR**

**RESULTS**

The population of fungi in various decayed natural cellulose samples including decayed wood and food is presented in Table 8. The results show that the number of colonies isolated ranged from 5.4× 107 to 5.0 × 109, and from 1.88 × 106 to 5.1 × 108 CFU/g, respectively (Table 7).Zone of clearance from this study ranged from 1.2 to 5.7cm with DW3 (Table 8). When the reaction pH was varied from 3 to 9, the enzyme activity varied from 41610 ± 1670mol to 63640 ± 15870μmol (Table 9). The highest activity was obtained at pH of 6.5. When the effect of temperature was investigated, the enzyme activity ranged from 42800.97 ± 182.4689 to 59,901.35 ± 4005.392μmol. The highest activity of 50,490μmol was obtained at a temperature of 50oC (Table 10). The effect of different buffer solutions on the cellulase activities showed that the activities ranged from 38264.81 ± 818.88 to 43098.18 ± 284.33μmol as shown in Table 11.The activity was highest in distilled water. The observed cellulase activity for the metal ions Zn, Mg, Mn, and Cu ranged from 26362.65 ± 455.36 to 45116.78± 227.46μmol (Table 12). Both Fe and Mn stimulated cellulose activity but addition of Mn resulted in the highest cellulase activity.

**4.1 Fungi population in samples of decayed wood and food:**

The petri dish labeled 103 had the highest number of colonies recorded at 220 × 103

Table 6: Fungi population in samples of decayed wood and food

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| S/N | Sample | Volume(ml) | Dilution factor | No of colonies | Cfu/ml |
| 1 | Decayed wood | 1 | 103 | 220 | 220 × 103 |
| 234 | Decayed woodDecayed foodDecayed food | 111 | 105103105 | 5611 | 56×1051 × 1031 × 105 |

**4.2 Zone of clearance of fungal isolates from various sources:**

DW3 with the highest zone of clearance was preliminarily identified as *Aspergilus* species, the identified *Aspergilus* species was further studied for Cellulase production and activity at various physical and chemical parameters

Table 7: Zone of clearance of fungal isolates from various sources

|  |  |  |
| --- | --- | --- |
| S/N | Isolate code | Zone of clearance (cm) |
| 1 | DF-3 | 0 |
| 2 | DF-5 | 0 |
| 3 | DW-3 | 5.7 |
| 4 | DW-5 | 2 |

DF: Decayed food; DW: Decayed wood.

**4.3 EFFECT OF pH ON CELLULASE ACTIVITY**

The pH optima of the enzyme was found to be 6.5 with the activity of 63637.6 ± 15877.25μmol, However *Aspergilus* spp. showed lowest activity at pH 3 with the activity of 41618.42 ± 1671.89μmol as shown in table (8)

Table 8: EFFECT OF pH ON CELLULASE ACTIVITY

|  |  |
| --- | --- |
| pH | CELLULASE ACTIVITIES ±S.D (μmol) |
| 3 | 41618.42 ± 1671.89 |
| 4 | 48591.01 ± 7392.709 |
| 4.5 | 42293.96 ± 375.3181 |
| 4.8 | 43587.97 ± 2978.73 |
| 5 | 43854.15 ± 2058.586 |
| 5.5 | 50110.98 ± 5675.324 |
| 6 | 42905.17 ± 1444.421 |
| 6.5 | 63637.6 ± 15877.25 |
| 7 | 44787.04 ± 2104.081 |
| 9 | 52676.45 ± 3184.547 |

Results are means ± standard deviation of replicate determinations

**4.4 EFFECT OF TEMP (°C) ON CELLULASE ACTIVITY**

Enzyme activity recorded at different temperatures showed that cellulase activity was maximum at 50°C with activity of 59901.89 ± 4219.525μmol

Table 9: EFFECT OF TEMP (°C) ON CELLULASE ACTIVITY

|  |  |
| --- | --- |
| TEMPERATURE(°C) | CELLULASE ACTIVITIES ±S.D (μmol) |
| 30 | 50490.35 ± 4005.392 |
| 40 | 47336.42 ± 8666.527 |
| 50 | 59901.89 ± 4219.525 |
| 60 | 42937.34 ± 1216.952 |
| 70 | 42800.97 ± 182.4689 |
| 80 | 43471.56 ± 1444.428 |

Results are means ± standard deviation of replicate determinations

* Enzyme activity recorded at different temperatures showed that cellulase activity was maximum at 50°C with activity of 59901.89 ± 4219.525μmol.

**4.7Effect of different buffer on cellulase activity**

* Distilled water recorded the highest activity with 43098.18 ± 284.33 while sodium acetate recorded the lowest with 38264.81 ± 818.88.

Table 10: Effect of different buffer on cellulase activity

|  |  |
| --- | --- |
| Buffer | CELLULASE ACTIVITIES ±S.D (μmol) |
| Na-acetate | 38264.81 ± 818.88 |
| Na-citrate | 42422.63 ± 261.58 |
| Na-phosphate | 41168.05 ± 420.82 |
| Distilled water | 43098.18 ± 284.33 |

Results are means± standard deviation of replicate determinations

**4.8Effects of Metal ions on *Aspergilus* cellulase activity**

Heavy metals such as Zinc and Magnesium reduced the specific activity of cellulase from 38530.21 ± 625.54 to 26965.52 ± 1398.93 and31268.10 ± 4321.88μmol while manganese, and iron at 10mM concentration were able to increase the *Aspergillus* species cellulase activity from 38530.21 ± 625.54 to44521.65 ± 272.95 and 40556.85 ± 125.10μmol, respectively (Table 11).

Table 11:Effects of Metal ions on *Aspergilus* cellulase activity

|  |  |
| --- | --- |
| Metals | CELLULASE ACTIVITIES ±S.D (μmol) |
| Mn | 45116.78± 227.46 |
| Mg | 26362.65 ± 455.36 |
| Zn | 34026.57 ± 307.08 |
| Fe | 43379.66 ± 1865.24 |
| Distilled water/ Control | 40291.45 ± 1455.79 |

**CHAPTER FIVE**

**DISCUSSION AND CONCLUSION**

**5.1 Screening for Hyper Producing Strains of *Aspergilus spp,* and identification**

The population of fungi in decayed wood and food ranged between 220 × 103, 56×105 and 1 × 103 to1 × 105CFU/g, respectively (Table 6).In addition, out of a total of 4isolates screened for cellulase production potential, only 2 showed good promises. Notably, isolate DW3and DW5, which had the highest zones of clearance of 5.7cm and 2cm, respectively (Table 7). The result does not correspond to the findings of Kluczek-Turpeinen *et al.* (2005). The morphological and biochemical properties of the cellulase producing fungal isolates showed that the 9 isolates studied were tentatively members of the genera: *Aspergilus* and *Trichoderma*.

**5.2 Effect of pH on *Aspergilus* cellulase activity**

The effect of various pH on the activities of cellulase was also studied. pH was varied between 3.0 and 9.0.The pH optima of the enzyme was found to be 6.5 with the activity of 63637.6 ± 15877.25μmol (table 8). Furthermore, the enzyme was active under acidic to alkali condition (pH 3 to 9). This means that they have potentials for application in pulp, paper, juice, fruit processing, and textile industries. Ogwuche *et al*. (2012) reported that cellulase from *Aspergillus* and *Rhizopus* species showed their highest activities at pH 7 and 6.5 respectively. Gilna and Khaleel (2011) have also reported that pH of 6.5 was the optimum for cellulase from *Aspergillus fumigatus*, Jecu (2000) also reported pH 6.0 as the optimum for cellulase obtained from *Aspergillus species*.

**5.3 Effect of Temperature on *Aspergilus* cellulase activity**

Cellulases are known to be active in a wide range of temperature (40- 90ºC) and pH (4 - 11). The optimum temperature of this cellulase was 50ºC with activity of 59901.89 ± 4219.525μmol (Table 9). The enzymes show high activity and stability within the temperature range of 30 to 80ºC studied. A similar finding has been reported by Hagihara *et al*., (2001). Furthermore, Saxena *et al*. (2011), reported the optimum temperature of *Bacillus* specie amylase to be 50ºC.

**5.4 Effects of Buffer solutions**

The activity of crude cellulase from *Aspergillus* species on several of buffer solutions was studied. The crude cellulase activities were 38264.81 ± 818.88μmol, 42422.63 ± 261.58μmol, 41168.05 ± 420.82μmol, 43098.18± 284.33μmol for sodium acetate, sodium citrate, sodium phosphate and distilled water respectively (Table 10). Surprisingly, distilled water recorded the highest activity in this study while Sodium acetate recorded the lowest activity.

**5.5 Effects of Metal ions on *Aspergilus* cellulase activity**

Effects of metallic ions on the *Aspergillus* species cellulase was investigated. Heavy metals such as Zinc and Magnesium reduced the specific activity of cellulase from 38530.21 ± 625.54 to 26965.52 ± 1398.93 and 31268.10 ± 4321.88μmol respectively. (Table 11). These metal ions inhibitory mechanism could be that they bind initially to active site before substrate thus reducing the chances of substrate binding. This establishes a competitive inhibition between the substrate and metallic ions for the active site of the enzyme. On the contrary, manganese, and iron at 10mM concentration were able to increase the *Aspergillus* species cellulase activity from 38530.21 ± 625.54 to44521.65 ± 272.95 and 40556.85 ± 125.10μmol, respectively (Table 11). Thus the manganese and zinc ion could be co-factors for *Aspergillus* cellulase. Also, manganese has been previously reported as a co-factor to *Bacillus*spcellulose (Orji *et al*. 2016). In a related report, it was shown that a cellulase enzyme from *Cellulomonas* was stimulated by Co2+ and Mn2+ while Hg2+ and Fe2+ reduced its activity Irfan *et al*, (2012).

**Conclusion**

The present study clearly shows that *Aspergilus species* can be successfully optimized and cultivated under solid state fermentation for the production of cellulase using Tiger nut bagasse as substrate. In other words, the Tiger nut bagasse waste can be turned into wealth. Local production of cellulase in Nigeria using local raw materials such as Tiger nut reduces enzyme cost, reduce the cost of products that require enzymes and thus increase the nation’s Gross Domestic Products.

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**APPENDIX**

**LAB FLOW**





