PRODUCTION OF ALPHA AMYLASE BY BACILLUS SUBTILIS USING MAIZE HUSK AS SUBSTRATE

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DEPARTMENT OF MICROBIOLOGY,
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AUGUST, 2017

DECLARATION

I declare that the work in this dissertation entitled "Production of alpha amylase by *Bacillus subtilis* using maize husk as substrate" has been performed by me in the Department of Microbiology, Ahmadu Bello University, Zaria. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this dissertation was previously presented for another degree or diploma at this or any other institution.

Abubakar MADIKA_____

Signature Date

CERTIFICATION

This dissertation entitled "PRODUCTION OF ALPHA AMYLASE BY BACILLUS SUBTILIS USING MAIZE HUSK AS SUBSTRATE" by Abubakar MADIKA meets the regulations governing the award of the degree of Master of Science in Microbiology of the Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

This piece of work is dedicated to Almighty Allah and my late mother Fatima Abdullahi.

ABSTRACT

Bacillus subtilis was isolated and characterized from soil samples of different locations namely flower bed, botanical garden and refuse dump site using conventional cultural and biochemical methods followed by microgen identification. *B. subtilis* isolates were screened for amylase production using starch agar plate method. Studies were conducted to produce α-amylase by the *Bacillus subtilis* using maize husk as substrate. Proximate composition of the maize husk substrate was determined following standard procedures described by Association of Official Analytical Chemists. Effects of incubation temperature, initial pH, incubation period and moisture content on the production of α-amylase were investigated. Alpha amylase was producedby the best α-amylase producing *B. subtlis* isolate using maize husk as substrate by solid state fermentation under predetermined optimum fermentation conditions. Nine (9) isolates were confirmed to be *B. subtilis* with percentage occurrence of 80% each from refuse dump site and botanical garden with only 20% from flower bed. All the *B. subtilis* isolates demonstrated amylase production ability while isolate RD4 from refuse dump site had the highest diameter of zone of starch hydrolysis (29mm). The proximate composition of the maize husk revealed percentages of ash (4.83%), crude protein (7.00%) and carbohydrates (60.37%) contents. All parameters optimized were found to have significantly ($p< 0.05$) influenced α-amylase production using maize husk. An overall yield of 51.65±0.13 U/g of α-amylase was obtained under predetermined optimum fermentation conditions (35^{o}C, pH 7.0, 72hours and moisture content of 1:2). *Bacillus subtilis* can readily be isolated from various soil types with frequency of 80% in soils from garden and refuse dumps. Maize husk contains sufficient organic nutrients for growth and amylase production by *B. subtilis*. The study reveals the potential of maize husk as substrate in the production of alpha amylase by *B. subtilis*.

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ABBREVIATIONS AND SYMBOLS

ANOVA	Analysis of Variance
AOAC	Association of Official Analytical Chemists
BG	Botanical garden
BSA	Bovin Serum Albumin
CFU/ml	Colony Forming Units per millilitre
CL	Corncob leaf
cm	Centimeter
DEAE	Di Ethyl Amino Ethyl
df	Degree of freedom
DNS	Dinitrosalicylic acid
E.C	Enzyme Commission
FB	Flower bed
g	Gram
g/l	Gram per litre
GRAS	Generally Recognized as Safe
H_2SO_4	Tetra-oxo-sulphate (VI) Acid
kg	Kilogram
KOH	Potassium Hydroxide
LB	Luria Bertani
min	Minutes
NaOH	Sodium Hydroxide
nm	Nanometer
OD	Optical Density
ONPG	Ortho-nitrophenyl Galactopyranoside
RD	Refuse dump site
RF	Retention Factor
rpm	Revolution per minute
RS	Rye straw
RSM	Response Surface Methodology
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
Sec	Seconds
SmF	Submerged Fermentation

SSF	Solid State Fermentation
TCA	Trichloroacetic acid
TLC	Thin Layer Chromatography
v/v	Volume per volume
VP	Voges-Proskauer
w/v	Weight per volume
WB	Wheat bran
WS	Wheat straw
%	Percentage
p	Probability
U/g	Unit per gram
mm	Millimeters
α	Alpha
β	Beta
γ	Gamma
^{o}C	Degree Celsius
kDa	Kilo Dalton
U/ml	Unit per millilitre
U/cfu	Unit per colony forming units

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background to the Study

Amylase is one of the most widely used enzymes in theindustry such as textile, paper, detergent, food, fermentation and pharmaceutical. It hydrolyzes starch which is used commercially for theproduction of sugar syrups which consists ofglucose, maltose, and higher oligosaccharides (Saxena and Singh, 2011).

Amylases are widely distributed in nature and derived from various sources such as plants, animals and microorganisms.Microbial amylases have successfully replaced chemical hydrolysis of starch in starch processing industries. Amylase had been obtainedfrom several fungi, yeasts, bacteria and actinomycetes, however, enzymes from fungal and bacterial sources had dominated applications in industrial sectors (Kalaiarasi and Parvatham, 2013). Fungal sources are confined to terrestrial isolates, mostly to *Aspergillus* species andone species of *Penicillium*namely;*P. brunneum* (Das *et al.*, 2011). Bacterial amylases are derived from *Bacillus subtilis*, *B. amyloliquefaciens, B. licheniformis* and *B. stearothermophilus*, *B. gavealeus, B. mesentericus, B. myocodes, B. polymyxa*, *B. vulgates* and *B. aterrimus* (Das *et al*., 2011). Both fungi and bacteria are widely used for the production of alpha amylases but bacteriaare preferred because of several characteristic advantages they offer. Some of whichincludepotential of *Bacillus* spp. to produce alpha amylases in solid and liquid state fermentation systems and ease of isolating thermostable enzymes from bacteria (Singh,2016).

Two major classes of amylases have been identified in microorganisms, namely α-amylase and glucoamylase. α-Amylases(endo-1, 4-α-D-glucan glucohydrolase, E.C. 3.2.1.1) are extracellular enzymes that randomly cleave the α-1,4-D glucosidic linkages

between adjacent glucose units in the linear amylose chain. Glucoamylase (exo-1, 4-α-D-glucan glucanohydrolase, E.C. 3.2.1.3) hydrolyzes single glucose units from the non-reducing ends of amylose and amylopectin in a step-wise manner (Ibrahim *et al.*, 2012).

Amylases have found wider applications in different industries such as the food, fermentation, textile, paper, detergent and pharmaceutical industries (Souza and Magalhaes, 2010).

The major advantage of using microorganisms for the production of amylases is the economical bulk production capacity independent of seasons and the fact that microorganisms are easy to manipulate to obtain enzymes of desired characteristics (Goma, 2013). Studies on bacterial amylase especially in the developing countries have concentrated mainly on *Bacillus* spp. probably because of the simple nature and nutritional requirements of these organisms (Oyeleke *et al.*,2010).

The aerobic, endospore-forming bacteria of the genus *Bacillus* had for long dominated the market for α-amylase along with the "liquefying" enzymes from *B. amyloliquefaciens*, *B. subtilis* and more recently *B. licheniformis* (Ubalua, 2014).

Agro wastes have been found to be good substrates for the cost effective production of several hydrolytic enzymes including alpha amylase and are thus attracting the attention of researchers for exploring different agro wastes as substrates for alpha amylase production (Shinde *et al*., 2014). In this regard, brans and flours of different grains and tubers, such as corn, rice, sorghum and wheat and peels of cassava and potato had been used inthe development of fermentation medium to increase the productivity of amylases from bacteria and fungi (Unakal *et al*., 2012).

Production of α-amylase had beencarried out through submerged fermentation (SmF) and solid state fermentation (SSF)(Saxena and Singh, 2011). In recent years, the technique of solid state fermentation (SSF) process had been developed and used more extensively. It offers several advantages over SmF and had been reported to be the most appropriate process for developing countries (Goma, 2013).Thus, solid state fermentation (SSF) is defined as the cultivation of microorganisms on moist solid supports, either on inert carriers or on insoluble substrates that can, in addition, be used as carbon and energy sources.The fermentation takes place in the absence or near absence of free water, thus being close to the natural environment to which microorganisms are adapted(Ibrahim *et al.*, 2012).

Themajor factors that affect microbial production of enzymes in SSF system include selection of a suitable substrate andmicroorganism, particle size and moisture content of the substrate, and inoculumconcentration.All of which involve the screening of a number of agro-industrial materialsas well as microorganisms for enhanced microbial growth and product formation (Saxena and Singh, 2011).

1.2 Statement of Research Problem of the Study

Growth conditions and nutrients promote high yields of microbial amylases. Carbon sources such as dextrin, fructose, glucose, lactose, maltose and starch are very expensive for commercial production of these enzymes (Unakal *et al*., 2012).

Production of alpha amylase via SmF system is known to cause certain potentialproblems which include the presence of product in lowconcentration, handling problems, reduction anddisposal of large volumes of water duringdownstream processing (Das *et al*., 2011).

There are many baking, pharmaceutical, brewing and food industries in Nigeria that utilizes amylase for their production processes. However, amylase enzymes are yet to be produced commercially in Nigeria, making the cost of procurement high as a result of importation (Ominyi, 2013).

Large quantities of agricultural residues accumulate every year which results not only in the deterioration of the environment, but also in the loss of potentially valuable materials which can be processed to yield a number of value added products such as food, fuel anda variety of chemicals (Damisa, 2007).

1.3 Justification for the Study

Amylase occupies amajor share of about 25% of the total world enzyme market owing to its high demand,thereby minimizing chemical hydrolysis of starch in theliquefaction process (Rajagopalan and Krishnan, 2008).Amylases are generally known to be of great significance in several different biotechnological applications. To meet these high and diversified demands, low cost production of amylase is required.

Synthetic medium tend to be very expensive and uneconomical, as such there is need to come up with more economically available agricultural and industrial by-products that can serve as good substrates for enzyme production through solid state fermentation (Goma, 2013).With the advent of biotechnological innovations especiallyin the area of fermentation technology and enzymeproduction, the use of SSF system on agro-wastes like maize husk, rice bran, banana waste, sugar cane bagasse could reduce the high cost of media generally usedin submerged fermentation for alpha amylase production.*Bacillus* species are frequently used for α-amylase productionwith the aid of SSF using wheat bran (Vijayabaskar *et al*., 2012).

Submerged fermentation (SmF) has been a traditional way of producing industrially important enzymes based on several factors associated with modelling and controlling of environmental factors such as the pH, temperature, level of aeration, and moisture content (Raul *et al*., 2014). However, SSF replaces SmF as it mimics the natural habitat of microorganisms. Solid state fermentation (SSF) is a better choice over SmF due to its simplicity of the technique, higher productivity, low capital investment, lower energy requirement, less water output, better product recovery and lack of foam built up (Couto and Sanrom'an, 2006; Tanyildizi *et al.*, 2007).

In Nigeria, local production of amylase will save about 200 million naira that is spent annually for the importation of amylase (Oyeleke and Oduwole, 2009). Hence, production of amylase locally in Nigeria is of great significance.

The optimization of fermentation conditions, particularly physical and chemical parameters are important in the development of effective fermentation system due to their impact on the economy and practical application of the process (Goma, 2013).

The utilization of Maize husk in this study may provide a cost effective substrate for α-amylase production.

1.4Aim of the Study

The aim of the study was to produce α-amylase with *Bacillus subtilis* using maize husk as substrate

1.5 Objectives of the Study

The specific objectives of this study were to:

1. determine the proximate composition of maize husk.
2. isolate and characterize *Bacillus subtilis* fromsoil samples.
3. screen the *Bacillus subtilis* isolates for amylase production.

4. determine the effects of someoptimization parameterson the production of α-amylase by the selected isolate.
5. quantify the α-amylase produced from maize husk using selected *B. subtilis* isolate by solid state fermentation under optimum conditions.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Amylases

Amylases are enzymes which hydrolyze starch molecules into simple sugar units of glucose and maltose. Alpha amylase is ubiquitous in distribution, with plants, bacteria and fungi being the major sources. Most of the microbial alpha amylases are hydrolases, and they contributed numerous common properties. But different reaction specificities have been observed across the family members. Structurally alpha amylase possesses barrel structures and is responsible for hydrolysis or formation of glucosidic bonds in the α-conformation (Mesfin, 2015).

Amylase enzyme is of great importance in biotechnological application ranging from food, fermentation, and textile to paper industries. Alpha amylase is a key enzyme in metabolism of spacious diversity of living organisms which utilize starch as carbon and energy sources. It can hydrolyze starch, glycogen and related polysaccharides by randomly cleaving internal α-1, 4 glucosidic linkages to produce different sizes of oligosaccharides (Asgher *et al*., 2007).

Of great significance are amylases ofmicrobial origin, such as those from bacterial andfungal sources, which have dominated applications in industrial sectors, as they meet process demands, and have successfully replaced the chemical hydrolysis of starch in starch-processing industries (Singh, 2016).

2.2 Classification of Amylases

Amylases are classified into three subtypes, viz., *α*-amylase, *ß*-amylase and *γ*-amylase. Unlike *ß*-amylase, *α*- and *γ*-amylases are produced in animal systems abundantly, and all these three enzymes are produced by plants, yeast, fungi and bacteria. Four groups

of starch converting enzymes have been identified viz., endoamylases, exoamylases, debranching enzymes and transferases. Endoamylases cleave α-1,4glycosidic bonds present in the amylose or amylopectin chain, the structural components of starch made of sugars in a random fashion.α-Amylase is anexample of endoamylase whereas ß-amylase and glucoamylase are exoamylases and cleave both α-1,4, and/or α-1,6 glycosidic bonds (Akkaya *et al*., 2012).

2.2.1Alpha amylase

Alpha amylase(EC3.2.1.1) is alternatively known as 1,4-α-D-glucan glucanohydrolase or glycogenase (Benjamin *et al*., 2013). The α-amylases are calcium metalloenzymes, i.e. require calcium ion for its function (Souza and Magalhaes, 2010). By acting at random locations along the starch chain, α-amylase breaks down long-chain carbohydrates, ultimately yielding maltose and maltotriose from amylose or maltose, glucose and limit dextrin from amylopectin (Rajagopalan and Krishnan, 2008). The hydrolytic products have α-configuration.α-Amylase tends to be faster acting than ß-amylase because it can act anywhere on the substrate (Liu *et al*., 2010).

2.2.2 Beta amylase

Beta amylase(EC3.2.1.2) is synonymous as 1,4-α-D-glucan maltohydrolase, glycogenase or saccharogen amylase synthesized by bacteria, fungi and plants (Benjamin *et al*., 2013). Working from the non-reducing end, ß-amylase catalyzes the hydrolysis of the second α-1,4 glycosidic bond, cleaving off two glucose units (maltose) at a time (Bijttebier *et al*., 2007). During the ripening of fruit, ß-amylase breaks starch into sugar, resulting in the sweet flavour of ripe fruits. ß-amylase appears prior to germination, whereas α-amylase and proteases appear once germination has begun. Amylase from the cereal grains is the key source to the production of malt. Many

microbes also produce amylase to degrade extracellular starches (Benjamin *et al*., 2013). Animal tissues do not possess ß-amylase, although it may be present in microorganisms contained within the digestive tract (probiotics).

2.2.3 Gamma amylase

Gamma amylase(E.C3.2.1.3) is also known as glucan 1,4-α-glucosidase, amyloglucosidase, exo-1,4-α-glucosidase, glucoamylase, lysosomal α-glucosidase or 1,4-α-D-glucan glucohydrolase. In addition to cleaving the last α-(1,4)-glucosidic linkages at the non-reducing end of amylose and amylopectin yielding glucose, γ-amylase also cleave α-1,6-glycosidic linkages (Tateno *et al*., 2007). Unlike the other forms of amylases, γ-amylase is best active in acidic environments (Kumar and Satyanarayana, 2009).

2.3 Sources of Alpha Amylase

2.3.1 Plant sources

Amylases are widespread in animals, fungi, plants, and are also found in the unicellular eukaryotes, bacteria and archaea (Da Lagea *et al*., 2007). Though plants and animals produce amylases, enzymes from microbial sources are generally used in industrial processes. This is due to a number of factors including productivity, thermostability of the enzyme as well as ease of cultivating microorganisms (Tiwari*et al*., 2015). The major advantages of the enzymatic route are the selectivity with its associated high yield and exclusively toward the desired product (Kim and Dale, 2004). Bacteria used in commercial production are the *Bacillus* spp. (Benjamin *et al.*, 2013).Others, such as *Escherichia* spp., *Pseudomonas, Proteus, Serratia* and *Rhizobium* also yield appreciable quantity of the enzyme (Oliviera *et al.*, 2007). *Aspergillus, Rhizopus, Mucor,*

Neurospora, Penicillium and *Candida* are some of the fungi that also produce extracellular amylases of commercial value (Gupta *et al*., 2003).

Plant sources had not been considered with enough significance as the source of these enzymes yet (Azad *et al*., 2009). The utilization of agricultural waste materials serves two functions: reduction in pollution and upgrading of these materials. Agricultural wastes are being used for both liquid and solid fermentation to reduce the cost of fermentation media. These wastes consist of carbon and nitrogen sources necessary for the growth and metabolism of organisms. These nutrient sources included pearl millet starch, orange waste, potato, corn, tapioca, wheat and rice as flours were used for α-amylase production (Tiwari *et al*., 2015). α-Amylases are being produced commercially in bulk from microorganisms and represent about 25-33% of the world enzyme market (Raul *et al.*, 2014). They had numerous applications including liquefaction of starch in the traditional beverages, baking and textile industry for desizing of fabrics (Souza and Magalhaes, 2010). Moreover, they have been applied in paper manufacture, medical fields as digestives and as detergent additives (Ajita and Thirupathihalli, 2014).

2.3.2 Animal sources

Ptyalin, a salivary α-amylase (α-1,4-α-D-glucan-4-glucanohydrolase; E.C.3.2.1.1) is one of the most important enzymes in saliva. The enzyme was first described in saliva by Leuchs in 1831. It consists of two families of isoenzymes, of which one set is glycosylated and the other contains no carbohydrate. The molecular weight of the glycosylated form is about 57 kDa; that of the non-glycosylated form is about 54kDa. Salivary amylases accounts for 40% to 50% of the total salivary protein and most of the enzyme are being synthesized in the parotid gland (80% of the total) (Tiwari *et al*., 2015). It is a calcium-containing metalloenzyme that hydrolyzes the α-1,4 linkages of

starch to glucose and maltose. It is known to be mainly involved in the initiation of the digestion of starch in the oral cavity. However, salivary α-amylase has also been shown to have an important bacterial interactive function (Azad *et al*., 2009).

2.3.3 Microbial sources

In spite of the wide distribution of amylases, microbial sources, mainly fungal and bacterial amylases, are used for the industrial production due to advantages such as cost effectiveness, consistency, less time and ease of process modification and optimization (Das*et al*., 2011). Fungal amylases have been widely used for the preparation of oriental foods (Ivanova *et al*., 2001). Among bacteria, *Bacillus* sp. is widely used for thermostable α-amylase production to meet industrial needs. *B. subtilis, B. stearothermophilus, B. licheniformis* and *B. amyloliquefaciens* are known to be good producers of α-amylase and these have been widely used for commercial production of the enzyme for various applications (Das*et al*., 2011). Similarly, filamentous fungi have been widely used for the production of amylases for centuries. As these moulds are known to be prolific producers of extracellular proteins, they are widely exploited for the production of different enzymes including α-amylase.

2.3.3.1 *Fungal amylases*

Fungi belonging to the genus *Aspergillus* have been most commonly employed for the production of α-amylase. With the development of genetic engineering, *Bacillus subtilis* is becoming an increasingly attractive host for cloning. The advantages of *B. subtilis* such as high secretion level and non-pathogenic safe (GRAS-generally recognized as safe) status for non-antibiotics strains have made it suitable for the production of heterologous enzymes (Tiwari *et al*., 2015).

Most reports about fungi that produce α-amylase have been limited to a few species of mesophilic fungi, and attempts have been made to specify the cultural conditions and to select superior strains of the fungus to produce on a commercial scale (Gupta *et al*., 2003). Fungal sources are confined to terrestrial isolates, mostly *Aspergillus* and *Penicillium* (Kathiresan and Manivannan, 2006). The *Aspergillus* species produce a large variety of extracellular enzymes, and amylases are the ones with most significant industrial importance (Tiwari *et al*., 2015). Filamentous fungi, such as *Aspergillus oryzae* and *Aspergillus niger*, produce considerable quantities of enzymes that are used extensively in the industry. *A. oryzae* has received increased attention as a favorable host for the production of heterologous proteins because of its ability to secrete a vast amount of high value proteins and industrial enzymes, e.g. α-amylase (Souza and Magalhaes, 2010). *Aspergillus oryzae* has been largely used in the production of food such as soy sauce, organic acid such as citric and acetic acids and commercial enzymes including α-amylase (Kammoun *et al.,* 2008). *Aspergillus niger* has important hydrolytic capacities in the α-amylase production and, due to its tolerance of acidity (pH <3), it allows the avoidance of bacterial contamination (Enshasy *et al.,* 2008). Filamentous fungi are suitable microorganisms for solid state fermentation (SSF), especially because their morphology allows them to colonize and penetrate the solid substrate (Rahardjo *et al.,* 2005). The fungal α-amylases are preferred over other microbial sources due to their more accepted GRAS status (Gupta *et al.,* 2003). The thermophilic fungus *Thermomyces lanuginosus* is an excellent producer of thermostable amylase (Jensen *et al.,* 2002; Kunamneni *et al.,* 2005).

2.3.3.2 *Bacterial amylases*

The production of microbial amylases from bacteria is dependent on the type of strain, composition of medium, method of cultivation, cell growth, nutrient requirements,

incubation period, pH, temperature, metal ions and thermostability. In fact, such industrially important microorganisms found within the genus *Bacillus*, can be exploited commercially due to their rapid growth rate leading to short fermentation cycles, capacity to secrete proteins into the extracellular medium and safe handling (Tiwari*et al.,* 2015).

α-Amylase can be produced by different species of microorganisms, but for commercial applications α-amylase is mainly derived from the genus *Bacillus*. Thermostability is a desired characteristic of most of the industrial enzymes. Thermostable enzymes isolated from thermophilic organisms have found a number of commercial applications because of their stability. As enzymatic liquefaction and saccharification of starch are performed at high temperatures (100-110), thermostable amylolytic enzymes have been currently investigated to improve industrial processes of starch degradation and are of great interest for the production of valuable products like glucose, crystalline dextrose, dextrose syrup, maltose and maltodextrins (Asgher *et al.,* 2007). *Bacillus subtilis, Bacillus stearothermophilus, Bacillus licheniformis* and *Bacillus amyloliquefaciens* are known to be good producers of thermostable α-amylase, and these have been widely used for commercial production of the enzyme for various applications (Prakash and Jaiswal, 2009). Thermostable α-amylases have been reported from several bacterial strains and have been produced using SmF as well as SSF (Saxena and Singh, 2011). However, the use of SSF has been found to be more advantageous than SmF and allows a cheaper production of enzymes (Sodhi *et al.,* 2005). The production of α-amylase by SSF is limited to the genus *Bacillus*, and *B. subtilis, B. polymyxa, B. mesentericus, B. vulgarus, B. megaterium* and *B. licheniformis* have been used for α-amylase production in SSF (Baysal *et al.,* 2008).

Currently, thermostable amylases of *Bacillus stearothermophilus* or *Bacillus licheniformis* are being used in starch processing industries (Tiwari *et al.,* 2015). Enzymes produced by some halophilic microorganisms have optimal activity at high salinities and could therefore be used in many harsh industrial processes where the concentrated salt solutions used would otherwise inhibit many enzymatic conversions (Prakash and Jaiswal, 2009). In addition, most halobacterial enzymes are considerably thermotolerant and remain stable at room temperature over long periods. Halophilic amylases have been characterized from halophilic bacteria such as *Chromohalobacter* sp. (Prakash and Jaiswal, 2009), *Halobacillus* sp., *Haloarcula hispanica*, *Halomonas meridian*and *Bacillus dipsosauri* (Souza and Magalhaes, 2010)

Bacillus is endowed to produce thermostable α-amylase and also large quantities of other enzymes. Indeed, 60% of commercially available enzymes are obtained from different species of *Bacillus* i.e *B. subtilis, B. licheniformis, B. amyloliquefaciens* and *B. stearothermophilus* (Tiwari *et al.,* 2015). Some *Bacillus* strains produce enzyme in the exponential phase, whereas some others in the mid stationary phase. Though, different *Bacillus* species have similar growth patterns and enzyme profiles, but their optimized conditions vary, depending upon the strain.

2.4 Production Methods

2.4.1 *α*-Amylase production by submerged fermentation (SmF)

Conventionally industry prefers SmF for the large scale production of microbial enzymes due to its ease in controlling the process parameters, for which commercial media formulations are used. *Bacillus* spp. secretes variety of enzymes including polysaccharases, proteases and nucleic acid hydrolyzing enzymes. Amylase from *B. subtilis* was considered as an industrial enzyme as early as 1917, but commercial

production of this enzyme began in the late 1940s with the introduction of submerged culture or fermentation (SmF) borrowed from antibiotic industry (Sivaramakrishnan *et al.,* 2006). Currently, a limited number of strains of selected species are used to prepare major seven enzymes including *α*-amylases on a large scale. *Bacillus*species like *B. amyloliquefaciens*, *B. licheniformis*, *B. stearothermophilus* and *B. subtilis* are the major producers of *α*-amylases (Benjamin *et al*., 2013).

Bacillus spp. grows at different rate with specificity to different substrates in culture medium (Ajayi and Fagade, 2006). Complex or semisynthetic medium seems to be better for amylase production. Most of the media formulations use soluble starch as the principal carbon source in liquid media, whose percentage normally varies between 1 to 2 (Riaz *et al.,* 2009) which may be in combination with peptone (Saui *et al.,* 2006) or yeast extract (Riaz *et al.,* 2009) or beef extract or triptone (Narang and Satyanarayana, 2001). Many authors evaluated efficacy of simple sugars such as lactose and glucose in combinations with nitrogen sources for amylase production using *Bacillus*. spp. (Saui *et al.,* 2006) and *B. licheniformis* (Tsurikova *et al.,* 2002), and their efficiency was not promising in comparison to soluble starch or extracted starch from agricultural products like white corn (Ajayi and Fagade, 2006). Efficiency of *B. licheniformis* to thrive on starch from hordium, pearl millet, rice, corn, gram and wheat starch have been evaluated for the production of *α*-amylase (Haq *et al*., 2005), of which pearl millet starch (1.5%) significantly enhanced amylase production, which was also reconfirmed independently by another group (Rao and Satyanarayana, 2007).

Application of response surface methodology (RSM) for the optimization of medium and growth parameters found great advancement in reducing labour and time. RSM was efficiently used to study the cumulative inter-active effect of the macronutrients (soybean meal, yeast extract and wheat bran) of the media and to optimize their

concentrations to enhance the production (up to 25%) of maltooligosaccharide-forming amylase from *B. circulans* GRS 313 (Benjamin*et al*., 2013), or another combination comprising starch, glycerol, yeast extract and peptone from *Bacillus* sp. (Tanyildizi *et al*., 2007), wheat bran and groundnut oil cake mixture (1:1 ratio) cultural parameters for the cultivation of *B. amyloliquefaciens* in liquid state (Gangadharan *et al.*, 2008).

Like medium, controlled aeration also play a major role in maximizing the yield of *α*-amylase, and in general, lower agitation rate would be better for maximal *α*-amylase synthesis. Mostly, maximum amylase production was noticed at 24hours in a batch process employing *B. licheniformis* (Bozic *et al.*, 2011). Normally, *Bacillus* spp. prefer neutral or slightly alkaline or a range between 6.8 and 7.2 pH for amylase production at the initial stage of fermentation.

Various authors use different units of amylase activity for the interpretations of results. Predominantly U/ml (units per millilitre) is being used for SmF, but some authors prefer U/cfu (units per colony forming units) (Benjamin *et al*., 2013). Established measure is the International Unit (IU) per ml culture medium in SmF, *i.e.*, micromoles of the product formed per minute (Smitha, 2010). For instance Ajayi and Fagade (2006) demonstrateddifferent growth profiles and substrate specificities of five species of *Bacillus*, and that the amylase production values ranged from 0.22×10^2 U/cfu by *B. circulans* to 0.912×10^2 U/cfu by *B. licheniformis* from corn starch and 0.01×10^2 U/cfu by both *B. megaterium* and *B. licheniformis* to 0.693×10^2 U/cfu by *B. subtilis* from soluble starch. It seems very difficult to compare the activities between U/cfu and U/ml, hence it is always preferred to use the units of enzyme activity in U/ml when SmF is the fermentation strategy. When liquid media supplements (like extracts of agricultural residues) are used for SmF, it would be easy to compare if the values are given as liquid equivalents, *i.e*., the control liquid medium, preferably a standard

medium like Luria Bertani (LB) or nutrient broth (Smitha, 2010). From SmF studies, it is evident that *B. licheniformis* and *B. subtilis* are the major producers of α-amylases. In general perspective and considering the thermostability, α-amylase from *B. licheniformis* is mostly preferred by the industry.

2.4.2 α-Amylase production by solid-state fermentation (SSF)

Current developments in biotechnology paved avenues for the novel applications of enzymes,accordingly the production strategies have been revolutionized. SmF has traditionally been used for the production of industrially important enzymes. In comparison, SSF with optimized production parameters seems to be better for enhanced amylase production (Smitha, 2010). Solid state fermentation (SSF) holds tremendous potential for the enhanced production of such industrially-significant enzymes. SSF has been defined as the fermentation process occurring in the absence or near-absence of free water (Benjamin*et al.*, 2013) and it has become a very attractive alternative to SmF for specific applications due to the recent improvements in reactor designs (Smitha, 2010). SSF processes generally employ a natural and very cheap raw material as sole source of carbon and energy. It can also be employed on inert material as solid matrix/support, which requires supplementing a nutrient solution containing necessary ingredients including carbon and nitrogen sources (Benjamin *et al.*, 2013).

SSF has emerged as a potential technology for the production of microbial products such as feed, fuel, food, industrial chemicals and pharmaceutical products (Ajita and Thirupathihalli, 2014). Its application in bioprocesses such as bioleaching, biobeneficiation, bioremediation, biopulping, has offered several advantages such as higher fermentation productivity, higher end products concentration, higher product stability, lower catabolic repression, coupled with cultivation of microorganisms

specialised for water- insoluble substrates and lower demand on sterility due to the low water activity used in SSF.

The success of SSF greatly depends on the appropriate incubation period, moisture level, suitable nitrogen source and pH requirements (Smitha, 2010).

In situ fermentation using microbes bearing GRAS label to enhance the palatability and flavour of vegetables, pulses and other agricultural raw food stuffs is getting more attention in these days. *B. subtilis* was found efficient to make Okpehe, a traditional fermented condiment in Nigeria; among other enzymes, amylase (13 U/ml) also contributed to the nutritional enrichment of Okpehe (Oguntoyinbo *et al.,* 2007). *B. subtilis* was also used to convert sterile dehulled cooked soybean into soy-daddawa (a condiment) by the α-amylase (48 h, 35°C). These studies show that *in situ* fermentation under controlled conditions for improving the organoleptic values of the raw food stuff, thereby increasing the price and shelf-life of the products which would in turn benefit the framers.

The activity of enzymes produced by SSF is normally expressed in U/gds (units per gram dry fermented substrate) (Benjamin *et al.,* 2013). However, when cross comparison of enzyme activities obtained from SmF and SSF is required, the authors may use confusing units of their choice, which may lead to false positive emphasis to the actual results.

2.5 Substrates for the Production of Amylase

A number of substrates have been investigated for use in SSF technique. The aim is to use substrates that are waste or by products of other processes in order to make the process of enzyme production environment friendly. Agricultural residues offer a

valuable substrate for amylase production by SSF. As far as SSF isconcerned, wheat bran seems to be the preferred substrate of interest for many investigators for *α*-amylase production (Shukla and Kar, 2006; Gangadharan *et al*., 2008; Vijayabaskar *et al*., 2012). Other agricultural residues like potato peel, banana peel, banana residue including whole plant after harvest (Unakal *et al*., 2012), residues generated during agricultural processing such as lentil husk (Baysal *et al.*, 2008), rice bran, wheat bran, sugarcane bagasse, black gram husk, green gram husk (Vijayabaskar *et al*., 2012), seem to be efficient substrates.

Wheat bran has been used as substrate for α-amylase production by *B.licheniformis* and *A.niger*. Studies on screening various combinations of substrates for optimum yield of enzyme by *B. amyloliquefaciens* have shown that wheat bran and groundnut oil cake in a ratio of 1:1 yield enzyme with activity of 1671 U/g (Ajita and Thirupathihalli, 2014). Banana waste has been used for its production by *B.subtilis* (Shaista*et al.*, 2003).

The nitrogen source used for production of α-amylase may be organic or inorganic. Few of the inorganic nitrogen sources include ammonium sulphate, ammonium chloride and ammonium hydrogen phosphate. Most commonly used organic sources of nitrogen include peptone, yeast extract and soyabean meal (Ajita and Thirupathihalli, 2014).

Maize husk is a by-product of maize processing and is abundantly available in northern parts of Nigeria and other parts of the world where maize is being cultivated as a staple food crop. It can be used as a substrate for amylase production.

2.6 Factors Affecting Alpha Amylase Production

2.6.1 Temperature

There are two temperatures that need to be in optimum range during production. They are temperature for the growth of the microbial source and optimum temperature at which maximum production of enzyme takes place. The optimum temperatures for both were studied in strains of *B. licheniformis* and *B. subtilis* 29. The optimum temperatures for growth and α-Amylase production were found to be 45°C to 46 °C and 50 °C, respectively. In *B. subtilis* 29 the optimum temperature for growth (42°C) was higher by 5°C than that of amylase formation (37°C) (Ajita and Thirupathihalli, 2014). α-Amylasesfrom thermophilic archaea are active at high temperatures. Studies by Jensen and Olsen (1992) and Bunni *et al.* (1989) reveal that optimal production of amylase is between 50–55°C for the thermophilic fungal cultures such as *Talaromyces emersonii*, *Thermomonospora fusca* and *Thermomyces lanuginosus*. *Thermococcus* α-amylases are optimally active at temperatures close to 80°C while *Pyrococcus* enzymes are optimally active around 100°C (Upgade *et al*., 2011). An optimum range of 95-100°C was observed to yield maximum enzyme activity in a study involving *Pyrococcus furiosus*and *Penicillium fellutanum* showed an optimum activity of 98±4.6 U/ml at a temperature of 30°C (Ajita and Thirupathihalli, 2014). An optimal temperature of 50^{o}C was observed when temperature was varied within a range of 30-90°C for production of α-amylase by *Aspergillus oryzae*(Thippeswamy *et al*., 2006). When solid state fermentation was carried out for production of the enzyme by *Penicillium janthinellum* in a range of 30-55°C, the optimum incubation temperature was found to be 45°C (Prakasham *et al*., 2007). The enzyme production increases with increase in temperature till it reaches the optimum. With further increase in temperature the enzyme production decreases. This may be due to the loss of moisture in the substrate which adversely

affects the metabolic activities of the microbes leading to reduced growth and decline in enzyme production (Prakasham *et al*., 2007). Theoptimum temperature for production of the enzyme by *Bacillus* spp. isolated from dhal industry waste was found to be 60°C (Ajita and Thirupathihalli, 2014). *Rhodothermus marinus*, a thermophilic marine microorganism exhibited optimal yield of thermostable α-amylase at 61°C (Anto *et al*., 2006). The α-amylase produced by *Clostridium acetobutylicum* was incubated at temperatures ranging from 30-60°C. The optimal activity was found to be at 45°C andmaximum α-amylase production by *Alteromonas haloplanktis* was found at an optimal temperature of 4°C (Ajita and Thirupathihalli, 2014).

2.6.2 Initial pH

Optimum pH is a critical factor for the stability of enzyme produced. Enzymes are pH sensitive and hence care must be taken to control the pH of the production process. *Pyrococcus furiosus* produces α-amylase which shows activity at an optimum pH of 6.5–7.5 (Ajita and Thirupathihalli, 2014). The production of extracellular amylase by the thermophilic fungus *Thermomyces lanuginosus* was investigated in solid state fermentation (SSF). The maximum enzyme activity was 534 U/g when wheat bran was used as the substrate with optimum pH conditions of 6.0 (Bhargav *et al*., 2008). *Bacillus amyloliquefaciens* produces the enzyme with an optimum pH of 7.0 (Ramachandran *et al*., 2004). *Halomonas meridiana* was studied for optimization of α-amylase production. The study revealed that the amylase exhibited maximal activity at pH 7.0, being relatively stable in alkaline conditions (Kathiresan and Manivannan, 2006). The optimum pH for production of the enzyme by *Bacillus* spp. isolated from dhal industry waste was found to be 6.5. *Aspergillus* species such as *A. oryzae*, *A. ficuum* and *A. niger* exhibit optimal production at pH 5.0–6.0 in SmF. *Saccharomyces cerevisiae* and *S. kluyveri* have shown maximum yield of enzyme at pH 5.0 and pH of

7.0 respectively (Ajita and Thirupathihalli, 2014). An initial pH range 7.5 to 8 was found to be optimal for enzyme production by *Rhodothermus marinus* (Anto *et al*., 2006). Thermophilic microorganisms such as *P. woesei* and *Thermococcus profundus* exhibited optimal production of α-amylase at pH value of 5.0 (Zaferanloo *et al*., 2014). *Clostridium thermosulfurogenes* yielded maximum enzyme at a pH of 7.0 (Zaferanloo *et al*., 2014). An optimum pH of 9.0 was observed to yield enzyme with specific activity of 138 U/mg in the fungi *Preussia minima* (Erdal and Taskin, 2010). During production of α-amylase from *P. fellutanum* the optimum pH was found to fall within the range of 6.0 to 7.0 (Erdal and Taskin, 2010).

2.6.3 Incubation period

This is a crucial factor in fermentation process. If the process is carried out for a time period shorter than the optimum duration the maximum yield cannot be obtained. The enzyme activity increases with increase in incubation time till it reaches the optimum duration. In most cases, the production of enzyme begins to decline if the incubation time is further increased. This could be due to the depletion of nutrients in the medium (Raul *et al.,* 2014) or release of toxic substances. *Bacillus subtilis,* a well-known producer of alpha amylase was studied for comparison between different fermentation hours and the study revealed a high yield of alpha amylase after 48 hours of fermentation (Gangadharan *et al*., 2006). Different incubation time durations were compared for yield of amylase from *Penicillium fellutanum* isolated from mangrove rhizosphere soil. The culture when incubated at 96 h, showed the maximum activity of 136 U/ml. This was a 2 fold increase in activity when compared to 24h incubation (68±2.3 U/ml) (Erdal and Taskin, 2010). In an attempt to produce α-amylase from *A. oryzae* using coconut oil cake as a substrate, the maximum activity was found to be 1752 U/gds which resulted after 72 h of incubation (Thippeswamy *et al*., 2006). An

optimum α-amylase activity of 62470 U/g was found after fermentation for 72 hours when produced by *Bacillus amyloliquefaciens* (Ajita and Thirupathihalli, 2014).

2.6.4 Moisture content

Optimum levels of initial moisture content may vary depending on the microbial source used. For fungal sources the moisture content required is less whereas bacterial sources need more moisture content for high yield of the enzyme (Ajita and Thirupathihalli, 2014). The optimum level can be obtained by determining the enzyme yield within a range of initial moisture content. In a study of production of α-amylase by *Penicillium janthinellum* the moisture content was varied within a range of 20-80% by varying the amount of salt solution used in moistening the substrate particles. The study revealed that with wheat bran as the substrate, the maximum yield of enzyme was of 295 U/gds with 60% of initial moisture content. The yield decreased to 220 U/gds at 80% (w/w) moisture content (Prakasham *et al*., 2007). For *Aspergillus oryzae* the maximum yield was found to be 9000 U/gds at an initial moisture content of 60% which declined drastically when the moisture content was increased to 70% (Ajita and Thirupathihalli, 2014). The optimal initial moisture content may also vary with substrate in addition to the microbial source used. This may be due to fact that substrates with better water holding capacity require lower moisture content for optimal production of the enzyme when compared to others. Production of α-amylase from *Penicillium chrysogenum* was carried out using various agricultural by-products like wheat bran (WB), corncob leaf (CL), rye straw (RS) and wheat straw (WS). Enzyme yields were 20, 34, 40 and 127 U/mL with CL, RS, WS and WB at 75, 55, 65 and 65% of initial moisture content, respectively.For *B. amyloliquefaciens*, studies were carried out to optimize the process parameters in which the initial moisture content was varied from 55- 90% for optimization. An optimal production of 3677 U/g of α-amylase was found at 85% of

initial moisture content using a combination of wheat bran and groundnut oil cake as the substrate (Ajita and Thirupathihalli, 2014). The enzyme yield usually increases with increase in initial moisture content reaching an optimal level followed by decrease in enzyme yield with further increase in moisture content. At low moisture content there is high water tension and low solubility of the nutrients which causes the low yield of enzyme. With increase in moisture content the swelling of substrate takes place which ensures better uptake of nutrients by the microbes. But with increase of moisture content further the enzyme yield decreases owing to many reasons. The inter particle distances reduce and can result in agglomeration of substrate particles. Also the reduction in gas volume and gas diffusion results in impaired oxygen transfer (Prakasham *et al*., 2007).

2.7 Industrial Applications of α-Amylase

α-Amylase is gaining increased attention due to its starch hydrolyzing properties and the activities that can be carried out owing to this property. There are many potential and widely used applications of this enzyme on the industrial front. Enzymes have replaced the previously used chemical methods of hydrolysis in various industrial sectors to make the process environment friendly and make processes easier (Ajita and Thirupathihalli, 2014).

2.7.1 Starch conversion

Starch is used in the production of fructose and glucose syrups (Souza and Magalhaes, 2010). This process involves three steps: Gelatinization, Liquefaction, and Saccharification. Gelatinization involves the dissolving of starch granules in water to form a viscous starch suspension. The amylose and amylopectin are dispersed into the water on dissolution. Liquefaction of starch is its partial hydrolysis into short chain

dextrins by α-amylase resulting in reduction of the viscosity of the starch suspension. Saccharification is the production of glucose and fructose syrup by further hydrolysis. This is carried out by glucoamylase which acts as an exo-amylase by cleaving the α-1, 4 glucosidic linkages from the non-reducing terminal. The action of pullulanase along with glucoamylase yields high glucose syrup. This high glucose syrup can then be converted into high fructose syrup by isomerization catalysed by glucose isomerase. The fructose syrup obtained is used as a sweetener, especially in the beverage industry.The hydrolysis of starch for this purpose was previously carried by acid hydrolysis followed by saccharification using enzymes. This method had many drawbacks. The acidic nature of the process required corrosion resistant material to be used for the equipment and the high temperatures would inactivate the thermolabile enzymes if the hot starch hydrolysate passes into subsequent steps. Hence, enzymatic hydrolysis is a preferred method. The α-amylase used in the liquefaction step can be produced from various microbial sources. Thermostable amylases can be employed for hydrolysis at a high temperature. *Bacillus Stearothermophilus, Bacillus amyloliquefaciens*, *Bacillus licheniformis* and *Pyrococcus furiosus* are few of the many microbial sources used to produce α-amylase that is used in starch conversion (Souza and Magalhaes, 2010; Ajita and Thirupathihalli, 2014).

2.7.2 Bakery industry

α-Amylase is added to the dough in bread baking process. This causes the starch to hydrolyze into small dextrins which can further be fermented by yeast. This increases the rate of fermentation. Also, the starch hydrolysis decreases the viscosity of the dough, thus improving its texture and increasing loaf volume by rising of dough. Once the baking is done, there may be changes during storage of baked products. All undesirable changes like increase of crumb firmness, loss of crispness of the crust,

decrease in moisture content of the crumb and loss of bread flavor together are called staling. The enzyme is also used as an anti-staling agent to improve the shelf life and softness retention of baked goods (Souza and Magalhaes, 2010; Ajita and Thirupathihalli, 2014). Though it has an anti-staling effect, a slight overdose may result in gummyness of the bread. This is caused due to production of branched dextrins (Chi *et al*., 2009). In such cases pullulanase is used in combination with amylase resulting in specific hydrolysis of compounds responsible for the gummy nature of amylase treated bread (Ajita and Thirupathihalli, 2014).

2.7.3 Detergent industry

Detergent industries are the primary consumers of enzymes, in terms of both volume and value (Souza and Magalhaes, 2010). The use of enzymes in detergents has increased with the changing methods of dishwashing and laundry. Consumers prefer to use cold water and mild conditions which requires the detergent to work in those imitations. Earlier the chemicals used in detergents caused harm when ingested and the conditions of dishwashing were very harsh. Hence, enzymes showed the industry an alternative path. The enzymes are environmentally safe and work at mild conditions. α-Amylase is used to digest the starch containing food particles into smaller water soluble oligosaccharides. Starch can also attract soil particles onto the clothes. Hence, removal ofstarch is also important to maintain the whiteness of clothes. The stability of α-amylase at low temperature and alkaline pH contributes to its extensive use in detergents.

The disadvantage of using α-amylase is the calcium dependency and oxidant sensitivity of the enzyme. This limitation can be overcome by using α-amylase from genetically modified organisms. Scientists from Novozymes and Genencore International, two

major suppliers of detergent enzymes, have worked on this. They replaced the oxidant sensitive amino acid residue *met* at position 197 by *leu* in B. licheniformis amylase which resulted in an amylase with improved resistance against oxidative compounds (Ajita and Thirupathihalli, 2014).

2.7.4 Desizing of textiles

Amylases are used in textile industry for desizing process.Sizing agents like starch are applied to yarn before fabricproduction to ensure a fast and secure weaving process. Starchis a very attractive size, because it is cheap, easily available inmost regions of the world, and it can be removed quite easily.Starch is later removed from the woven fabric in a wet-processin the textile finishing industry. Desizing involves the removalof starch from the fabric which serves as the strengtheningagent to prevent breaking of the warp thread during theweaving process. The α-amylases remove selectively the sizeand do not attack the fibres (Ahlawat*et* al., 2009). Amylase from *Bacillus*stain was employed in textile industries for quite a long time.

2.7.5 Paper industry

The use of α-amylases in the pulp and paper industry is forthe modification of starch of coated paper, i.e. for the production of low-viscosity, high molecular weight starch (Ajita and Thirupathihalli, 2014).The coating treatment serves to make the surface of paper sufficiently smooth and strong, to improve the writing quality of the paper. In this application, the viscosity of the natural starch is too high for paper sizing and this can be altered by partially degrading the polymer with α-amylases in a batch or continuous processes. Starch is a good sizing agent for the finishing of paper, improving the quality and erasebility, besides being a good coating for the paper. The size enhances the stiffness and strength in paper (Souza and Magalhaes, 2010). Examples of amylases

obtained from microorganisms used in paper industry includes Amizyme® (PMP Fermentation Products, Peoria, USA), Termamyl®, Fungamyl, BAN® (Novozymes, Denmark) and α-amylase G9995® (Enzyme Biosystems, USA) (Ajita and Thirupathihalli, 2014).

2.7.6 Fuel alcohol production

Ethanol is the most utilized liquid biofuel. For the ethanolproduction, starch is the most used substrate due to its low price and easily available raw material in most regions of the world (Chi *et al.,* 2009). In this production, starch has to be solubilized andthen submitted to two enzymatic steps in order to obtainfermentable sugars. The bioconversion of starch into ethanolinvolves liquefaction and saccharification, where starch isconverted into sugar using an amylolytic microorganism orenzymes such as α-amylase, followed by fermentation, wheresugar is converted into ethanol using an ethanol fermentingmicroorganism such as yeast *Saccharomyces cerevisiae* (Ajita and Thirupathihalli, 2014).In order to obtain a new yeast strain that can directly produce ethanol from starch without the need for a separate saccharifying process, protoplast fusion was performed between the amylolytic yeast *Saccharomyces fibuligera* and *S. cerevisiae* (Chi *et al.*, 2009). Amongbacteria, α-amylase obtained from thermoresistant bacteria like*Bacillus licheniformis* or from engineered strains of*Escherichia coli* or *Bacillus subtilis* is used during the first stepof hydrolysis of starch suspensions (Sanchez and Cardona, 2008).

2.8Measurement of Enzyme Activity

The enzyme activity is determined by measuring the reducing sugars released as a result of the action of α-amylase on starch. Another method is to measure the extent of

hydrolysis by reading the absorbance of starch-iodine complex. Few of the commonly used methods for enzyme assay include:

2.8.1 Dinitrosalicylic acid method (DNS)

In the dinitrosalicylic acid method, aliquots of the substrate stock solution are mixed with the enzyme solution. Followed by 10 min of incubation at 50°C, DNS reagent is added to the test tube and the mixture is incubated in a boiling water-bath for 5 min. After cooling to room temperature, the absorbance of the supernatant is measuredat 540 nm. The A540 values for the substrate and enzyme blanks are subtracted from the A540 value for the analyzed sample (Gusakov *et al.,* 2011; Ajita and Thirupathihalli, 2014). In a study on alkalophilic α-amylase from *Bacillus* strain GM8901, the enzyme assay was done by measuring the reducing sugars by DNS method and the activity was found to be a maximum of 0.75 U/ml after incubation of 24 h (Gusakov *et al.,* 2011).

2.8.2 Nelson – Somogyi (NS) method

In the NS method, an aliquot of stock solution of substrate is heated at 50°C for 5min. Preheated (50°C for 5 min) enzyme solution is added to the substrate. This reaction mixture is incubated at 50°C and the reaction is carried out for 10min. After incubation Somogyi copper reagent is added to terminate the reaction. This is then incubated in boiling water bath for 40 min and cooled to room temperature. Nelson arsenomolybdate reagent is then added and incubated for 10 min at room temperature. Finally, water is added and the mixture is centrifuged at 13,000 rpm for one min and absorbance of supernatant is read at 610 nm (Ajita and Thirupathihalli, 2014). Haloalkaliphilic α-amylase isolated from archaebacterium *Natronococcus sp.* strain Ah-36 was subjected to NS method of enzyme assay and activity of 0.01 U/ml was recorded at the end of 35

h of incubation. The maximum activity was found to be 0.12 U/ml after 90 h and was retained even after 110 h (El-Safety and Ammar, 2004).

2.8.3 Determination of activity using iodine

The hydrolytic activity of α-amylase can be determined based on the principle that starch and iodine react to form a blue colored complex. On hydrolysis of starch this complex changes to a reddish brown colored one. The absorbance can be read after the enzyme substrate reaction has been terminated. This gives a measure of the extent of hydrolysis of starch by α-amylase(Ajita and Thirupathihalli, 2014).

2.8.3.1 Dextrinizing Activity

In a study involving characterization of thermophilic α-amylase from *P. furiosus*, the crude enzyme sample is incubated at 92°C with soluble starch to carry out the reaction for 10 min. The reaction is terminated by cooling the reaction mixture in ice water. Iodine (KI, Iodine) is added to form a colored complex with the starch in the reaction mixture. This complex is diluted with water in order to bring the color to a measurable range that can be read at 600 nm (Haki and Rakshit, 2003).

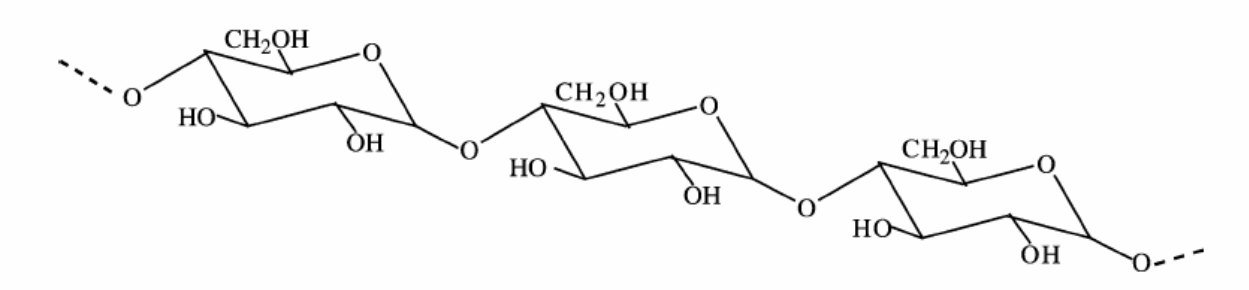
2.8.3.2 Indian Pharmacopoeia Method

This method is employed to measure the enzyme activity in terms of the amount of starch (grams) digested by enzyme of a given volume. In this method various dilutions of enzyme solution in incubated with starch at 40°C for 1 h after forming the colored complex by addition of iodine, the tube that shows no blue color is used to calculate the enzyme activity (Gupta *et al.*, 2003).

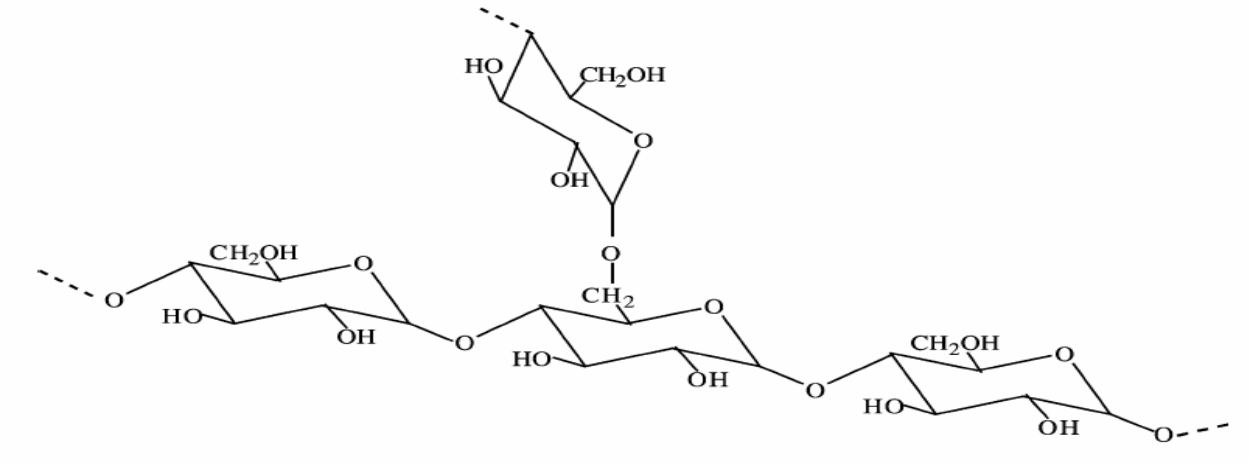
2.9Starch

Starch is an important constituent of the human diet and, for this purpose, is used chemically and enzymatically processed into a variety of different products such as starch hydrolysates, glucose syrups, fructose, maltodextrin derivatives or cyclodextrins, used in food industry. In addition to that, the sugars produced can be fermented to produce ethanol. In spite of the large number of plants able to produce starch, only a few plants are important for industrial starch processing. The major industrial sources are maize, tapioca, potato, and wheat, butlimitations such as low shear resistance, thermal resistance,thermal decomposition and high tendency towardsretrogradation limit its use in some industrial food applications(van Der Maarel *et al.,* 2002; Agrawal *et al.,* 2005; Goyal *et al.,* 2005). Among carbohydrate polymers, starch is currentlyenjoying increased attention due to its usefulness in differentfood products. Starch contributes greatly to the texturalproperties of many foods and is widely used in food andindustrial applications as a thickener, colloidal stabilizer,gelling agent, bulking agent and water retention agent (Jaspreet-Singha *et al.*, 2007).

Starch is a polymer of glucose linked to another one through the glycosidic bond. Two types of glucose polymers are present in starch: amylose and amylopectin (Figure 2.1). Amylose and amylopectin have different structures and properties. Amylose is a linear polymer consisting of up to 6000 glucose units with α-1,4 glycosidic bonds. Amylopectin consists of short α-1,4 linked to linear chains of 10–60 glucose units and α-1,6 linked to side chains with 15–45 glucose units.



A. Structure of amylose



B. Structure of amylopectin

Figure 2.1: Glucose polymers present in starch: amylose (A) and amylopectin (B) (Muralikrishna and Nirmala, 2005)

Granule bound starch synthase can elongate maltooligosaccharides to form amylose and is considered to be responsible for the synthesis of this polymer. Soluble starch synthase is considered to be responsible for the synthesis of unit chains of amylopectin. α-Amylase is able to cleave α-1,4glycosidic bonds present in the inner part of the amylose or amylopectin chain (Sorensen *et al.,* 2004; Tester *et al.,* 2004; Muralikrishna and Nirmala, 2005).Starch is hydrolyzed into smaller oligossaccharides by α-amylase, which is one of the most important commercial enzyme processes. Amylases find application in all the industrial processes such as in food, detergents, textiles and inpaper industry, for the hydrolysis of starch (Souza and Magalhaes, 2010).Saccharide composition obtained after hydrolysis of starch is highly dependent on the effect of temperature, the conditions of hydrolysis and the origin of enzyme. Specificity, thermostability and pH response of the enzymes are critical properties for industrial use (Kandra, 2003).

2.10Purification of α-Amylase

Enzymes used for industrial applications are usually crude preparations and require less downstream processing. Whereas the enzymes used in clinical and pharmaceutical industry need to be highly purified. Also, when used for study of structure function relationships and biochemical properties the enzymes have to be in purified form (Ajita and Thirupathihalli, 2014).

Purification methods commonly employed are precipitation, chromatography and liquid-liquid extraction depending on the properties of the enzyme desired. A combination of the above methods is used in a series of steps to achieve high purity. The number of steps involved in purification will depend on the extent of purity that is desired (Sivaramakrishnan *et al.,* 2007). The crude extracellular enzyme sample can be

obtained from the fermented mass by filtration and centrifugation. In the case of intracellular enzymes, raw corn starch may be added followed by filtration and subsequent steps.

The crude amylase enzyme can be precipitated and concentrated using ammonium sulphate precipitation or organic solvents. The precipitated sample can be subjected to dialysis against water or a buffer for further concentration.This can be followed by any of the chromatographic techniques like ion exchange, gel filtration and affinity chromatography for further separation and purification of the enzyme. In a method of purification of the enzyme produced by *Aspergillus falvus var. columnaris*, the enzyme was precipitated followed by dialysis and then column chromatography (Ajita and Thirupathihalli, 2014).In a study of amylase production by *Preussia minim*, the sample was precipitated using trichloroacetic acid (TCA)/acetone and applied to a Sephadex G-200 gel filtration column. The resulting fractions were pooled and further applied to a DEAE-Sepharose ion exchange column (Erdal and Taskin, 2010). In a study of purification and characterization of an extracellular α-amylase from *Clostridium perfringens* Type A, crude enzyme concentrate was prepared by precipitation with polyethylene glycol. This concentrated sample was then separated by DEAE Sephacel chromatography resulting in three distinct amylolytic peaks. Fractions from a single peak were then collected and chromatographed on the same column, followed by separation of obtained active fractions on a Sephacryl S-100 HR column (Demirkan, 2011).

Purification of α-amylase produced by a mutant *Bacillus subtilis* strain was done in a series of steps employing precipitation with 80% ammonium sulfate, TSK Toyopeal column chromatography, ultrafiltration, dialysis and SP Sepharose column chromatography (Ballschmiter *et al.,* 2006). In case of thermotolerant amylases, the cell

extract obtained after centrifugation which is free of cell debris can be subjected to high temperatures, in order to denature thermolabile proteins. For purification of α-amylase produced by *Thermotoga maritima* MSB8, this step was followed by anion exchange chromatography. The purity was then analysed by using SDS-PAGE. The purity can also be analysed by size exclusion chromatography wherein the molecular weight of the purified protein can be determined (Ajita and Thirupathihalli, 2014).

2.11Characterization of α-Amylase

Once the enzyme has been purified, characterization of the enzyme is carried out. This can be done by determining the hydrolysates obtained after enzyme action on starch by PAGE. The purified enzyme sample along with molecular markers like bovin serum albumin (BSA) (67 kDa) and ovalbumin (43 kDa), are run on the gel. The resulting bands are then stained using staining agents like coomassie brilliant blueand silver nitrate (Mukesh *et al.,* 2012) and visualized. Various types of electrophoretic units are available for PAGE (Singhania *et al.,* 2009). The electrophoretic separation also indicates the purity and homogeneity of the enzyme obtained. In a study of α-amylase produced by *Bacillus* MNJ23 strain the molecular mass of the enzyme was characterized by SDS-PAGE (12% acrylamide gel) using Bio-Rad miniprotean II electrophoresis unit (Singhania *et al.,* 2009). Characterization of the enzyme produced by *Clostridium acetobutylicum* ATCC 824 was carried out by SDS-PAGE in a Phast Gel gradient (10 to 15%) with the Pharmacia Phast system with standard proteins ranging from 14,400 to 94,000 in molecular weight for comparison (Mukesh *et al.,* 2012). For determination of molecular weight of purified enzyme produced by mutant *Bacillus subtilis*, SDS-PAGE (10%) was carried out (Ballschmiter *et al.,* 2006).

The molecular mass of the enzyme can also be determined using size exclusion chromatography. A study on intracellular α-amylase produced by *Streptococcus bovis* 148 showed the appearance of a single band on a SDS-PAGE gel which confirmed the purification to homogeneity. The molecular mass after purification was found to be 57,000 Da. The molecular mass was determined as 55,000 Da from gel filtration method. This indicated that the intracellular α-Amylase exists in a monomeric form (Ajita and Thirupathihalli, 2014).

Isoelectric focusing is another method used in addition to the gel based characterization technique. The isoelectric point of the enzyme can be determined by using protein markers of broad range of pI of values (Mukesh *et al.*, 2012). Characterization by this method was used for α-amylase produced by *C. thermohydrosulfuricum* E 101-69. The crude enzyme sample was concentrated using ultrafiltation. This was followed by isoelectric focusing on a gel with pI range of 3.5-5.0. The Pharmacia low-pI-calibration-kit was used for standards (Nielsen and Borchert, 2000). For determination of isoelectric point of α-amylase produced by *Clostridium acetobutylicum* ATCC 824, Phast Gel IEF 3-9 with the Pharmacia Phast system with protein markers (Pharmacia) of pI 3 to 10 as standards was used (Mukesh *et al.*, 2012).

2.12 The Genus *Bacillus*

Bacillus is a genus of Gram-positive, rod-shapedbacteria and a member of the phylum Firmicutes. *Bacillus* species can be obligate aerobes or facultative anaerobes. They test positive to catalase test (Graumann, 2012). They are ubiquitous in nature and includes both free-living (nonparasitic) and parasiticpathogenic species. Under stressful environmental conditions, the bacteria can produce oval endospores to which the bacteria can reduce themselves and remain in a dormant state for very long periods.

These characteristics originally defined the genus, but not all such species are closely related, and many have been moved to other genera of the Firmicutes (Madigan and Martinko, 2005). *Bacillus* species exhibit quite diverse physiological properties such as the ability to degrade many different substrates derived from plant and animal sources including cellulose, starch, proteins (Lutz *et al.*, 2006).

Members of the genus *Bacillus* areheterogeneous and they are very versatile in their adaptability to the environment. Several factors influence the nature of their metabolic processes and enzymes produced. A great deal of attention is being given to thermophilic and extremely thermophilic microorganisms and their enzymes (Oyeleke and Oduwole, 2009).

Many species of *Bacillus*produce a large variety of extra cellular enzymes, such as amylases,which have significant industrial importance such as in food, paper, detergent, textile and pharmaceutical industries.

2.12.1 *Bacillus subtilis*

Bacillus subtilis are rod-shaped, Gram-positive bacteria that are naturally found in soil and vegetation. They grow in the mesophilic temperature range with the optimal temperature between 25-35 degrees Celsius. They form stress-resistant endospores that enable them survive under harsh environmental conditions (Bandow *et al*., 2002). *Bacillus subtilis* are motile by means of flagella. This motility occurs on surfaces, for example on agar plates, rather than in liquids (Schaechter *et al.*, 2006). *Bacillus subtilis* are considered strictly aerobic, meaning that they require oxygen to grow and they cannot undergo fermentation. However, recent studies show that they can indeed grow in anaerobic conditions making them facultative aerobes. The bacteria can make ATP in

anaerobic conditions via butanediol fermentation as well as nitrate ammonification (Marino, 2001). They are catalase positive that is produces catalase, an enzyme that is responsible in the catalysis of the decomposition of hydrogen peroxide to water and oxygen, and superoxide dismutase, an enzyme that catalyzes the breakdown of superoxide into oxygen and hydrogen peroxide (Bandow *et al*., 2002).

Bacillus subtilis secrete enzymes such as amylase, protease, pullulanase, chitinase, xylanase, lipase, among others. These enzymes are produced commercially and this enzyme production represents about 60% of the commercially produced industrial enzymes (Morikawa, 2006).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Collection of Samples

3.1.1 Maize husk

One (1) kg of maize husk sample wasprocured from a maize milling station in Samaru, Sabon Gari Local Government Area of Kaduna State. The maize husk sample was packaged in a clean polythene bag, labelled appropriately and brought to the Industrial/Food Research Laboratory, Department of Microbiology, Ahmadu Bello University, Zariafor analyses.

3.1.2 Soil

Fifteen (15) soil samples were collected from different locations namely, flower bed around the Department of Microbiology (FB), refuse dump site within the campus (RD) and the botanical garden of Department of Biological Sciences, Ahmadu Bello University, Zaria (BG). Five (5) samples (10 grams each) were separately collected from each location at a distance of 300 meters interval and a depth of 5-10cm. Each sample was packaged in a sterile bottle using a hand trowel and labelled appropriately (Ubalua, 2014). The soil samples were brought to the Industrial/Food Research Laboratory, Department of Microbiology, Ahmadu Bello University, Zaria for further analyses.

3.2 Proximate Analyses of the Sample

Eight grams (8g) of the dried unfermented maize husk sample was used to carry out proximate analyses at the Product Development Research Programme, Institute of Agricultural Research, Ahmadu Bello University, Zaria in order to determine the total

carbohydrate content, the crude protein content, crude fat, crude fibre, ash content and moisture content as the percentage composition of the substrate according to standard methods described by Association of Official Analytical Chemists (AOAC, 2000).

3.2.1 Determination of moisture content:

Cruciblewas washed and dried to a constant weight in an oven at 100^{o}C. It was later removed and cooled in a dessicator and weighed (**W1**). Two grams of the ground (powder)sample was placed in the weighed crucible (**W2**). The crucible containing the sample was kept in an oven for 3hr, the sample was removed and cooled in the dessicator and weighed (**W3**).

% moisture content wascalculated as: $\frac{\mathbf{W2-W3}}{\mathbf{W2-W1}} \times 100$

3.2.2 Determination of ash content

Crucible was cleansed and dried in an oven, after which it was cooled in a dessicator and weighed (**W1**). Two grams of the ground (powder) sample was placed in the crucible and weighed (**W2**). It was then transferred into a muffle furnace (SXL Model) set at 550^{o}C for 2hrs. Thereafter, the crucible was removed, cooled in the dessicator and weighed (**W3**).

% Ash of the sample was calculated as: $\frac{\mathbf{W3-W1}}{\mathbf{W2-W1}} \times 100$

3.2.3 Determination of Crude Fibre Content

Two grams of the sample was placed in a beaker containing 1.3ml of H_2SO_4 per 100ml of solution and boiled for30min, the residue was filtered and washed with hot water, then transferred to another beaker containing 1.2g of NaOH per 100ml of solution and boiled again for another 30min, the residue was washed with hot water and dried in an

oven and weighed (**C2**), the weighed sample was then incinerated in a muffle furnace set at a temperature of about 550^{o}C, removed, allowed to cool and weighed as **C3.**

% Crude Fibre was calculated as: $\frac{\mathbf{C2-C3}}{\mathbf{W}}$ **x** 100

3.2.4 Determination of crude fat

A clean boiling flask of 250ml capacity was dried in an oven and transferred into a dessicator and allowed to cool. Empty filter paper labelled **W1** was weighed and recorded. Two grams (2g) of the sample was weighed into the labelled thimble (filter paper) and recorded as **W2.** The boiling flask was filled with petroleum ether after which the soxhlet apparatus was assembled and allowed to reflux for 8hr. The sample was then removed and transferred to an oven to dry and then to a dessicator to cool and then weighed and recorded as **W3.**

% Crude Fat of the sample was calculated as: $\frac{\mathbf{W2-W3}}{\mathbf{W2-W1}}$ x 100

3.2.5 Determination of crude protein

Crude protein was determined by Kjeldhal method. The nitrogen content in the sample was determined by using Kjeltech apparatus based on Kjeldhal's AOAC Method 46-10 (AOAC, 2000). The percentage protein was calculated by multiplying nitrogen with the conversion factor of 6.25.

a. Digestion

Two grams of the sample was placed into a Kjeldahl flask and a catalyst (Kjeltabs Cu/3.5 containing 3.5g of K_2SO_4 and 0.4g of $CuSO_4$. $5H_2O$) was added followed by 15ml of concentrated Sulphuric acid. The tube was placed in a digestion box and the fume cupboard closed. The content of the tube was heated until the solution assumes a

green colour and then cooled after which each ofthe digest was transferred with several washings into 100ml with distilled water.

b. Distillation

The digest was first steamed through the Markham distillation apparatus for 15min. Under the condenser, a 100ml Erlenmeyer flask containing 10ml of 4% boric acid was placed and 2 drops of bromocresol purple added. Ten millilitres of 40% NaOH was added to 10ml of the digest after which the NaOH-sample mixture was distilled and the distillate collected in the boric acid-indicator mixture until it turns green. This was then titrated with 0.01N hydrochloric acid.

c. Titration

The solution in the receiving flask was titrated with 0.01N hydrochloric acid. The Nitrogen content and hence the protein content of the sample was calculated.

% protein content was calculated as: $\frac{(V_2 - V_1) \times 1.4}{W \times 6.25}$

Where: V_1 = Initial reading, V_2 = Final reading, W = Initial weight of the maize husk sample, 6.25 = conversion factor

3.2.6 Determination of Total carbohydrate

Total carbohydrate content wasobtained by difference as stated below:

Total carbohydrate= 100- (% moisture + %Ash + % Protein + %Fat)

3.3 Isolation and Characterization of *Bacillus subtilis*

3.3.1Media preparation

3.3.1.1Nutrient agar:

A weighed amount (8.4g) of dehydrated powder of nutrient agar medium was added to 300ml of distilled water and mixed thoroughly. The mixture was gently heated and brought to boiling to dissolve, then autoclaved for 15 min at 15 psi pressure and at 121°C then allowed to cool. The mixture was then poured into sterile Petri dishes and allowed to set.

3.3.1.2Motility medium:

A weighed amount (3g) of dehydrated powder of motility medium was added to 150ml of distilled water and mixed thoroughly. The mixture was gently heated and brought to boiling to dissolve, 10ml each was then dispensed in cleaned test tubes, then plugged with cotton wool and autoclaved at 121^{o}C for 15 min at 15 psi pressure. The medium was allowed to cool and then stored in refrigerator at 4^{o}C until required.

3.3.1.3Citrate Agar:

A weighed amount (3.64g)of dehydrated powder of Simmon's citrate agar was added to 150ml of distilled water and mixed thoroughly. The mixture was gently heated and brought to boiling to dissolve, 10ml each was then dispensed in cleaned universal bottles and autoclaved at 121^{o}C for 15 min at 15 psi pressure. The medium was slanted and allowed to cool and then stored in refrigerator at 4^{o}C until required.

3.3.1.4 Starch Agar:

A weighed amount (5.04g) of dehydrated powder of starch agar medium was added to 180ml of distilled water and mixed thoroughly. The mixture was gently heated and brought to boiling to dissolve, then autoclaved for 15 min at 15 psi pressure and at

121°C and allowed to cool. The mixture was then poured into sterile Petri dishes and allowed to set.

3.3.2 Isolation of *Bacillus subtilis* from the soil:

Tengrams (10g) of each soil sample was suspended in 90ml of sterile distilled water. The soil suspension was heat shocked at 60^{o}C for one hour in a water-bath to kill non-spore forming organisms. Then the soil suspensions were inoculated by streaking on nutrient agar medium. The inoculatedplates were incubated aerobically at 37^{o}C for 24hrs and examined for the appearance of colonies. The colonies that exhibited cultural characteristics typical of *Bacillus* species were sub-cultured onto nutrient agar slants for subsequent identification.

3.3.3 Identification of *Bacillus subtilis*

Identification of*Bacillus subtilis* was primarily based on the taxonomic comparison and microgen identification system. The characteristic morphological, cultural and biochemical properties were observed (Bergey, 2004; Cowan and Steel, 2003).

3.3.3.1Cultural characterization

Nutrient Agar Medium:

The plates of the nutrient agar were examined for size, pigmentation, form, margin and elevation of the colonies.

3.3.3.2Morphological characterization

Morphological characteristics such as the cell shape, cell arrangement as well as the Gram's reaction of the organism were determined by Gram staining technique. Endospore-staining technique was also carried out to morphologically characterize the isolates.

Gram Staining:

A smear was prepared from the 24 hour culture of the colonies, where a well separated colony (pure culture) was placed directly into a drop of normal saline on a clean, dried, grease-free slide, smeared and then allowed to air-dry. The smear was heat-fixed by passing the slide over a Bunsen flame for three quick successions.The slide was flooded with crystal violet solution and allowed to stand for one minute, then washed with slow-running tap water and then flooded with Gram's iodine (mordant) and allowed to act for also one minute. The slide was washed with tap water and decolourized with 95% alcohol for 30sec. The slide was rinsed with tap water and then counter-stained with safranin for another 30sec. The slide was finally rinsed with slow-running tap water, allowed to air-dry and then examined microscopically under oil immersion objective lens (100x) after adding a drop of immersion oil for the presence of Gram positive rods (Cowan and Steel, 2003).

Endospore Staining:

A smear was prepared from the 24 hour culture of the colonieson a clean, dried, grease-free slide and then allowed to air-dry. The slide was flooded with 5% aqueous malachite green and steam for one min, then, washed under running tap water. The slide was then counterstain with 0.5% aqueous safranin for 15 seconds and rinse with water, drain to dry and examined for the presence of spore, its position and shape. Spore-forming rods appearing green were observed. This distinguishes *Bacillus*spp. from other Gram positive but non-spore forming soil bacteria (Cowan and Steel, 2003).

3.3.3.3Biochemical characterization

Carbohydrate fermentation test:

A series of test tubes containing sterile nutrient broth with 1% each of membrane-filter-sterilized (0.45µm) single fermentable sugar were prepared. The tubes wereinoculated with the test organisms sub-cultured on nutrient agar slants following cultural and morphological characterisation. The sugar fermentation tubes were incubated at 37^{o}C for 24 hours. At the end of the incubation period, all tubes were examined for acid following the addition of methyl red indicatorand then compared with the control for interpretation (Cowan and Steel, 2003). A total of four (4) sugars were used for the fermentation tests, these include; Glucose, Mannitol, Arabinose and Xylose.

Catalase test:

Using an inoculating needle, the 24 hours culture of each of the isolates (from the slant) was picked and placed separately on a clean, grease-free slide already containing three drops of 3% H_2O_2. The slides were immediately observed for bubbling (Effervescence) which indicated a positive test (Cowan and Steel, 2003).

Motility test:

This test was carried out by inoculating the motility medium with the suspected *B. subtilis*colonies. A stab was made with a straight wire to a depth of about one third the total volume of the medium. The culture was then incubated at 37^{o}C for 24hrs. If the culture turns cloudy (turbid) after incubation, it means the organism is motile but if growth is restricted to the line of inoculation and the rest of the culture remains clear, then the organism is non-motile(Cowan and Steel, 2003).

Citrate utilization test:

This was carried out by inoculating the suspected colonies of *B. subtilis* on Simmons' citrate agar slant and the inoculated slant was then incubated at 37^{o}C for 48hrs and examined for development of a deep blue colour which indicates a positive reaction (Cowan and Steel, 2003).

Urease test:

Urease test was carried out by inoculating urea agar slant with the suspected colonies of *B. subtilis*and the slant was then incubated at 37^{o}C for 24hrs and examined for the development of bright pink or red colour indicates a positive reaction (Cowan and Steel, 2003).

Indole test:

Indole test was carried out by inoculating the suspected colonies of *B. subtilis* into 1% peptone water and then the inoculated peptone was incubated at 37^{o}C for 24hours. After 24hours of incubation, 3 drops of Kovac's reagent was added, shaken and examined. A positive reaction is indicated by the development of a red colour in the reagent layer above the broth while a negative reaction is indicated by a yellow colour (Cowan and Steel, 2003).

Methyl Red test:

Test tubes containing 5ml of MR-VP broth each were inoculated with the suspected colony of *Bacillus subtilis*using wire loop. The tubes were incubated at 37^{o}C for 48hrs. After incubation, about 3ml of the cultured broth was transferred to a small test tube to which three drops of methyl red indicator was added. Formation of red colour on addition of the indicator signifies a positive methyl red test and a yellow colour signifies a negative test (Cowan and Steel, 2003).

Voges-Proskauer test:

Test tubes containing 5ml of MR-VP broth each were inoculated with the suspected colony of *Bacillus subtilis*using wire loop. The tubes were incubated at 37^{o}C for 48hrs. After incubation, about 3mL of the cultured broth was transferred to a small test tube to which three drops of 40% potassium hydroxide (KOH) was added followed by the addition of five drops of 5% α-naphthol in ethanol. The mixture was shaken and the tubes placed in a sloping position. Development of red colour starting from the liquid-air interface within 15 min indicated a positive VP test and no colour change occurs in a VP negative test (Cowan and Steel, 2003).

Nitrate reduction test:

Test tubes containing 5mL of nitrate broth each with inverted Durham tubes were inoculated with the suspected colony of *Bacillus subtilis*using a straight wire. The tubes were incubated at 37^{o}C for 24hrs. After 24hrs of incubation,10 drops of reagent A (sulphanilic acid) was added followed by 10 drops of reagent B (α-napthol amine) and examined. A deep red colour shows the presence of nitriteand thus, shows that nitrate has been reduced and indicates apositive reaction.

To tubes not showing a red colour within 5 min, powdered zinc (up to 5 mg/ml of culture) was added and allowed to stand and then examined for red colour which indicates nitrate present in the medium (i.e. notreduced by the organism) and absence of red colour indicates nitrateabsent in the medium (i.e. reduced by the organism tonitrite, which in turn was itself reduced) (Cowan and Steel, 2003).

Starch hydrolysis:

This was carried out by inoculating suspected colonies of *B.* subtili*s* on starch agar. The inoculated plates were incubated at 37^{o}C for 24hrs. After incubation, the plates

werethen flooded with Lugol's iodine and examined for zone of hydrolysis (Cowan and Steel, 2003).

3.4Microgen Identification

Suspending broth andmicrowell strips were allowed to returnto room temperature before inoculation. Colonies from an 24hours pure culture were removed using a sterile loop and emulsified in a vial of *Bacillus* Suspending medium (5ml), mixed thoroughly by vortex until the suspension is equivalent to 2.0 McFarland standard and the particulates were allowed to settle prior to inoculating the strips. The microwell strips were removed from the foil pouch and placed in the holding tray. The adhesive strips sealing the microwells were carefully peel back.Using a sterile Pasteur pipette 4 dropsof the bacterial suspension was transferred toeach well of both microwell strips.After inoculation, well 21 (arginine) was overlayed with 3 drops of mineral oil**.**The top of the microwells were sealed with the adhesive strips peeled back earlier and incubated at37°C for 24 hours and 48 hours**.** After 24 hours, the adhesive strip was peel back and all positive results for wells one to 18(carbohydrates) were recorded with reference to the control well that was orange in colour compared to the control well was scored as positive. The arginine, ortho-nitrophenyl galactopyranoside (ONPG) and citrate were read against the colour chart and recorded. The adhesive strips were resealed and reincubated at 37°C for 24 hours. After 48 hours incubation, the appropriate reagents were added to the following microwells in the second microwell strip: two drops of Kovac's reagent was added to well 19 and read after 60 seconds. Formation of a pink/red colour indicates a positive result.One drop each of the Voges-ProskauerI (VP I) and VP II reagents were added to well 23 and read after 30 minutes. Formation of a red colour indicates a positive result. The nitrate reduction test was performed on well 20 after reading and recording the ONPG result. One drop each of Nitrate A and Nitrate

B reagents were added to the well and read after 60 seconds. Formation of a red colour indicates a positive result (Microgen Bioproducts Ltd, U.K.).

3.5Screening of the *B. subtilis* Isolates for Amylase Production

The amylase production potential of the test isolates was determined by using the starch agar plate method. The identified *B.subtilis* isolates were inoculated centrally on starch agar medium. The inoculated plates were then incubated aerobically at 37^{o}C for 24hrs. After the incubation period, the cultured plates were flooded with Lugol's iodine solution to observe the zone of hydrolysis around the colonies. The diameter formed after the addition of iodine solution was measured in millimetres using a transparent ruler to represent the amylolytic activity (Oyeleke *et al*., 2011).

3.6Standard Curve of Glucose

Seven test tubes were taken, six of which were labelled 1 to 6 for different glucose concentrations and the seventh was labelled blank. An aliquot of 1.8 ml of starchsolution was placed in each tube and 0.2 ml of different dilutions (0.5, 1, 1.5,2,2.5 and 3mg/ml) of glucose was added intothe six test tubes respectively and 0.2 ml of buffer was added in the blank test tube. Three millilitres ofdinitrosalicylic acid (DNS) reagent was added to each tube and mixed thoroughly by vortex. Then, the tubes were immersed in boiling water-bath for 15 minutes and were allowed to coolto room temperature. Six millilitres ofdistilled water was added to all the test tubes. Absorbance was read at 540nm and a graph was plotted for values ofglucose concentration against corresponding absorbance.The standard curve was used to extrapolate the corresponding concentrations of the absorbance values gotten (Appendix I).

3.7Alpha Amylase Production by Solid State Fermentation (SSF)

3.7.1 Inoculum preparation:

The selected *B. subtilis* isolate with the best production potentialwas inoculated into nutrient broth with the following composition in (g/l): peptone, 5; Beef extract, 3; NaCl, 5 , followed by incubation at 37^{o}C for 24hours to obtained a standardized inoculum of 3.5 x 10^5 CFU/ml with 0.5 optical density (OD) at 600nm (Saxena and Singh, 2011).

3.7.2 Preparation of medium for SSF

For α-amylase production under solid state fermentation, mineral salt solution(MSS) was used as the fermentation medium and comprised the following per litre: 0.1g, KH_2PO_4; 0.1g $(NH_4)_2SO_4$; 0.25g, NaCl; 0.01g, $MgSO_4$ and 0.01g, $CaCl_2$. The initial pH was adjusted to 7.0 with 1M NaOH.

3.7.3 Solid state fermentation

Ten millilitres of the mineral salt solution was mixed with 5g of maize husk substrate in a 250 ml cotton-plugged Erlenmeyer flask in duplicate and labelled appropriately. The contents were homogenized and the initial pH adjusted to 7.0, then sterilized at 121^{o}C for 15 min. After which, the flasks were inoculated with 1.0 ml of freshly prepared inoculum (3.5 x 10^5 CFU/ml) and incubated aerobically at 35°C for 72hrs (Goma, 2013).

3.8Parameters Optimized for α-Amylase Production

The following parameters were used in order to determine the optimum conditions for α-amylase production by*Bacillus subtilis*:

3.8.1 Effect of incubation temperature on α-amylase production

The effect of incubation temperature on α-amylase production was determined using temperature values of 20-40^{o}C at 5^{o}C interval to find the metabolically favorable temperature of *B. subtilis* to optimally synthesizeα-amylase.

3.8.2 Effect of initial pH on α-amylase production

Various pHof 5, 6, 7,8 and 9 were used to determine the optimum pH for α-amylasehyper production under predetermined incubation temperature.

3.8.3 Effect of incubation period on α-amylase production

The effect of incubation period on the α-amylase production was determined by varying the incubation period from 24 to 120hours that is 1-5 days to find the optimum fermentation period for maximum α-amylase production under predetermined incubation temperature and initial pH.

3.8.4 Effect of moisture content on α-amylase production

Different moisture contents (substrate: moisture) of 1:1, 1:2, 1:3, 1:4 and 1:5 were used to determine the optimum moisture content for maximumα-amylaseproduction under predetermined incubation temperature, initial pH and incubation period.

3.9 Alpha AmylaseAssay

3.9.1 Enzyme extraction

After fermentation, 50 ml of 0.1 M phosphate buffer (pH = 7) was added and the flasks were placed in a shaker at 150 rpm for 1h. They were thenfiltered through a muslin cloth followed by centrifugation at 10,000 xg for 20 min. The clear supernatant was subjected to amylase assay (Goma, 2013).

3.9.2 Amylase enzyme assay

Amylase activity was determined by spectrophotometric method as described by Bernfield (1955). According to the procedure, 1.0 ml of enzyme solutionwas taken into test tube and 1.0 ml of substrate (1% w/v starch) in 0.1 M phosphate buffer (pH 7.0) was added and incubated at 50°C for 30 min in water-bath. Then, 2.0 ml dinitrosalicylic acid (DNS) reagent was added to each tube to stop the reaction and kept in boiling water-bath for 5 min. The content was thencooled to room temperature and the volume made up to 10 ml with distilled water and absorbance was read at 540 nm. One unit (U) of α-amylase activity is defined as the amount of enzyme that releases 1μmol of reducing sugar as glucose per minute under 50°C for 30 min and expressed as U/g of dry substrate (Goma, 2013).

3.9.3 Identification of the type of amylase by sugar analysis on thin layerchromatography (TLC)

To identify the type of amylase produced based on starch hydrolysate, thin layer chromatography wascarried out. Exactly 0.9 ml 1% soluble starch was mixed with 0.3 ml crude enzyme solution and incubated for 30 minutes at 80^{o}C in a water-bath. The hydrolysate was then spotted on TLC plate along with standard known sugar (glucose and maltose) solutions. A one dimensional ascend was done using a solvent system (v/v) of butanol: ethanol: water (5:3:2). After ascend, air-dried TLC plate was sprayed with 50% (v/v) Methanol-H_2SO_4mixture and heated for 10 min at 100^{o}C. The dark brown spot that appeared was identified by comparing with the standards (Muluye, 2006).

3.10Statistical Analysis

Data obtained from the optimization parameters were subjected to statistical analysis by comparison of means through one way analysis of variance (ANOVA). Data were presented in Tables and Figures.

CHAPTER FOUR

4.0RESULTS

4.1 Proximate Composition of Maize Husk

The proximatecompositionof the dried unfermented maize husk sample presented in Table 4.1revealed moisture (10.60%), ash (4.83%), fat(17.20%), crude protein (7.00%), crude fibre (3.45%) and carbohydrate contents (60.37%).

4.2 Isolation and Identification of *Bacillus subtilis*

Isolates with colonies typical of*B. subtilis* were observed on nutrient agar medium (Appendix II). Eleven isolates were tentatively confirmedto be *B. subtilis* based on cultural, microscopic as well as biochemical characteristics as shown in Table 4.2.However, only nine isolates were confirmed to be *B. subtilis* based on microgen identification system as shown in Table 4.3. The percentage occurrence of the *Bacillus subtilis* isolates in the three soil samples is presented in Table 4.4 with soil samples from flower bed having the least isolation rate of 20%, while soil from botanical garden and refuse dump site had isolation rate of 80% each.

4.3 Amylase Production by *Bacillus subtilis*Isolates

The nine isolates were screened andfound to be capable of producing amylase which was identified by zone of clearance signifying hydrolysis of starch on agar plate (Appendix III).The isolate RD4 (from refuse dump) gave the highest (29mm) zone of starch hydrolysis while isolates BG1 and BG3 (from botanical garden) gave the least (15mm each) zone of starch hydrolysis (Table 4.5).

Table 4.1: Proximate Composition of Maize Husk

Parameter	**Percentage (%)**
Moisture	10.60
Ash	4.83
Fat	17.20
Crude Protein	7.00
Crude Fibre	3.45
Carbohydrate	60.37

Table 4.2: Cultural, Microscopic and Biochemical Characteristics of the *Bacillus* Isolates

Isolate's Code	Growth on NA	GRM	SS	Biochemical Characteristics									Sugar Fermentation				Tentative Identity
				SH	M	C	Cat	U	I	MR	VP	NR	Glu	Man	Ara	Xyl	
BG1	Creamy, opaque colonies	G+ve rods	oval+	+	+	+	-	-	-	+	+		+	+	+	+	*B. subtilis*
BG2	Creamy, opaque colonies	G+ve rods	oval+	+	+	+	-	-	-	+	+		+	+	+	-	*B. subtilis*
BG3	Creamy, opaque colonies	G+ve rods	oval+	+	+	+	-	-	-	+	+		+	+	+	+	*B. subtilis*
BG4	Creamy, opaque colonies	G+ve rods	oval+	+	+	+	-	-	-	+	+		+	+	-	+	*B. subtilis*
BG5	Creamy, opaque colonies	G+ve rods	oval+	+	+	+	-	-	-	+	+		+	+	+	+	*B. subtilis*
RD1	Creamy, opaque colonies	G+ve rods	oval+	+	+	+	-	-	-	+	+		+	+	+	+	*B. subtilis*
RD2	Creamy, opaque colonies	G+ve rods	oval	+	+	+	+	-	-	-	+	+		+	+	+	+	*B. subtilis*
RD3	Creamy, opaque colonies	G+ve rods	oval	+	+	+	+	-	-	-	+	+		+	+	+	+	*B. subtilis*
RD4	Creamy, opaque colonies	G+ve rods	oval	+	+	+	+	-	-	-	+	+		+	+	+	+	*B. subtilis*
FB1	Creamy, opaque colonies	G+ve rods	oval	+	+	+	+	-	-	-	+	+		+	+	+	+	*B. subtilis*
FB4	Creamy, opaque colonies	G+ve rods	oval	+	+	+	+	-	-	-	+	+		+	+	+	+	*B. subtilis*

BG; Botanical garden, **RD**; Refuse dump, **FB**; Flower bed, **NA**; Nutrient agar, **GRM**; Gram reaction and Morphology, **SS**; Spore Staining, **SH**; Starch Hydrolysis, **M**; Motility, **C**; Citrate Utilization, **U**; Urease, **I**; Indole, **MR**; Methyl red, **VP**; Voges-Proskauer, **NR**; Nitrate Reduction, **Glu**; Glucose, **Man**; Mannitol, **Ara**; Arabinose, **Xyl**; Xylose, **G+ve;** Gram positive.

Table 4.3: Identity of *Bacillus subtilis* Isolates based on Microgen

Isolate's Code	Octal Code	Probability (%)	Identity
BG1	70710427	99.55	*B. subtilis*
BG2	70710427	99.55	*B. subtilis*
BG3	70710427	99.55	*B. subtilis*
BG4	70710427	99.55	*B. subtilis*
RD1	73710427	99.68	*B. subtilis*
RD2	70710427	99.55	*B. subtilis*
RD3	70710427	99.55	*B. subtilis*
RD4	70710427	99.55	*B. subtilis*
FB4	73710427	99.68	*B. subtilis*

BG; Botanical garden, **RD**; Refuse dump, **FB**; Flower bed

Table 4.4: Occurrence of *Bacillus subtilis* in Various Soil Samples Collected

Sampling Location	Number of Samples Collected n = 15	Number Positive (%)
Botanical garden	5	4(80)
Refuse dump site	5	4(80)
Flower bed	5	1(20)

n = Total number of samples collected

Table 4.5: Amylase Production by *Bacillus subtilis* Isolates

Isolate Code	Diameter of Zone of Starch Hydrolysis (mm)
BG1	15
BG2	17
BG3	15
BG4	20
RD1	22
RD2	20
RD3	22
RD4	29
FB4	22

BG; Botanical garden, **RD**; Refuse dump, **FB**; Flower bed

4.4 Optimum Conditions Affecting Alpha Amylase Production by *Bacillus subtilis*

4.4.1 Effect of incubation temperature on alpha amylase production by *Bacillus subtilis*

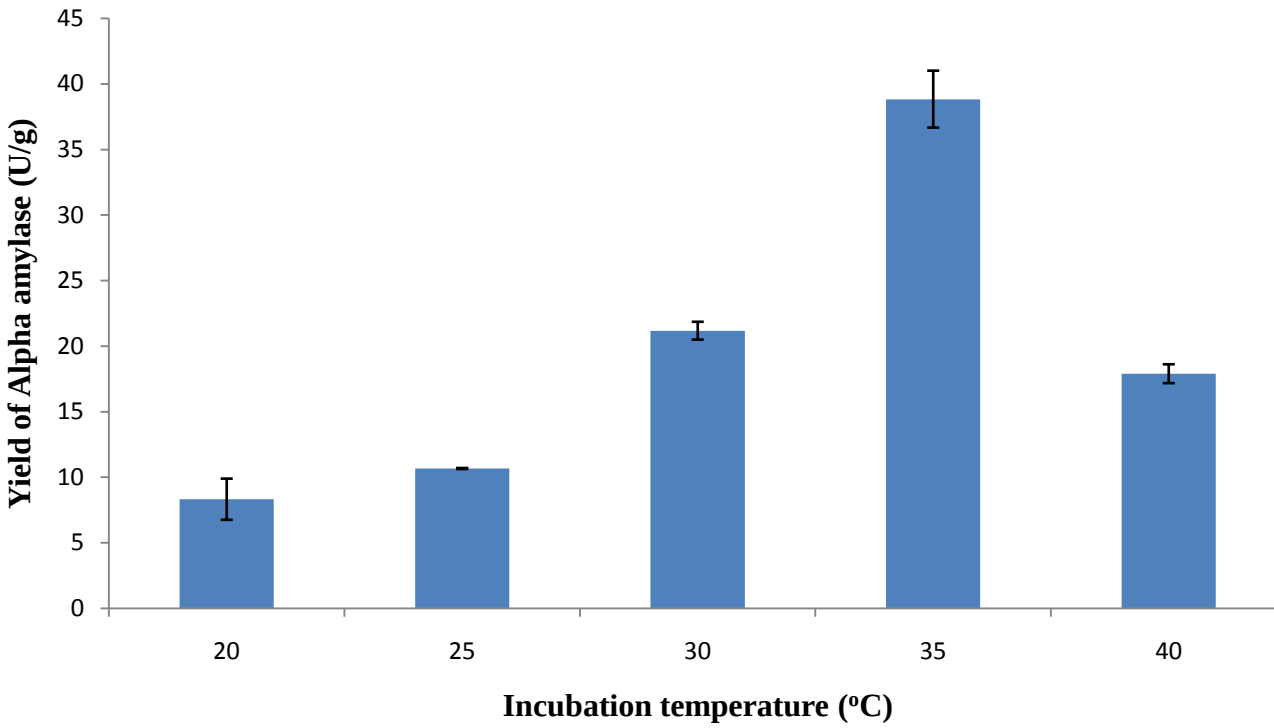
Figure 4.1 shows effect of different incubation temperatures (20, 25, 30, 35 and 40^{o}C) on α-amylase production by *Bacillus subtilis* at initial pH 7.0,moisture content of 1:2for 72hrs. Highest α-amylase (38.83±2.17 U/g) was produced at 35^{o}C. There was an increasing trend in α-amylase production as the temperature increases up to 35^{o}C after which further increase in temperature resulted in decreaseα-amylase production.

4.4.2 Effect of initial pH on alpha amylase production by *Bacillus subtilis*

Effect of various levels of initial pH (5, 6, 7, 8 and 9) on α-amylase production by *Bacillus subtilis* at 35^{o}C, moisture content of 1:2 for 72hrs is presented in Figure 4.2. Alpha amylase production of 39.97±0.72 U/g was observed at pH 7.0. Further decrease in α-amylase production was observed with increasing pH.

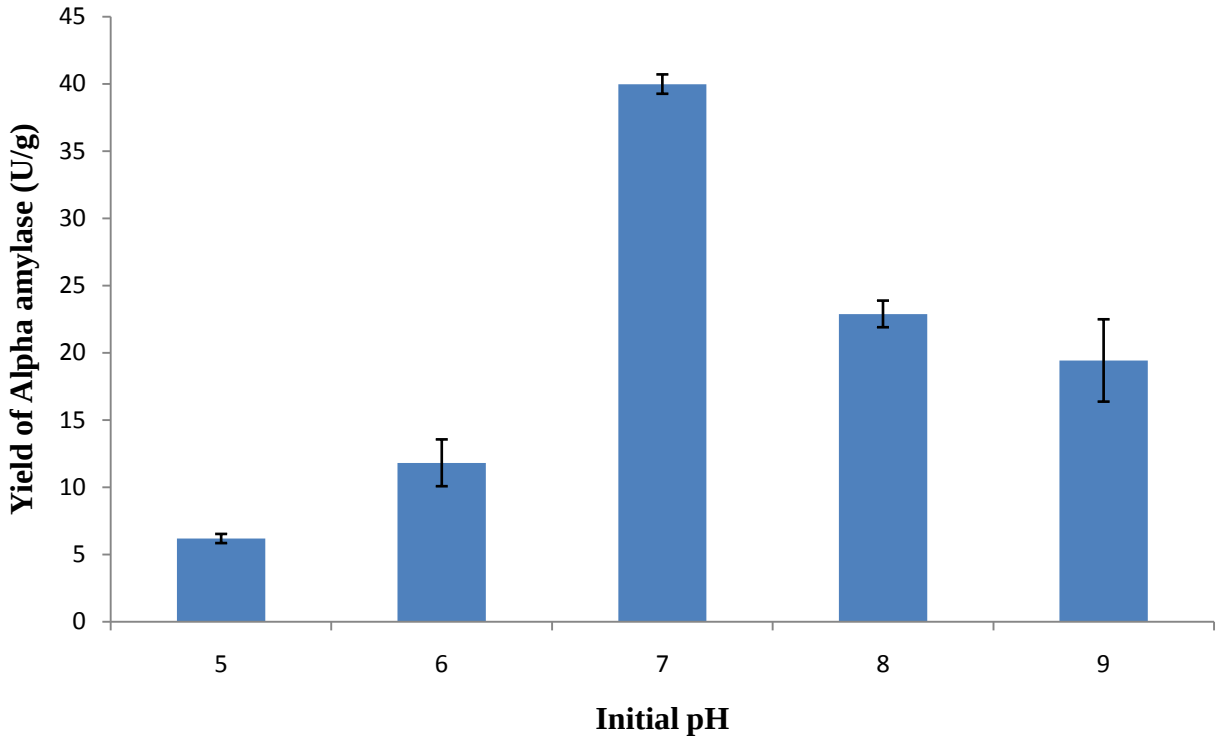
4.4.3 Effect of incubation period on alpha amylase production by *Bacillus subtilis*

Figure 4.3 reveals effect of various incubation periods (24, 48, 72, 96 and 120 hours) on α-amylase production by *Bacillus subtilis* at 35^{o}C and initial pH 7.0 and moisture content of 1:2. Highest α-amylase production was observed after 72 hours of incubation (47.98±2.51 U/g). With increasing incubation period, a decrease inα-amylaseproduction was observed.



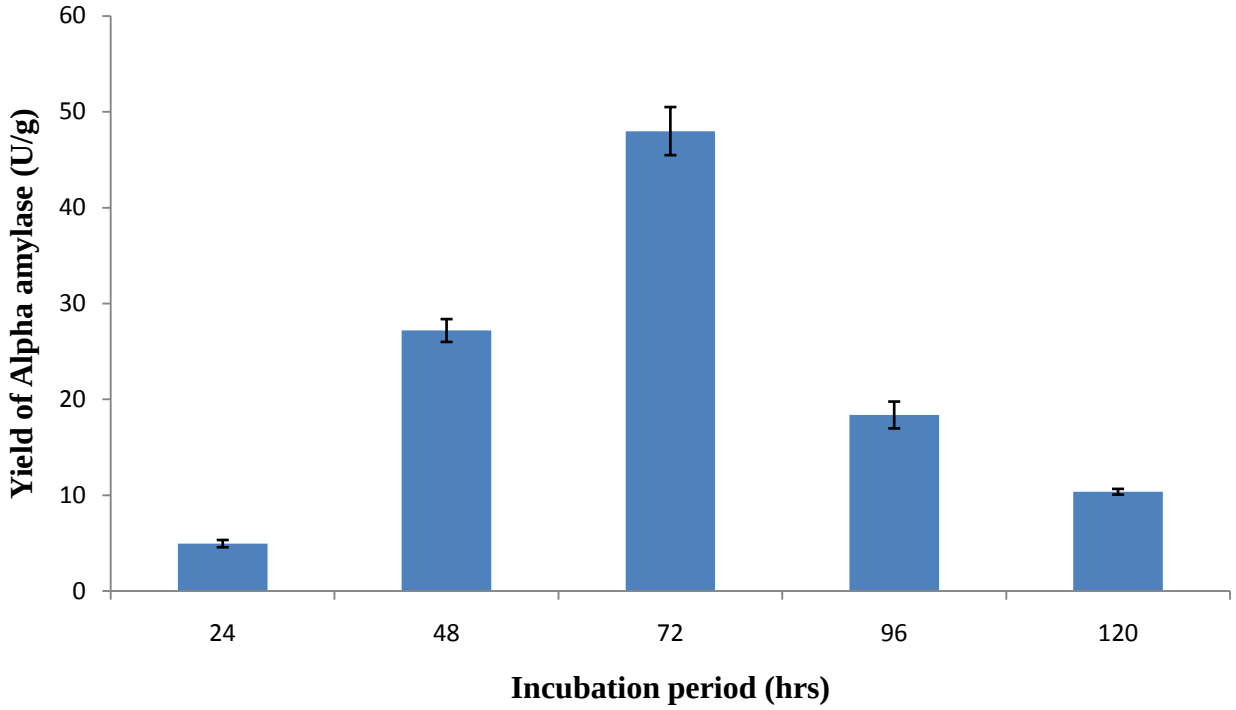
p= 0.041df=9 ***f=177.925***

Figure 4.1: Effect of Incubation Temperature on Alpha Amylase Production by *Bacillus subtilis*at Initial pH 7.0, Moisture Content of 1:2 for 72 hours



p=0.012df=9 ***f=118.614***

Figure 4.2: Effect of Initial pH on Alpha Amylase Production by *Bacillus subtilis* at 35^{o}C, Moisture Content of 1:2 for 72 hours



p=0.034df=9 ***f=287.178***

Figure 4.3: Effect of Incubation Period on Alpha Amylase Production by *Bacillus subtilis*at 35^{o}C and Initial pH 7.0 and Moisture Content of 1:2

4.4.4 Effect of moisture content on alpha amylase production by *Bacillus subtilis*

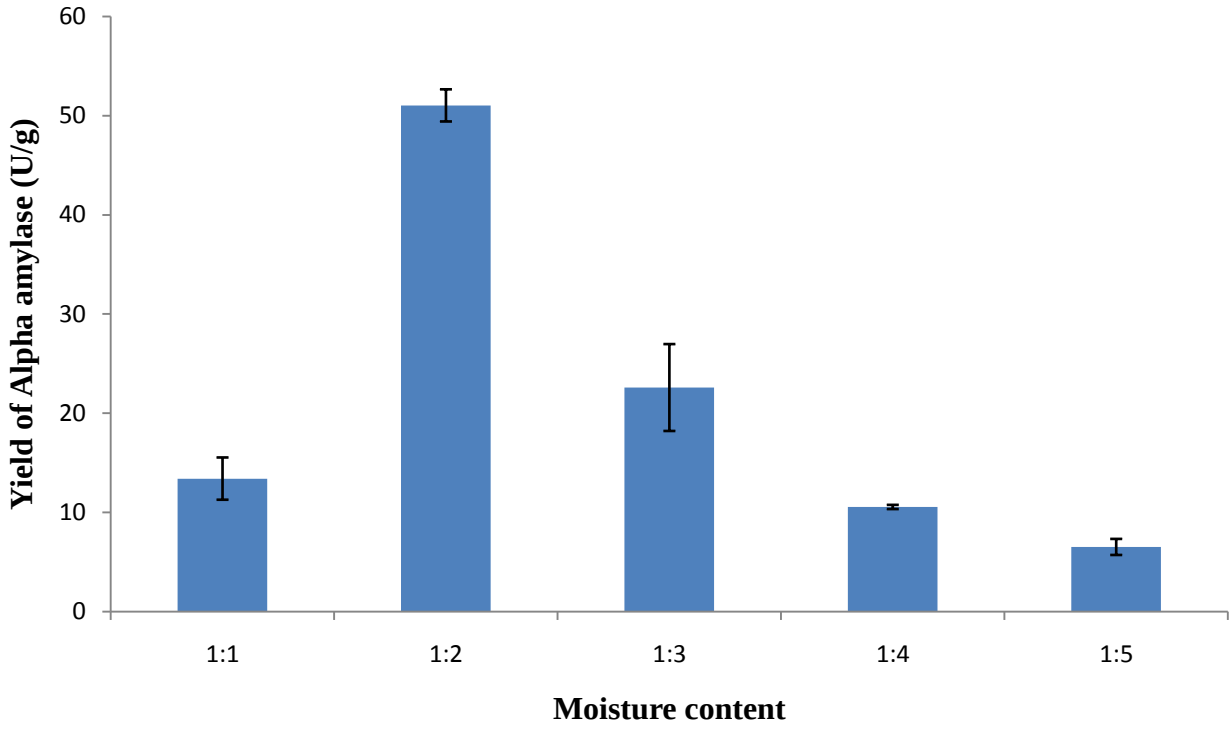
Effect of different moisture contents (1:1, 1:2, 1:3, 1:4 and 1:5) on α-amylase production by *Bacillus subtilis* at 35^{o}C, initial pH 7.0 and incubation period of 72 hours is presented in Figure 4.4. Highest α-amylase production was observed at moisture content of 1:2 (51.02±1.62 U/g) after which decrease inα-amylaseproduction was observed with increasing moisture content.

4.5Yield of Alpha Amylase Produced by *Bacillus subtilis* Using Maize Husk under Optimum Fermentation Conditions

Optimum α-amylase yield of 51.65±0.13 U/g as presented in Table 4.6 was obtained from maize husk substrate with the selected *B. subtilis* isolate under predetermined optimum fermentationconditions of incubation temperature (35^{o}C), initial pH (7.0), incubation period (72hrs) and moisture content of 1:2 (substrate : water).

4.6 Identification of the Type of Amylase by Sugar Analysis on Thin Layer Chromatography (TLC)

The spots developed on TLC plate for identification of the type of amylase produced are shown on Plate 4.1. The product of starch hydrolysis labelled S corresponds to the spot developed by maltose labelled M with a retention factor of 0.48 indicating the amylase produced was α-amylase.



p=0.013df=9 ***f=118.305***

Figure 4.4: Effect of Moisture Content on Alpha Amylase Production by *Bacillus subtilis*at 35^{o}C, Initial pH 7.0 and Incubation Period of 72 hours

Table 4.6: Yield of Alpha Amylase Produced by *Bacillus subtilis* Using Maize Husk under Optimum Fermentation Conditions

Parameter (Optimum value)	**Yield of Alpha Amylase** (U/g)
Incubation Temperature (35^{o}C)	38.83 ± 2.17
Initial pH (7.0)	39.97 ± 0.72
Incubation Period (72hrs)	47.98 ± 2.51
Moisture Content (1:2)	51.02 ± 1.62
Overall Optimum Production	51.65 ± 0.13

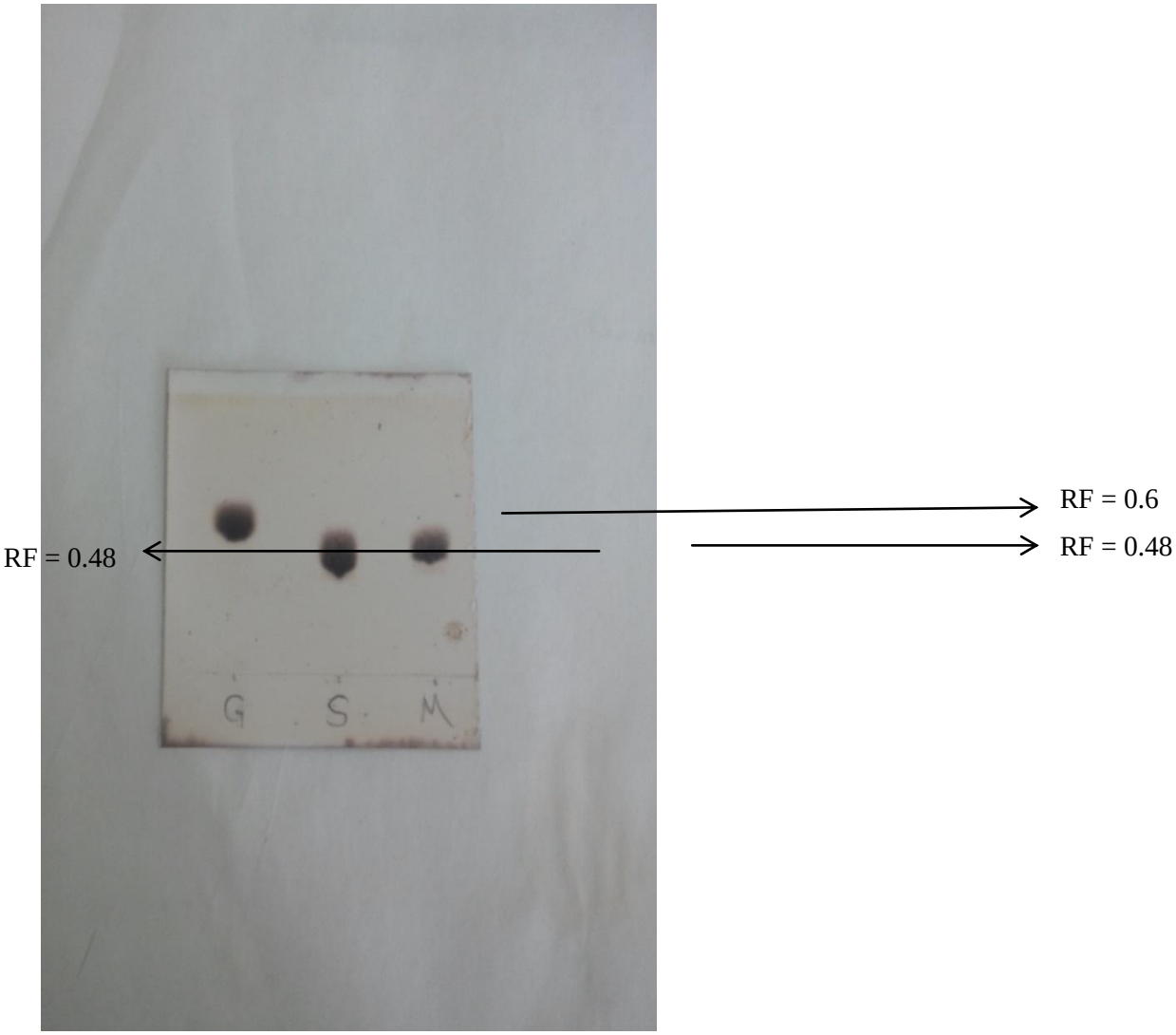


Plate 4.1: Spots of Sugars on TLC Plate for Identification of Type of Amylase Produced

G= Glucose,**S** = Starch Hydrolysis Product**, M** = Maltose

CHAPTER FIVE

5.0 DISCUSSION

Alpha Amylase has been in increasing demand world-wide due to its crucial role of starch hydrolysis and the applications of this hydrolytic action (Ajita and Thirupathihalli, 2014). To meet the growing demands in the industry, it is necessary to improve the performance of the system and thus increase the yield without increasing the cost of production (Gangadharan *et al*., 2008).

Proximate composition of the maize husk revealed percentages of crude protein (7.00%), carbohydrates (60.37%) and ash (4.83%) contents. The crude proteinshowed the potential of the maize huskas a nitrogen source required for growth and efficient enzyme expression, whereas the carbohydrate content indicates high content of fermentable sugars required for growth and enzyme production by the organism. The ash content indicates the richness of the substrate in minerals required for growth of the organism. These might have accounted for theα-amylase production (51.65±0.13 U/g) obtained in this study.The result is similar to the finding of Enyisi *et al*. (2014) on the chemical and nutritional value of maize and maize products.

Out of the fifteen soil samples collected,a percentage occurrence of 80% was obtained with soil from botanical garden and refuse dump site whereas soil from flower bed had the least percentage occurrence of 20%. This might be due to the richness in organic matter content of the humic soil from the refuse dump and botanical garden, whereas, the flower bed might have little organic matter as nutrient to the organism.This agrees with the findings of Bello (2016) who reported a higher isolation rate (50%) from organically-rich soil than from nutrient poor soil (20%).This is also similar with the findings of Ubalua (2014) who reported a higher occurrence of *B. subtilis* from garden

soil. Only nine (9) isolates were confirmed to be *B. subtilis* based on microgen identification. The reduction in the number of confirmed isolates could be due to the sensitivity of the microgen system of identification. Similarly the differences observed in the octal code of the isolates could be due to strain variations.

Bacillus species are considered to be the most importantsources of α-amylase and have been used frequently for enzyme production using SSF.Out of nine (9) isolates confirmed to be*B. subtilis* and screened for amylase production ability, the isolate from refuse dump (RD4) had the highest zone of starch hydrolysis (29mm) whereas, the lowest zone of starch hydrolysis (15mm) was observed with isolates named BG1 and BG3 from botanical garden.This observed variationmight be due to differences in the metabolic capabilities of the different isolates.This result is in agreement with the work of Ubalua (2014) where out of the 13 isolates observed, isolate CNS_3 (*Bacillus subtilis*) demonstrated highest starch hydrolyzing ability. Similar result was also reported by Vijayalakshmi*et al.* (2012) where their isolate KC3 had the highest zone of starch hydrolysis (23mm) on starch agar plate.The occurrence ofamylolytic organisms in soil agrees with the earlier report of Omemu *et al*. (2005), which described soil as a repository of amylase.

Optimization of fermentation conditions, especially physical and chemical parameters are important in the development of fermentation processes due to their impact on the economy and practicability of the process (Goma, 2013). In this study, physicochemical parameters such as incubation temperature, initial pH, incubation period and moisture content were investigated for enhanced α-amylase production.

Temperature is known to influence protein denaturation, enzyme inhibition and cell growth, thus plays a significant role in biological process. In this study, the effect of

incubation temperature on α-amylase production by *Bacillus subtilis* under SSF revealed that 35°C was the optimum(38.83±2.17 U/g)among the tested incubation temperatures. Decrease in α-amylase production as observed beyond the optimum temperature (35^{o}C) might be due to growth reduction and enzyme inactivation or suppression of cell viability. The decrease inα-amylaseproduction as observed below the optimum temperature might be as a result of reduction in the metabolism of the microorganism and consequently, the enzyme synthesis caused by low temperature values(Mazutti *et al*., 2007).The yield of α-amylase produced by *Bacillus subtilis* at the different incubation temperatureswas found to be statistically significant ($P< 0.05$). The result of this study is similar tothe work ofSingh (2016) who observed a maximum α-amylase production by*Bacillus amyloliquefaciens*MTCC-610 using agro-byproducts under solid state fermentation at 35^{o}C. This is also similar to the findings of Irfan *et al*. (2011) who reported 35^{o}C as the optimum incubation temperature for α-amylase production by *Bacillus subtilis*using potato peel.Sajitha*et al*. (2011) reported the optimum incubation temperature for the production of α-amylase by *Bacillus* sp. as 35°C using starch as substrateand Goma (2013) who observed a maximum α-amylase production by *Bacillus subtilis* NCTC-10400 and *Bacillus cereus* ATCC 14579 under solid state fermentation at 35^{o}C using wheat bran. However, Shinde *et al*. (2014) reported 30^{o}C as the optimum incubation temperature by *Bacillus licheniformis* using potato as substrate whereas Riaz *et al*. (2003) and Ashraf *et al*. (2005b) reported 40^{o}C as the optimum incubation temperature for *Bacillus subtilis* and *Bacillus licheniformis*using pearl millet and wheat bran as substrates respectively.

Alpha amylase production by microbial strains strongly depends on the extracellular pH as it influences the transport of various components across the cell membrane. The initial pH of the fermentation media may change since organic acids are produced

during fermentation, however, it is known that agro industrial substrates possess unique buffering action, and therefore have advantages for enzyme production (Goma, 2013). Maximum production of α-amylase by *B. subtilis* occurred at pH of 7.0 (39.97±0.72 U/g).This might be due to the physiological nature ofpH 7.0 at which best metabolic functions are carried out.The enzyme yieldsharply increased from pH 5 to pH 7 while a decrease in the enzyme yield was observed from pH 8 to 9. This might bebecause the metabolic activities of microorganisms are very sensitive to pH.The yield of α-amylase produced by *Bacillus subtilis* at the different initial pH of the fermentation mediumwas found to be statistically significant ($P< 0.05$).The results of this finding are in line with that of Goma (2013) who observed a maximum α-amylase production of 232.95 and 180.8 U/gby *Bacillus subtilis* NCTC-10400 and *Bacillus cereus* ATCC 14579 respectively using wheat branat initial pH of 7.0. Similar results were reported byShaista *et al*. (2003) who also found maximum production of amylase enzyme at pH 7 and comparatively lower production was observed at pH 8 when *Bacillus subtilis* was used in SSF of banana peels.WhileHaq *et al*. (2003) and Shinde *et al*. (2014) reported7.5– 8.0 and pH 6.5to be the best for α-amylase production by *Bacillus subtilis*using wheat bran as substrate and *Bacillus licheniformis*using potato peelas substrate respectively.

The incubation time is governed by culture characteristics and also based ongrowth rate (Singh, 2016).In this study, the effect of incubation period on α-amylase production by *Bacillus subtilis* under SSF revealed that 72hwas the optimum(47.98±2.51 U/g)among the tested incubation periods.Beyond this period, the α-amylase production started to decrease. This might be due to the deficiency of nutrients present in the medium. It might also be due to proteolysis of α-amylase (Shafique *et al*., 2009). The yield of α-amylase produced by *Bacillus subtilis* at the different incubation periodswas found to be

statistically significant (P< 0.05).This result is in agreement with that of Singh (2016) who reported maximum α-amylase production by *Bacillus amyloliquefaciens*MTCC-610 using agro-byproducts under solid state fermentation at 72h. Similar results were reported by Gangadharan *et al*. (2006) who found 72h as the optimum fermentation period for alpha amylase production by *Bacillus amyloliquefaciens*by SSF using wheat bran and ground nut oil cake and Anto *et al*. (2006) who observed maximum enzyme yield after 72h by *B. cereus* with wheat bran as substrate and decreased with further incubation. Contrary results were obtained by Irfan *et al*. (2011) who recorded 48h of fermentation period as best for the alpha amylase secretion by SSF using potato peel and Riaz *et al*. (2003) who found maximum production after 48h by *Bacillus subtilis* with pearl millet as substrate.These variations in the fermentation period might be due to thedifferent microbial strains and different substrates used during fermentation processes.

Moisture content is a critical factor in SSF processesand has been found to have great influence on microbial growth and biosynthesis and secretion of different metabolites(Saxena and Singh, 2011).Maximum production of α-amylase by *B. subtilis*was attained when the substrate: moisture ratio was 1:2 (51.02±1.62 U/g). The observed variations in the α-amylase produced with varying moisture contents could be explained by the fact that higher moisture levels can cause a reduction in enzymeyield due to steric hindrance of the growth of strain due to reduction in porosity (interparticle spaces) of the solidsubstrate, thus interfering with oxygen transfer (Perez-Guarre *et al*., 2003).Similarly, lower moisture contentof the substrate causes reduction in solubility of the nutrients, low degree of swelling and high water tension (Balkan and Ertan, 2007).The yield of α-amylase produced by *Bacillus subtilis* at the different moisture contentswas found to be statistically significant (P< 0.05). The result of this study is

similar to the work of Goma (2013) who reported maximum α-amylase production when the substrate: moisture ratio wasmaintained at 1:2. It also agrees with the work of Anto *et al*. (2006) who reported substrate: moisture ratio of 1:2 as the optimum moisture content for α-amylase production by *B. cereus* MTCC 1305 using solid state fermentation using wheat bran as substrate.

In this study, the total amount of α-amylase produced at optimum conditions by *Bacillus subtilis* using maize husk was 51.65±0.13 U/g.This shows that agricultural residues such as maize huskcould be a good substrate for the production of α-amylase. The result is similar with the findings of Goma (2013) who reported 74.60 and 56.66 U/g of alpha amylase by *B. subtilis* NTCC-10400 and *B. cereus* ATCC-14579 respectively using wheat bran as substrate and also Anto *et al*. (2006) who reported 94±2 U/g of alpha amylase by *B. cereus* MTCC 1305 under solid state fermentation using wheat bran as substrate. However, the result is higher than the 17±1.20 U/g of α-amylase reported by Kalaiarasi and Parvatham (2013) using maize bran as substrate but lower than 5400 U/g amylase reported to be produced by*Bacillus*spp.with mustard oil seed cake as the substrate (Saxena and Singh, 2011).

The product of starch hydrolysis was found to appear as a spot with a retention factor (RF) of 0.48 which is the same as that of standard maltose. Thus, indicating the amylase produced was alpha amylase. This is in agreement with the report of Muluye (2006) who stated that *Bacillus* spp. produce mainly α-amylase.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

From the observations made in this study, the following conclusions are drawn:

Maize husk contains organic nutrients in quantities sufficient to support the growth of *B. subtilis* and amylase production.

Bacillus subtilis can readily be isolated from various soil types with frequency of 80% in soil samples from botanical garden and refuse dump and 20% in samples from flower bed.

All the isolates of *B. subtilis* obtained from the three soil sources demonstrated the ability for amylase production.

Amylase production by *B. subtilis* using maize husk as substrate occurs when the solid state fermentation process was conducted at 35^{o}C, initial pH 7.0, 72hrs and moisture content of 1:2.

An overall yield of 51.65±0.13 U/g of α-amylase using maize husk as substrate was obtained.

6.2 Recommendations

Based on the observations made in this study, the following recommendations are made:

1. Genetic improvement of the organism should be carried out to enhance alpha amylase production.
2. Further studies should be carried out with the aim of enhancing the production of α-amylase from maize husk using *B. subtilis* by optimizing the medium composition.

3. Agrowastes and residues should be tested for their potentials to serve as substrates for α-amylase production by *Bacillus subtilis*.

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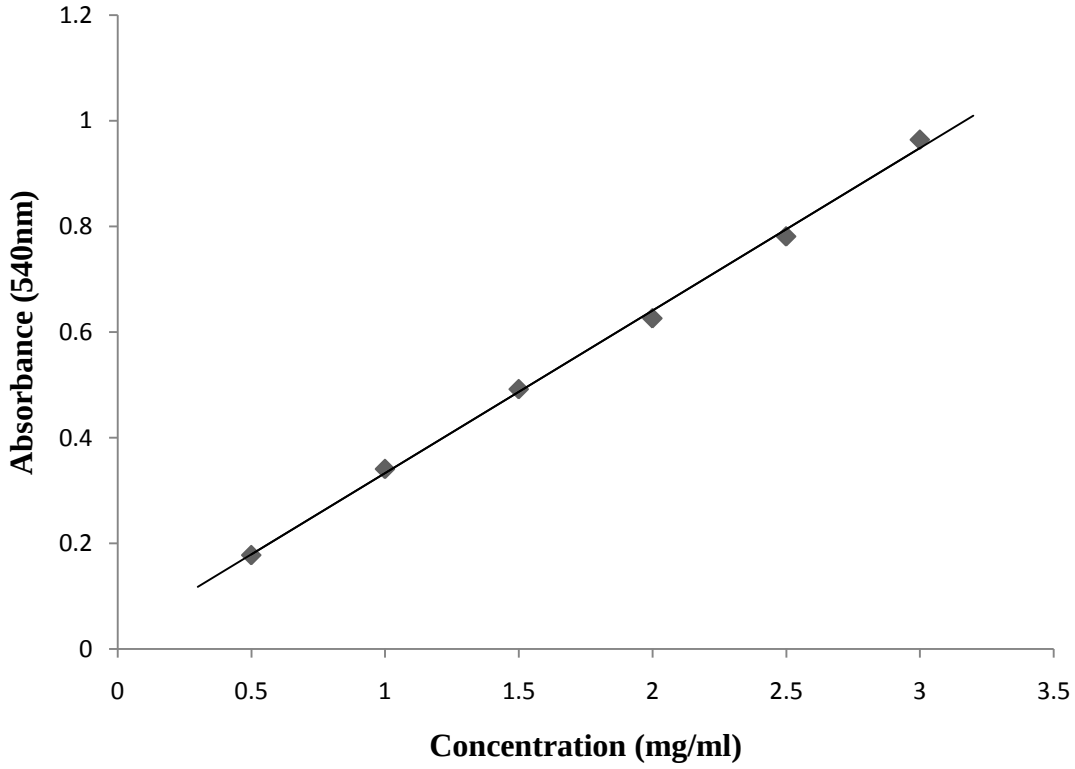
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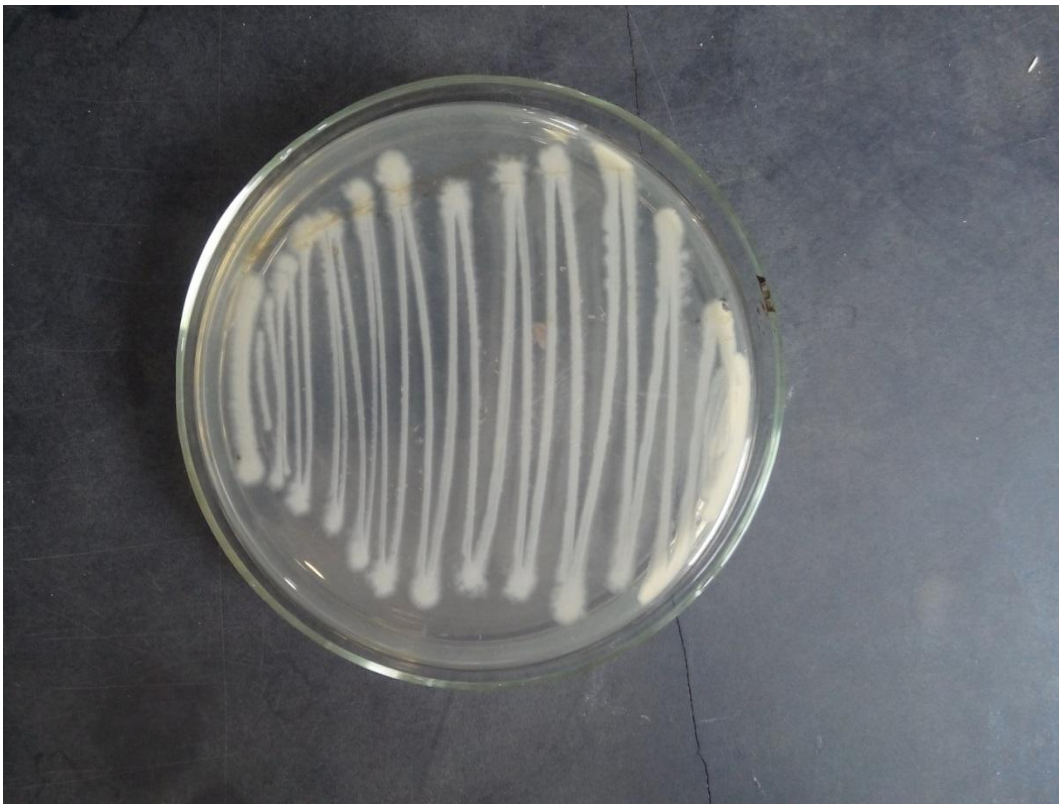
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APPENDICES

Appendix I: Glucose Standard Curve



Appendix II: Colonial Morphology of *B. subtilis* on Nutrient Agar



Appendix III: Zone of Starch Hydrolysis Shown by *B. subtilis*

