ISOLATION AND SCREENING OF *STREPTOMYCES* SPECIES FOR ENHANCED AMYLASE PRODUCTION

BY

Martina NATHANIEL

DEPARTMENT OF MICROBIOLOGY,
FACULTY OF LIFE SCIENCES, AHMADU BELLO UNIVERSITY,
ZARIA, NIGERIA

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MARTINA NATHANIEL
[B.Sc. (Hons) Microbiology, A.B.U – 2011]
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NIGERIA

JUNE, 2017

DECLARATION

I declare that the work in this dissertation entitled "Isolation and screening of *Streptomyces*spp. for enhanced amylase production"has been carried out 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.

Martina NATHANIEL Date

CERTIFICATION

This dissertation entitled "ISOLATION AND SCREENING OF *STREPTOMYCES*SPPFOR ENHANCED AMYLASE PRODUCTION"by Martina NATHANIEL 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.

Prof. S.A. Ado

Chairman, Supervisory Committee Signature Date

Prof. I.O. Abdullahi

Member, Supervisory Committee Signature Date

Prof. I.O. Abdullahi

Head of Department Signature Date

Prof. S.Z. Abubakar

Dean, School of Postgraduate Studies Signature Date

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DEDICATION

This piece of work is dedicated to God Almighty for His faithfulness and divine favour granted me, which has enabled me to finish this work successfully. To God alone is all the Glory.

ABSTRACT

In this work, optimization of culture conditions for enhanced amylase production by five *Streptomyces* strains isolated from the agricultural soil from Ahmadu Bello University dam were investigated. *Streptomyces* isolates were identified by Gram staining, morphological and physiological observations and those identify were; *Streptomyces narbonensis, Streptomyces michiganensis, Streptomyces orientalis, Streptomyces*spp (S4), *Streptomyces* spp (S5). Upon screening for amylase production, only *Streptomyces michiganensis* was able to produce amylase with diameter of clear zones of starch hydrolysis of 38.3±0.45mm, hence it was used for further production of amylase under solid state fermentation. Determination of parameters such as period of fermentation, pH, temperature, moisture content and inoculum size were also carried out in this study. Highest amylase produced was observed at 72h incubation period with amylase activity of 8.5±21U/ml and 12.1±0.35U/ml using wheat bran and soluble starch respectively. The optimum period of fermentation, pH, temperature, initial moisture content and inoculum size were 48h (17.7±0.41U/ml), pH 6 (12.2±0.28U/ml), 35°C (11.2±1.13U/ml), 30% (7.9±0.21U/ml) and 2ml of 1.0 x10^6spores/ml (7.9±0.21U/ml) respectively. There was a significant increase in enzyme production after optimization of fermentation condition, with maximum yield of 17.7±0.41U/ml recorded at 48h.Wheat bran could serve as a good substrate for amylase production by *Streptomyces michiganensis*.

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CHAPTER ONE

1.0 INTRODUCTION

1.1 Background of the Study

Enzymes are single or multiple chain protein that act asbiocatalysts with the ability to promote specific chemical reactions under mild conditions in most living organisms (Aneja, 2003). They are present in the cell of living organisms in minute amounts and are capable of speedingup chemical reactions (associated with life processes),without themselves being altered after the reaction (Oyeleke and Oduwole, 2009).

Like other proteins, they consist of long chains of amino acids that fold to produce a three-dimensional product and each unique amino acid sequence produces a specific structure, which has unique properties. Enzymes are responsible for many essential biochemical reactions in microorganisms, plants, animals, and human beings.They differ in function in that they have the unique ability to facilitate biochemical reactions without undergoing change themselves, which makes enzymes unique.The molecule that an enzyme acts on is known as its substrate, which is converted into a product or products(Mojsov, 2012). Some enzymes require small non-proteinmolecules, known as cofactors, in order to function as catalysts (Jenkins, 2003).

A large number of processes in the areas of industrial, environmental and food biotechnology utilize enzymes at some stage or the other. Current developments in biotechnology are yielding new applications for enzymes (Frolova *et al*., 2004 ; Patil, 2008). Amylases are among the most important enzymes and are of great significance in present day biotechnology. This industrial enzyme constitute about 25-33% of the world market and has almost completely replaced chemical hydrolysis of starch in the starch processing industry (Rajagopalan and Krishnan 2008; Azad *et al*., 2009).

Acid chemical hydrolysis of starch has many limitations: it is non-specific, lacks ways ofcontrolling saccharide composition, requires high refining cost and is lessenvironmentally friendly. The application of enzymes for this process has avoided these limitations (Crabb and Shetty, 1999).

Amylases are universally distributed throughout the animal, plant and microbial kingdom. Over the past few decades, considerable research has been undertaken with extracellular alpha amylase being produced by a wide variety of microorganisms. The major advantage of using microorganism for amylase production are the economic bulk production capacity of microbes and they can easily be manipulated to obtain enzyme with desired characteristics(Behal *et al*., 2006 ; Zhenming *et al*., 2007).

Amylase stands out as an enzyme useful in food, brewing, textile, detergent and pharmaceutical industries (Pandey *et al*., 2000a ; Rosell *et al*., 2001). They are mainly employed for starch liquefaction, production of maltose, oligosaccharide mixtures, high fructose syrup and maltotetrose syrup. They are applied during detergent production to improve cleaning effect and are also used for starch de-sizing in textile industry (Lonsane and Ramesh, 1990). Amylasesare also used for the clarification of beer or fruit juices and pretreatment of animal feed toimprove the digestibility of fiber (Ghorai *et al*., 2009).

A wide range of microorganisms such as fungi and bacteria are used for the industrial production of amylases. However, *Streptomyces* exhibit remarkable capacity for synthesis of secondary metabolites and uses numerous extracellular hydrolytic enzymes to degrade organic material in their natural habitat. *Streptomyces* are regarded as a natural source of biocatalytic enzymes. They catalyse transesterification reactions and more importantly are stable under various storage conditions (Simkhada, 2009). There are various reports on alpha amylase production by Actinomycetes. A promising *Streptomyces clavifer* and *Streptomyces* spp.(Hoque *et al*., 2006 ; Kar and Ray, 2008 ; Yassien and Asfour, 2011) had been reported.

Amylase can be produced either by submerge fermentation (SmF) (Aguilar *et al*., 2002) or solid state fermentation (SSF) (Viswanathan and Surlikar, 2001). Although, the conventional amylase production was carried out by SmF, SSF system offers advantages such as high volumetric productivity, high concentration of product with less effluent generation and utilization of simple fermentation equipments (Pandey *et al*., 1999; Pandey *et al*., 2000b). Since the substrates used in SSF are mainly from agro-industrial residues, this process also resolve the pollution problem that arises because of their disposal (Pandey *et al*., 2000c ; Pandey *et al*., 2000d).

In this study, solid state fermentation was carried out using wheat bran as substrate for production of amylase by*Steptomyces* spp. and various fermentation conditions such as pH, temperature, inoculum size, moisture content and period of fermentation were optimized to enhance amylase production.

1.2 Statement of Research Problem

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

Problems associated with the production of this enzyme in developing counties such as Nigeria is the high cost of production (Oyeleke and Oduwole, 2009).

Numerous agricultural residue generated represent one of the most important resources. Accumulation of these residues causes deterioration of the environment and huge loss of potentially valuable nutritional constituent which when processed could yield food, feed, fuel, chemicals and mineral (Bisaria, 2000). Agricultural residues when dumped in open environment constitute health hazards due to pollution (Barton, 1999). Also, due to great commercial value of amylase in biotechnologicalapplications ranging from food, fermentation,textile to paper industries, there is greater need for increase inamylase production and a search for more efficient processes (Wolfgang, 2007).

1.3 Justification of the Study

In Nigeria, the local production of amylase willsave about 200 million naira that is spent annually on the importation of amylase (Ayansina *et al*, 2017).

The search for a cost effective production process that will also bring about high yield of the product will help in reducing the cost of production and also meet up with the market demand. Since the substrates used in solid state fermentation are mainly fromagro-industrial residues which are cheap and readily available, this process can be used for amylase production. This will not only reduce the production cost but also resolve pollution problems that arises as a result of their disposal (Pandey *et al*., 1999).

1.4 Aim of the Study

The aim of this study was to isolate *Streptomyces* spp. and evaluate its amylase production potential with the view to optimize the fermentation conditions for enhanced amylase production.

1.5 Objectives of the Study

The specific objectives were to;

1. Isolate and identify *Streptomyces* spp.from soil.
2. Screen isolates of *Streptomyces*spp. for amylase production.
3. Produce amylase using wheat bran as substrate under solid state fermentation.
4. Determine the optimum fermentation conditions for enhanced production of amylase by selected species.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Actinomycetes

Actinomycetes are aerobic, gram-positive bacteria that have high GC content in their DNA (69-73%). They form extensive branching substrate, aerial mycelia with numerous pigmentations and widely distributed in soil (Oskay *et al*., 2004).

2.2 Genus Streptomyces

Streptomyces is the largest genus of Actinobacteria and the type genus of the family Streptomycetaceae (Hong *et al*., 2009). Over 500 species of *Streptomyces* bacteria have been described *(*Lee *et al*., 2005). *Streptomyces* species are chemoorganotrophic, filamentous gram-positive bacteria but not acid-alcohol fast, not fungi and occur in the same habitats as fungi and are superficially similar (Ikeda *et al*., 2003). They have genomes with high guanine-cytosine content 69-78% (Kavitha *et al*., 2010).The filaments and spores are very small, usually 1 µm or less in diameter (Willemse *et al*., 2011). The spores are formed by the fragmentation of the filaments and are borne in straight, wavy, or helical chains (Chater, 1993).The spores are heat sensitive with exposure to temperature of 70°C for 10 minutes proving lethal (Dorokhova *et al*., 1970).

The colonies are slow-growing and often have a soil-like odour because of the production of a volatile metabolite, geosmin (Juttner and Watson, 2007). In the early stages of development, colonies are relatively smooth surfaced but later, they develop a weft of aerial mycelium that may appear floccose, granular, powdery, or velvety (Ambarwati *et al*., 2012).

They produce a wide variety of pigments which are responsible for the colour of the vegetative and aerial mycelia (Flardh and Buttner, 2009).

Their peptidoglycan cell wall contains major amounts of L- diaminopimelic acid (L-DAP). They have no mycolic acids but contain major amounts of saturated, iso- and anteiso-fatty acids. They also possess either hexa- or octahydrogenated menaquinones with nine isoprene units as the major isoprenolog, and have complex polar lipid patterns that typically contain diphosphatidyl glycerol, phosphatidyl ethanolamine, phospha-tidyl inositol, and phosphatidyl inositol mannosides (Cummins and Harris, 1958).

2.3The Life Cycle of Streptomycetes

The life cycle of Streptomycetes starts when a spore settles in a nutrient rich medium. This stimulates the spore to exit its dormant state and undergo germination and form germ tubes which grow by tip elongation and the cells don’t undergo binary fission. Through extension and branching, the germ tubes give rise to a network of filaments which grow into and across the surface of an agar plate. This network is called the substrate mycelium (Flardh and Buttner, 2009).

As the colony continues to grow, the mycelium in the center of the colony starts to differentiate. Differentiation results in the formation of a new cell type, the aerial hyphae. When growth of the spiraling, multi-genomic aerial hyphae stops, the aerial hyphae undergo synchronous cell division giving rise to monoploid compartments each of which will develop into a resistant spore (Kieser *et al*., 2000).

The life cycle of a *Streptomyces* spp. has been studied by the Robinow HC1-Giemsa method of nuclear staining, and is described in the subsequent manner: (1) initial nuclear division phase; (2) primary mycelium; (3) secondary mycelium (Including aerial); (4) the formation of spores. The primary mycelium develop after the initial nuclear division phase and produce side divisions, then later gave rise to single swellings in the hyphae. These swellings grow to form large round cells, each of which contains many nuclei. The secondary mycelium develops, a part of which become aerial and terminates in the form of chains of spores (Mc Gregor, 1954).

2.4 Streptomycetes Habitats

Although streptomycetes are widely distributed in soil, water and other natural environments (Seong *et al*., 2001;Singh *et al*., 2006), their population in an ecosystem is determined by numerous physical, chemical and biological factors (Kharat *et al*., 2009). Identification of novel ecological systems is therefore crucial for the discovery of novel Streptomycetes (Wang *et al*., 1999).

Streptomycetes make 40% of soil bacteria (Boone *et al*., 2001). Under dry and alkaline conditions *Streptomyces* spp. are the numerous microbial population in soil and because of their filamentous form, they impact the strength of soil texture and protect it from wind and water erosion (Vetsigian and Roy Kishony, 2011). The percentage of *Streptomyces* spp. in total microbial population has positive correlation with the depth of soil and they can even be obtained from the horizon C of the soil (Kim *et al*., 2004). Distribution of Streptomycetes in water and soil depends on food stress, temperature, pH, moisture, salinity, soil texture and climate (Locci, 1989). Although soil is the most important Streptomycetes habitat (Mokrane *et al*., 2013), other habitats are:

2.4.1 Hay and other Organic Material

Mesophilic and Thermophilic *Streptomyces* spp. can degrade many natural substrates, cotton textiles, plastics, rubber and paper (Subbarao, 1999). Most of streptomycetes subjected as indigenous microbes isolated from soil have the capability to grow and successfully remove and use different organic compound (Rahmansyah *et al*., 2012). Streptomycetes are capable of degrading cellulose, lignocellulose, chitin and different organic compounds in biogeochemical cycles (Horn *et al*., 2012).

2.4.2 Fresh Water and Marine Habitats

Streptomycetes can also be found in fresh water and marine habitat in addition to drinking water systems with drainage after heavy rainfall (Rowbotham and Cross, 1977). The isolation of Streptomycetes from marine environments has been an abundant area of investigation in the past decade (Remya and Vijayakumar, 2008). Of the marine inhabitants studied, marine invertebrates, particularly sponges, are of excessive interest for discovering novel Streptomecetes (Selvakumar *et al*., 2010).

In recent times, the marine derived actinomycetes have become documented as a source of novel antibiotic and anticancer agent (Baskaran *et al*., 2011).

2.4.3Plants

Streptomycetes play a minor role as plant pathogens such as *Streptomyces scabies*, *S. ipomoea*, *S. turgidiscabies, S. aureofaciens, S. acidiscabies* and *S. tumescans* causing gall potato, soil rot or pox, pitted scab, netted scab, gall potato in acidic soil and root gall respectively (Fatope *et al*., 2000). On the other hand, some species of *Streptomyces* act as biological control of pest (Rugthaworn, 2007). Biological control is an environmentally sound and effective means of reducing or mitigating pests and their effects through the use of microorganisms. For example, biological control of sunflower stem rot(Aghighi *et al*., 2004; Baniasadi, 2009) and potato common scab(Kalantarzadeh, 2006).

2.4.4 Animal and Humans

Although a small number of clinical Streptomycetes have been isolated so far, their role as pathogens and infectious disease cannot be ignored (Korn-Wendisch and Kutzner, 1992). Streptomycetes are uncommon pathogens, though infections in humans, such as mycetoma, can be caused by *Streptomyces somaliensis* and *Streptomyces sudanensis* (Quintana *et al*., 2008).

2.5 Growth Requirements of Streptomycetes

Streptomycetes are aerobes, chemoorganotrophic bacteria and they need organic carbon source, inorganic nitrogen sources, and mineral salts and don’t need vitamins and growth factors (Lee and Demain, 1997). *Streptomyces* growthrequirements has been investigated byMost *Streptomyces* spp are mesophiles that grow in temperatures between 10-37°C (Deeble *et al*., 2005) but three species *Streptomyces thermonitrificans, S.thermovulgaris* and *S.thermoflavus* are thermophiles that grow in temperatures between 45-55°C (Srivibool *et al*., 2004). Streptomycetes grow in pH ranging between 6.5 and 8.0 (Cabello *et al*., 2003). Streptomycetes are not only more resistant to drought and form arthrospore. They also require less moisture than other bacteria and are very sensitive to water logged conditions (Subbarao, 1999). Some reports reveal that drained soils (e.g sandy loam, calcareous soils) have more *Streptomyces* than heavy clay soils (Sujatha *et al*., 2005).

2.6 Metabolites Produced by Streptomycetes

A primary metabolite is directly involved in normal growth, development, and reproduction in an organism. It usually performs a physiological function in the organism. Primary metabolites are typically present in many organisms or cells (Demain, 1980).

Secondary metabolites are organic compounds that are not directly involved in the normal growth, development, or reproduction of an organism (Shomurat *et al.*, 1979). Therefore secondary metabolites differ from primary metabolites (e.g. amino acids and nucleotides) in four ways: 1) they are not essential for growth, 2) their production is dependent on growth conditions like type of culture media, 3) they are often produced as groups of closely related molecules, and 4) it is often possible to overproduce these components (Drew and Demain, 1977).

The most important characteristic of Streptomycetes is their ability to produce secondary metabolites with antibacterial, antifungal, antiviral and antitumoral properties. Two species of *Streptomyces* by the name of *Streptomyces griseus* and *Streptomyces coelicolor* are used for industrial production of Streptomycin and novel antibiotics such as dihydrogranticin respectively. Doxorubicin as anticancer agents (Mukhtar *et al*., 2012) and Rapamycin as immune modulatory agents are secondary metabolites produced by *Streptomycete*s (Ying and Marta, 2001).

Another metabolite of *Streptomycetes* known as the ‘Geosmin’and sidrophore are responsible for the earthy odor (Sanglier *et al*., 1993). However volatile product secreted by *Streptomyces* may also be responsible for the specific smell (Bais *et al*., 2012).

2.7 Usefulness of *Streptomyces* spp. In Biotechnology

In recent years, biotechnology researchers have begun using *Streptomyces* spp for heterologous gene expression of proteins. Traditionally, *Escherichia coli*was the species of choice to express eukaryotic genes since it was well understood and easy to work with (Brawner e*t al*., 1991).

According to Binnie *et al*. (1997), expression of eukaryotic protein in *E. coli* may be problematic. Sometimes, proteins do not fold properly, which may lead to insolubility, deposition in inclusion bodies and loss of bioactivities of product. Though *E. coli* strains have secretion mechanisms, these are of low efficiency and result in secretion into the periplasmic space, whereas secretion by a gram positive bacterium like *Streptomyces* spp results in secretion directly into the extracellular medium. In addition *Streptomyces* spp have a more efficient secretion mechanism than *E. coli*. The properties of the secretion system is an advantage for industrial production of heterelogous expressed protein because it simplifies subsequent purification steps and may increase yield. This properties among others make *Streptomyces* spp. an attractive alternative to other bacteria such as *E. coli* and *Bacillus subtilis*.

2.8 Amylase

Amylases are important enzymes employed in the starchprocessing industries for the hydrolysis ofpolysaccharides such as starch into simple sugarconstituents (Mitchell and Lonsane, 1990; Akpan *et al*.,1999). The amylase family ofenzymes is of great significance due to its wide area ofpotential application.

Starch degrading enzymes like amylase havereceived great deal of attention because of theirperceived technological significance and economicbenefits. Evidences of amylase in yeast, bacteria andmoulds have been reported and their properties documented (Adebiyi andAkinyanju, 1998;Akpan *et al*., 1999; Buzzini and Martini, 2002).

2.9 Classification of Amylase

2.9.1 α-Amylase

α-Amylases (endo-1,4-α-D-glucan glucohydrolase, EC 3.2.1.1) are extracellularenzymes that randomly cleave the 1,4-α-D-glucosidic linkagesbetween adjacent glucose units in a linear amylose chain.These are endoenzymes that split the substrate in the interiorof the molecules and are classified according to theiraction and properties. For example, amylases that producefree sugars are termed ‘saccharogenic’ and those thatliquefy starch without producing free sugars are known as‘starch-liquefying’ (Pandey *et al*., 2000a).

The α-amylases are calciummetalloenzymes, completely unable to function in the absence of calcium, α-amylase breaksdown long-chain carbohydrates by acting at random locations along the starch chain,ultimately yielding maltotriose and maltosefrom amylose, or maltose, glucose and "limitdextrin" from amylopectin. α-amylase tends to be faster-acting than β-amylase because it can act anywhere on the substrate. In human physiology, both the salivary and pancreatic amylases are α-Amylases. Also found in plants (adequately), fungi (ascomycetes and basidiomycetes) and bacteria (*Bacillus*) (Das *et al*., 2011).

2.9.2 β-Amylase

Pandey *et al*. (2000a) stated that β-Amylase (*α*-1,4-glucan maltohydrolase,EC 3.2.1.2) is usually of plant origin, but a few microbialstrains are also known to produce it. It is an exo-actingenzyme that cleaves non-reducing chain ends of amylose,amylopectin and glycogen molecules. It hydrolyses alternateglycosidic linkages, yielding maltose (β-anomeric form). Sinceβ-amylase is unable to by-pass *α*-1,6-glycosidic linkagesin amylopectin, it results in incomplete degradation of themolecule, yielding 50–60% maltose and a β-limit dextrin.

During the ripening of fruits,β-amylase breaks down starch into maltose, resultingin the sweet flavor of ripe fruit. Both α-amylaseand β-amylase are present in seeds; β-amylaseis also present in an inactive form prior togermination, whereas α-amylase and proteasesappear once germination has begun. Animal tissues do not contain β-amylase (Das *et al*., 2011).

2.9.3 Glucoamylase (GA)

GA(synonyms amyloglucosidase, ‘glucogenic enzyme’, ‘starchglucogenase’ and ‘γ-amylase’; exo-1,4-α-D-glucan glucanohydrolase,EC 3.2.1.3) hydrolyses single glucose units fromthe non-reducing ends of amylose and amylopectin in astepwise manner. Unlike α-amylase, most glucoamylases arealso able to hydrolyse the 1,6-α-linkages at the branchingpoints of amylopectin, although at a lower rate than 1,4-linkages. Thus glucose, maltose and limit dextrins are theend products of GA action (Pandey *et al*., 2000a).

Unlike the other forms of amylase, γ-amylase is most efficient in acidic environmentsand has an optimum pH of 3 (Das *et al*., 2011).

2.10 Fermentation Method Used for Production of Amylase

To meet the demand of industries, low-cost mediumis required for the production of α-amylase. Both solid state fermentation (SSF) and submerged fermentation (SmF) could be used forthe production of amylases, although traditionally thesehave been obtained from submerged cultures because of ease of handling and greater control of environmentalfactors such as temperature and pH. Synthetic mediahave been mostly used for the production of bacterial amylasethrough SmF (McTigue *et al*., 1995; Haq *et al*., 1997; Haddaoui *et al*., 1999; Hamilton *et al*., 1999).

The contents of synthetic mediasuch as nutrient broth, soluble starch, as well as othercomponents are very expensive and these could be replacedwith cheaper agricultural by-products to further reduce the cost of the medium. SSF resembles naturalmicrobiological processes such as composting andensiling, which can be utilized in a controlled way toproduce a desired product. SSF has been used for longto convert moist agricultural polymeric substrates such as wheat, rice, soy and cassava into fermented food productsincluding industrial enzymes (Rahardjo *et al*., 2005).

SSF is generallydefined as the growth of microorganisms on moist solidsubstrates with negligible free water (Pandey *et al*., 2001). The solid substratemay provide only support or both support andnutrition. SSF constitutes an interesting alternative sincethe metabolites so produced are concentrated and purificationprocedures are less costly (Pandey, 1992; Nigam and Sindh, 1995; Chadha *et al*., 1997;Pandey *et al*., 2000a).

SSF is preferredto SmF because of simple technique, low capitalinvestment, lower levels of catabolite repression and end-product inhibition, low waste water output, better productrecovery, and high quality production (Lonsane *et al*., 1985). Amongthe different substrates used for SSF, wheat bran hasbeen reported to produce promising results (Haq *et al*., 2003; Kunamneni *et al*., 2005). Othersubstrates such as sunflower meal, rice husk, cottonseedmeal, soybean meal, and pearl millet and rice bran havebeen tried for SSF (Baysal *et al*., 2003).

SSF technique is generally confined to the processesinvolving fungi. However, successful bacterial growth in SSF is known in many natural fermentations (Ramesh and Lonsane, 1991). Theproduction of α-amylase by SSF is limited to the genus*Bacillus*. *Bacillus subtilis, B. polymyxa, B. mesentericus, B. vulgarus,B. coagulans, B. megaterium* and *B. licheniformis* have been used for α-amylase production in SSF (Babu and Satyanarayana, 1995).

Research on the selection of suitable substratesfor SSF has mainly been centered around agro-industrialresidues due to their potential advantages forfilamentous fungi, which are capable of penetrating intothe hardest of these solid substrates, aided by the presenceof turgor pressure at the tip of the mycelium (Ramachandran *et al*., 2004).

2.11Factors Affecting the Production of Amylase

The production and stability of α-amylase in the medium is affected by a variety ofphysicochemical factors. In spite of expression's possibility under a wide range of culturingconditions, α-amylase could be denatured under some conditions. Many proteins easilyaggregate into so-called inclusion bodies during expression in bacterial systems (Espargaro *et al*., 2008). Inhibition of protein aggregation during fermentation/expression can beachieved by adjusting the production conditions (Hao *et al*., 2007; Bahrami *et al*., 2009)**.**

2.11.1 Incubation Period

With regards to incubation period, many investigators have found that extracellular α-amylase production is growth associated (Murthy *et al.*, 2009;Asoodeh *et al.*, 2010;Abou Dobara *et al.*, 2011).

The changes in productivity of extracellular enzymes can be attributedto the differences in the timing of induction of separate components of the enzyme system,the inhibition by products of substrate hydrolysis and differential inactivation by proteasesand/or variation in the pH during cultivation conditions (Tuohy and Coughlan, 1992; Wang *et al*., 1993).

The accumulation of sugars over a critical concentration in the medium iswell documented to inhibit the enzyme production (Wang *et al*., 2006; Dona *et al*., 2010).

2.11.2 Temperature

Among the physical parameters, the temperature of the medium plays an importantrole in α-amylase production and stability. Generally, the influence of temperature onamylase production is related to the growth of the organism. Hence, the optimum temperature depends on whether the culture is mesophilic, thermophilic or psycrophilic.Among the fungi and actinomycetes, most amylase production studies achieved theoptimum yields within the range of 25°C- 40°C (Gupta *et al*., 2003).

However, thermophilic fungi such as *Thermomyces lanuginosus* (Mishra &Maheshwari, 1996), and actinomycetes,namely; *Thermomonospora fusca* (Busch & Stutzenberger, 1997) and *Thermoactinomycesvulgaris* (Abou Dobara *et al*., 2011) have been reported to produce α-amylase optimally at 50°C, 55°C and 55°C, respectively. On the other hand, it has been produced at a wider rangeof optimal temperature by bacteria reaching to 90°C in *Thermococcale* and *Sulfolobus* species(Leuschner & Antranikian, 1995; Sunna *et al*., 1997).

2.11.3 pH

Also, the pH values were reported toserve as an indicator of the initiation and end of enzyme synthesis (Friedrich *et al*., 1989)because the change in pH affects α-amylase stability in the medium (Calamai *et al*., 2005). It is worth noting that the α-amylase active site consists of a large number of charged groups(Lawson *et al*., 1994; Strokopytov *et al*., 1996; Uitdehaag *et al*., 1999) which explains the reason why most α-amylases have optimum pH in the acidic to neutral range (Pandey *et al*., 2000a;Sun *et al*., 2010;Bozic *et al*., 2011).

2.11.4 Substrate Concentration

In general, amylase activity is connected with the substrate utilization. The inducible nature of α-amylase has been assured in different microorganisms (Aiyer, 2004; Ryan *et al*., 2006;Asoodeh *et al*., 2010;Abou Dobara *et al*., 2011). α-amylase production also appearedto be subjected to catabolite repression by maltose and glucose, like most other inducibleenzymes that are affected by substrate hydrolytic products (Bhella & Altosaar, 1988;Morkeberg *et al*., 1995). However, α-amylase synthesis by *Bacillus* strains was reported tonot be subjected to catabolite repression by monosaccharides (Kalishwaralal *et al*., 2010).

Gupta *et al*. (2003) have classified xylose and fructose as strongly repressive to α-amylase synthesis.Addition of starch to the medium has normally been employed for the production of α-amylase from various microorganisms as reported in the literature.

2.11.5 Particle Size of Substrate

In SSF, particle size of the substrate affects growthof the organism and thereby influences the enzyme production. The adherence and penetration of microorganismsas well as the enzyme action on the substrateclearly depend upon the physical properties of the substratesuch as the crystalline or amorphous nature, theaccessible area, surface area, porosity, particle size, *etc*.In all the above parameters, particle size plays a majorrole because all these factors depend on it (Pandey, 1991; Nandakumar, 1996).

Smallersubstrate particles have greater surface area for growthbut inter-particle porosity is lower. For larger particlesizes, the porosity is greater but the saturated surfacearea is smaller. Hence, determination of particle size correspondingto optimum growth and enzyme productionis necessary (Pandey, 1991).

A mathematical model has been described,which enables the quantification and predictionof the extent of degradation of the substrate particleswith fermentation time for any given initial size of substrateparticles. Studies on the release of α-amylase have beenrecorded and a gradual increase was noticed up to 72hfor *Aspergillus niger*and 36 h for *Bacilluscoagulans*. The reduction ofparticle size was up to 60 % at the end of the fermentationtime, 72 and 36 h for*A. niger*and *B. coagulans*,respectively. Wheat bran having particle sizes between500–1000 μm gave better enzyme yields by *B. subtilis*when compared with those with larger particles (Baysal *et al*., 2003).

2.11.6 Nitrogen Source

Nitrogen source as a basal component of the medium is a major factor affecting α-amylaseproduction. Its effect was not only as a nitrogen source but also as a metal ion source and apH controller as well. Many investigators had recorded that organic nitrogen sources supported maximum alpha-amylase production by various bacteria (Saxena *et al*., 2007;Mrudula & Kokila, 2010;Abou Dobara *et al*., 2011).

The increase in α-amylaseproduction fostered by organic nitrogen sources could be attributed to a high nutritional aminoacids and vitamins content. However, various inorganic salts have been reported to supportbetter production in fungi (Gupta *et al*., 2003). As a metal ion source, ammonium chloridewas found to enhance the production of α-amylase by *T. vulgaris*, where chloride is astabilizer, over that of other ammonium salts (Abou Dobara *et al*., 2011). In addition, thesame authors also reported different productivity of α-amylase by using sodium nitratefrom potassium nitrate.

2.11.7 Moisture Content

Moisture is one of the most important parameters inSSF that influences the growth of organisms and hence enzyme production. Too low or too high moisture levelsof the substrate affect the growth of the microorganismresulting in lower enzyme production. High moisturecontent leads to reduction in substrate porosity,changes in the structure of substrate particles and reductionof gas volume. Bacteria are generally known to requireinitial moisture of 70–80 %.

α-Amylase productionby *Bacillus licheniformis* M27 was found to be highest with 65 % initialmoisture content in an SSF system. Significant decreasein enzyme production was observed with highincrease in moisture content, which was due to the decreasein the rate of oxygen transfer. Studies indicatedthat enzyme titres could be increased significantly by agitationof the medium with high moisture content (Ramesh and Lonsane, 1990).

Athermotolerant*B. subtilis* required initial moisture of 30% for its growth and maximum enzyme production (Baysal *et al*., 2003).Moisture content of 65 % was required by *B. coagulans*for optimum alpha-amylase production on wheat bran (Nandakumar *et al*., 1996), A*spergillusoryzae* (on wheat bran) and *Streptomyces rimosus* (on amixture of sweet potato and peanut meal residue) requiredan initial moisture content of 65 % for maximumenzyme yield in SSF (Yang and Wang, 1999; Ramachandran *et al*., 2004). Maximum alpha-amylase productionby *Thermomyces lanuginosus* ATCC 58157 by SSFon wheat bran was achieved at 90 % moisture content (van der Maarel*et al*., 2002).

2.12Starch and its Source

Starch is a polymer of glucose linked to one another through the C1 oxygen, known as theglycosidic bond.Starch and starch-containing substrates are wide spread in nature and also in industrialpraxis. They can predominantly find their application in many industrial processes.

Starch occurs mainly in the seeds, roots and tubers of higher plants. Some algae produce asimilar reserve polysaccharide called phytoglycogen. Plants synthesize starch as a result ofphotosynthesis. It is synthesized in plastids as a storage compound for respiration duringdark periods. It is also synthesized in amyloplasts found in tubers, seeds, and roots as along-term storage compound. In these latter organelles, large amounts of starch accumulateas water-insoluble granules. The shape and diameter of these granules depend on thebotanical origin. Regarding to commercial starch sources, the granule sizes range from 2–30µm (maize starch) to 5–100 µm (potato starch) (Robyt, 1998).

A variety of different enzymesare involved in the synthesis of starch. Sucrose is the starting point of starch synthesis. It isconverted into the nucleotide sugar ADP-glucose that forms the actual starter molecule forstarch formation. Subsequently, enzymes such as soluble starch synthase and branchingenzyme synthesize the amylopectin and amylose molecules (Smith, 1999).

Starch-containing crops form an important constituent of the human diet. Besides the directuse of starch-containing plant parts as a food source, starch is harvested and chemically orenzymatically processed into a variety of different products such as starch hydrolysates,glucose syrups, fructose, starch or maltodextrin derivatives, or cyclodextrins. In spite of thelarge number of plants able to produce starch, only a few plants are important for industrialstarch processing. The major industrial sources are maize, tapioca, potato, and wheat (Goyal *et al*., 2005).

2.13 Structure of Starch

Starch is a polymer of glucose linked to one another through the C1 oxygen by a glycosidic bond. Two types of glucose polymers are present in starch: (i) amylose and (ii) amylopectin.While amylopectin is soluble in water, amylose and the starch granule itself are insoluble incold water.Amylose is a linear polymer consisting of up to 6000 glucose units with α, 1-4 glycosidic bonds. The number of glucose residues, also indicated with the term DP (degree ofpolymerization), varies with the origin. The relative content of amylose and amylopectinvaries with the source of starch. The average amylose content in most common starches, e.g.in barley, corn and potato, is 20-30% (Marc *et al*., 2002).

Amylopectin consists of short α, 1-4 linked linear chains of 10–60 glucose units and α,1-6linked side chains with 15–45 glucose units. The average number of branchingpoints in amylopectin is 5% (Thompson, 2000), but varies with the botanical origin. Thecomplete amylopectin molecule contains about 2,000000 glucose units, thereby being one ofthe largest molecules in nature (Marc *et al*., 2002). The most commonly accepted model ofthe structure of amylopectin is the cluster model, in which the side chains are ordered inclusters on the longer backbone chains (Thompson, 2000).

In general, Zhu *et al*. (2011) suggested that the internal part of amylopectin is critical to the physical behavior of granular starch. The diameter of starch granules ranges from 2 to 100µm (Whistler & Daniel, 1985) depending on its source. The orientation of the starch chains is thought to be perpendicular to the granule surface (French, 1984). Native starch is partly crystalline. The crystallinity ofnative starch varies between 15 and 45% depending on the origin and pretreatment (French,1984). According to the currently accepted concept, amylopectin forms the crystallinecomponent whereas amylose exists mainly in the amorphous form (Zobel, 1992; Hanashiro *et al*., 1996;Marc*et al*., 2002;). Structural studies have shown that native starch hascrystalline polymorphism. In x-ray diffraction, cereal starch typically gives A-type patternsof monoclinic symmetry, and tuber starch gives B-type patterns of hexagonal symmetry(Imberty *et al*., 1991;Gerard*et al*., 2000). The crystal lattice of B-type starch contains morewater molecules than the A- structures, which is proposed to be the reason for higherstability of the A- structure. Both structures' molecular conformations are practicallyidentical. They have left-handed double helices with parallel strands. Double helices containsix glucose units per turn in each chain and the glucose units are in a chair conformation. Within the double helix, there are inter-chain but no intra-chain hydrogen bonds. In addition, parallelly packed double helices are connected through a hydrogen bondingnetwork.

2.14 Compositions of Wheat Bran

Wheat bran, a by-product of flour milling is composed ofthe pericarp and the outermost tissues of the seed,including the aleurone layer. It constitutes almost 10%of the total weight of wheat milled for flour. On moisturefreebasis, bran contains about 17% protein and 70%carbohydrates, about 80% of which is cellulose andhemicellulose. Most of the bran protein and othernutrients are contained in the aleurone cells (Saunders*et al*., 1972). Ranum (2000) found that bran is mainlycellulose with very little gluten, so there is not much itcan be used for other than being a source of fibre, but itdoes contain higher vitamin and mineral contents, soflour made with a higher extraction rate tends to be morenutritious.

2.15 Role of Wheat Bran in Fermentation Industries

Many agro-industrial by-products are replacing the synthetic and expensive substrates for the production of biotechnological products. Among the agro-industrial substrates, wheat bran is one of the most attractive alternatives to synthetic medium in fermentation processes (Pandey, 1992). The coarse variety of wheat bran is an efficient substrate due to its heat dissipation, better air circulation, loose particle binding and efficient penetration by mycelia and it is cheaper than fine bran so it is a better prospect economically in fermentation industry (Malathi and Chakraborty, 1991). Almost every type of enzyme can be produced by fermentation of wheat bran both by utilizing solid state fermentation (SSF) and submerged fermentation (SmF) systems.

Nowadays wheat bran is widely used in solid state fermentation for the production of secondary metabolites and other industrial products because it reduces pollution effects (Pandey *et al*., 1999). Wheat bran is a potential candidate in fermentation industry because of its unique properties listed below:

2.15.1 Water Retention Capacity

Apart from the presence of important nutritional components, physical characteristics of wheat bran also play vital role in fermentation processes. Wheat bran has the ability to retain high moisture content in SSF. This ability of wheat bran promotes the fungal growth just as in the natural environmental conditions. Wheat bran proved a suitable substrate for the growth of *Trichoderma harzianum, Trichoderma viride, Trichoderma koningii,* and *Trichoderma polysporum* by SSF. There was no need for provision of additional nutrients in Wheat bran medium for the production of *Trichoderma* spores (Cavalcante *et al*., 2008).

2.15.2 Complex Substrate

Complex nature of wheat bran lies in its unique nutrient composition. The higher starch content of wheat bran i.e., 75.6% as compared to other agro-industrial wastes such as rice bran (coarse waste 71.1%> rice powder 55.8%> medium waste 48.6%> fine waste 34.2%) can be correlated for higher amylase production (Ellaiah *et al*., 2002). Wheat bran can be used as an inducer for a multitude of enzymes such as CMCase, xylosidase, glucosidase, L-arabinofuranosidase, amylase, protease, pectinolytic enzymes, rennet, alpha galactosidase, lipase, invertase and phytase (Maheswari and Chandra, 2000; Sindhu *et al*., 2009; Soarse *et al*., 2010; Javed *et al*., 2011).

Xylanase production on commercial scale can also be achieved by using wheat bran as a substrate as it is an agro-economical inducer due to its high xylan content (12.65% of dry material) (Kulkarni *et al*., 1999; Subramanyan and Prema, 2002). Battestin and Macedo (2007) studied tannase production by *Paecilomyces variotii* and reported that the presence of important mineral contents of WB was essential for the mold growth resulting in an 8.6 fold increase in tannase production.

2.15.3 Nitrogen Source

Naturally, higher amount of the nitrogen also requires no or little addition of other nitrogen supplements in Wheat bran containing medium. Elevated nitrogen content of wheat bran makes it suitable for the production of enzymes such as protease, amylase and glucoamylase. Increase in the production of acid protease with an increase in the nitrogen content of Wheat bran has been reported by Vishwanatha *et al*. (2009).

Supplementation of Wheat bran with additional protein sources such as soy flour, defatted sesame flour, casein and peptone facilitate acid protease production. Although Wheat bran alone can be used as an efficient nitrogen source but supplementation of Wheat bran with glucose, peptone, yeast extract, KH_2PO_4 and CaO resulted in the highest spore production 1.7×10^{11} spores/g dry substrate (Vishwanatha *et al*., 2009).

2.15.4 Biofuel Production

To deal with the problem of depletion of fossil fuel reserves with each passing years, researchers are now focusing on bioethanol production from natural substrates to meet energy challenges of the millennium (Shafiee and Topal, 2009). Wheat milling byproducts are now being focused for fermentative production of bioethanol (Palmarola-Adrados *et al*., 2005; Hawkes *et al*., 2008; Manikandan and viruthagiri, 2009).

Manikandan and Viruthagiri (2009) investigated the simultaneous saccharification and fermentation of Wheat bran that resulted in the highest ethanol concentration of 23.1 g/L after 48h of fermentation. Palmarola-Adrados *et al*. (2005) converted the complex polysaccharides in Wheat bran to sugar rich feedstock for conversion to ethanol. The overall sugar yield by combined hydrolysis method (acid treatment and enzymatic hydrolysis) reached 80% of the theoretical and it consisted of 13.5 g arabinose, 22.8 g xylose and 16.7 g glucose per 100 g starch-free bran.

Moreover, Wheat bran can also act as potential substrate for biobutanol production, which may be used as a replacement for gasoline (Liu *et al.*, 2010).Fermentative bio-hydrogen production from carbohydrate-rich substances can be achieved through anaerobic digestion by bacteria (Pam *et al.*, 2006). Acid treatment of Wheat bran followed by anaerobic digestion with mixed anaerobic culture resulted in maximum hydrogen yield of 128.2 ml/g total volatile solid (TVS) and hydrogen production rate of 2.50 ml/ (g-TVS h). Maximum hydrogen content was 62% with negligible methane production (Pan *et al*., 2008).

Treatment of Wheat bran with NaOH and H_2O_2 and then fermentation with mixed culture in sewage sludge produced 22 and 31 m^3 H_2 per ton dry weight assuming that all the sugar is hexose. Fermentation of unhydrolysed wheat feed is also known to improve H_2 yield (Hawkes *et al*., 2008).

2.15.5 Bioremediation

The removal of heavy metal ions from abandoned industrial sites is a major challenge for decontamination and rehabilitation of industrial wastewaters. Presence of heavy metal ions such as Cu (II), Pb (II), and Cd (II) is a potential threat for human health. The use of lingo-cellulosic compounds from wheat bran for the removal of these heavy metal ions offers a cheap and flexible substrate. Wheat bran contains lignin, cellulose and fatty acid units whose functional group content (hydroxylic, carboxylic and phenolic) is ideal for ion fixation. It can be used as a natural filter for decontaminating industrial effluent containing heavy metals. This method can be a cheap alternative to conventional pollution control methods for wastewater by use of synthetic resins for heavy metal adsorption (Dupont *et al*., 2003).

2.16 Uses of Amylase

The amylase family ofenzymes is of great significance due to its wide area ofpotential application. Interestingly, the first enzyme producedindustrially was an amylase from a fungal source in1894, which was used as a pharmaceutical aid for the treatmentof digestive disorders (Crueger and Crueger 1989). Amylases find potentialapplication in a number of industrial processes such as;

2.16.1 Food Industry

Amylasesare extensively employed in processed-foodindustry such as baking, brewing, preparation of digestive aids,production of cakes, fruit juices and starch syrups (Couto and Sanromán, 2006). For decades, microbial α-amylaseshave been widely used in the baking industry (Hamer, 1995; Si, 1999).Theseenzymes can be added to the dough of bread to degrade thestarch in the flour into smaller dextrins, which are subsequentlyfermented by the yeast.Besides generatingfermentable compounds, α-amylasesalso have an anti-stalingeffect in bread baking, and they improve the softness retentionof baked goods, increasing the shelf life of these products (Gupta *et al.*,2003; M.J.E.C. van der Maarel *et al.*, 2002;Sahlstrom and Brathen, 1997).

The addition of α-amylaseto the doughresults in enhancing the rate of fermentation and the reductionof the viscosity of dough, resulting in improvements in thevolume and texture of the product. The most widespread applications of α-amylasesare inthe starch industry, which are used for starch hydrolysis in thestarch liquefaction process that converts starch into fructose and glucose syrups (Nielsen and Borchert, 2000).

The hydrolysis of starch may be carried out using either acid or enzyme as catalyst.Acid conversion has, however, many limitations: it is non-specific, lacks ways ofcontrolling saccharide composition, requires high refining cost and is lessenvironmentally friendly. The application of enzymes for this process has avoidedthese limitations (Crabb and Shetty, 1999). Conversion of starch into sugar, syrups anddextrins forms the major part of the starch processingindustry.Amylasesare also used for the clarification ofbeer or fruit juices, or for the pretreatment of animal feed toimprove the digestibility of fiber (Gavrilescu and Chisti, 2005; Ghorai *et al*., 2009; M.J.E.C. van der Maarel *et al*., 2002).

2.16.2 Textile Industry

Amylasesare 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. In textile weaving, starch paste is applied for warping.This gives strength to the textile at weaving. It alsoprevents the loss of string by friction, cutting andgeneration of static electricity on the string by givingsoftness to the surface of string due to laid down warp. After weaving the cloth, the starch is removed and thecloth goes to scouring and dyeing. The starch on cloth isusually removed by application of α-amylase(Hendriksen *et al*., 1999).

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. The enzymatic desizing of cotton with α-amylasesis state-of-the-art since many decades (Marcher *et al*., 1993).

The amylose is bioconverted to 100% by the α-amylaseinto glucose whereas the amylopectin is converted to 50% into glucose and maltose. Bio-desizing is preferred due to their high efficiency and specific action. Amylasesbring about complete removal of the size without any harmful effects on the fabric besides ecofriendly behavior. The α-amylasesremove selectively the sizeand do not attack the fibres (Ahlawat *et al*., 2009; Feitkenhauer ,2003).Amylasefrom *Bacillus*strain was employed in textile industries for quite a long time.

2.16.3 Paper Industry

The use of α-amylasein pulp and paper industry is in the modification of starchesfor coated paper, i.e. for theproduction of low-viscosity, high molecular weight starch. As for textiles, sizing of paper with starch is performed to protect thepaper against mechanical damage during processing (Gupta *et al*., 2003; M.J.E.C. van der Maarel *et al*., 2002;Bruinenberg*et al*., 1996).The coating treatment serves improves the quality ofthe finished product, enhances stiffness, and elasticity of paper (Gupta *et al*,. 2003; Bruinenberg *et al.*, 1996).Because starch isadded to paper at a temperature range of 45- 60 □C, and the viscosity of the naturalstarch is too high for paper sizing partial degradation of this polymer is essential.α-amylaseis employed for this purpose (Gupta *et al*., 2003).

2.16.4 Detergent Applications

The demand for α-amylasefor use in laundry and automatic dishwashing is veryhigh.The use ofenzymes in detergents formulations enhances the detergentsability to remove tough stains and making the detergentenvironmentally safe.These enzymes are used in detergents for laundry andautomatic dishwashing to degrade the residues of starchy foodssuch as potatoes, gravies, custard, chocolate, etc. to dextrinsand other smaller oligosaccharides (Mukherjee *et al*., 2009; Olsen and Falholt 1998).Removal of starch from surfaces is also important in providing a whiteness benefit, since starch can bean attractant for many types of particulate soils.90% of all liquid detergents contain these enzymes (Gupta *et al*., 2003). Alkaliphilic *Bacillus* strains often produce enzymes active at alkaline pH,including alkaline α-amylase(Horikoshi, 1996).

2.16.5 Beverage Alcohol and Fuel Ethanol production

In beer industries, microbial amylases are used to aid cereal amylase in theproduction of fermentable sugar. Ethanol is the most utilized liquid biofuel. Over the past decades, there has been anincreasing interest in fuel ethanol as a result of increased environmental concern andhigher crude oil prices.Ethanol fuels can be derived from renewable resources such as agricultural crops and by products. For ethanolproduction, starch is the most used substrate due to its lowprice and easily available raw material in most regions of the world (Chi *et al*., 2009).

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* (Moraes*et al*., 1999).

2.16.6 Treatment of Starch Processing Waste Water

Starch is also present in waste produced from foodprocessing plants. Starch waste causes pollutionproblems. Biotechnological treatment of food processing wastewater can produce valuable products such asmicrobial biomass protein and also purifies the effluent (Friendrich *et al*., 1987; Kingspohn *et al*., 1993).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Sample Collection

Exactly 100g of agricultural soil samples were taken at a depth of 5cm at four different points around Ahmadu Bello University dam and made into composite sample. These soil samples were collected in clean polythene bags. Five hundred grams (500g) of wheat bran obtained from mills located at Samaru market, in Zaria was collected in clean polythene bag. All the samples were appropriately labeled and immediately transported to the laboratory for further investigation.

3.2 Sample Preparation

The soilsamples were sun-dried and pretreated with heat at 40°C for 7 days prior to isolation of the organism (Chakraborty *et al*., 2012). Wheat bran sample was washed, sun dried and grinded into smaller particle size.Five hundred grams (500g) of the wheat bran sample was soaked in 5L of 2% solution of NaOH for 2h at room temperature according to the method of Irfan*et al*. (2010), after which the sample was autoclaved at 121°C for 15 min. The sample was then filtered and solid residues were washed up to neutrality, dried and stored for further use.

3.3 Isolation of Streptomyces spp.

One gram of soil sample was suspended in 10 ml of 5mM phosphate buffer (pH 7.0) and centrifuged for 1 min. After a 10-fold serial dilution, 0.1ml of the suspension was spread on Starch Casein Agar plates(Starch -19g, Casein -0.3g, KNO_3-2g, $NaCl_{2-}$2g, K_2HPO_{4-}2g, $MgSO_4.7H_2O$- 0.05g, $CaCO_{3-}$ 0.02g, $FeSO_4.7H_2O$- 0.01g, Agar -20g, Distilled H_2O-1L) supplemented with nystatin (50µg/ml) and tetracycline (100µg/ml) to inhibit fungal and bacterial growth respectively and then incubated at 28°C for 14 days. After which colonies suspected to be

Streptomyces spp. were selected and streaked on starch casein agar slants. The slants were incubated at 28°C for 14 days after which it was used for further studies (Mrudula and Kokill, 2010).

3.4 Characterization and Identification of the Isolates.

Isolates were identified and characterized using Gram staining reaction, morphological and physiological properties.

3.4.1 Gram Staining of the Isolates

A smear was made from a colony of the actinobacteria and fixed on a glass slide which was then covered with crystal violet stain for 60secs and washed off with clean water. This was followed by treatment with Lugol's iodine for 60secs and then washed off. The smear was decolorized with acetone and washed off immediately with water. The slide was finally covered with neutral red stain for two minutes and washed off. Then wiped, air dried and examined using the 40 objective, then the 100 objective with oil immersion (Cheesbrough, 2000).

3.4.2Morphological Characterization of the Isolates

The morphology of the spore bearing hyphae and spore chain of the isolate were determined by direct microscopic examination according to the method of Suneetha *et al.* (2011). The standard culture media used were; yeast extract- malt extract agar, oatmeal agar and inorganic salt-starch agar. Each of the medium was poured into seven plates and held for a minimum of 24h at 28°C to promote moderate drying and check sterility before inoculation, onto the agar surface near an edge of the petri dish, 0.1ml of inoculum was placed, which serves as a pool of inoculum. A flamed sterile wireloop was used to make five equally spaced streaks across the plate, dipping the loop into the pool of inoculum prior to each streak and cross-hatch streaks were also made. Plates were incubated in the dark at 28°C and observed after 7, 14 and 21 days respectively.

The plates used for the morphological studies were also used for the determination of colour of pigmentation of mature, sporulating aerial surface growth, colour of substrate mycelium, and diffusible soluble pigments other than melanin.

3.4.3 Physiological Characterization of the Isolates

Physiological characteristic was determined according to themethod of Hamedo and Makhlouf (2013). Peptone iron agar and tyrosine agar slants were used for the determination of melanin production. Using a sterile wire loop, a heavy inoculum of spore and aerial mycelium was picked and streaked on the surface of the agar slant. Cultures were incubated at 30°C and examined after 2 and 4 days.Utilization of carbon sources was investigated. Using L-arabinose, sucrose, D-xylose, I-inositol, D-mannitol, D-fructose, Rhamnose, Rhafinose and cellulose, which were added to the basal salt medium at 1.0% (w/v) with growth recorded after 10-16 days.

3.5 Screening for Amylase Activity of the Isolates

The screening of *Streptomyces* spp isolated for amylase production was performed according to the method of Maheswari and Soundariya (2012). The actinomycetes isolates was spot inoculated on a sterile starch agar medium supplemented with griseofulvin (50mg/ml), solublestarch- 10.0g, polypeptone- 5.0g , KH_2 PO_4 -1.0g, $MgSO_4$ $7H_2O$ - 5.0g and agar- 15g . The plate was incubated at 28°C, for 48h after which the plate was flooded with iodine. Clear zone of hydrolysis around the colony indicated the presence of the amylase with diameter of clear zone around the colony recorded. The isolate that showed the highest amylase activity was selected for amylase production.

3.6 Enzyme Production

3.6.1 Preparation of Inoculum

To a two weeks old culture slant, 10mls of tween-80 solution was added with the spores dislodged using an inoculation needle. Spores in the solution were collected in a sterile flask and the suspension was diluted appropriately to the required spore density of $1.0x10^6$spores/ml (Sivaramakrishnan *et al*., 2007).

3.6.2 Production of Amylase under Solid State Fermentation

The method of Maheswari and Soundariya (2012) was used. The static experiments was conducted in 250ml of Erlenmeyer flask containing 50g of substrate (Wheat bran) moistened with mineral salt solution of the following composition; sodium citrate-2.5g, KH_2 PO_4 -5.0g,NH_4NO_3 -2.0g, (NH_4) SO_4-4.0g, $MgSO_4$-0.2g to 50% moisture content. The flasks were plugged with cotton and sterilized by autoclaving for 15 minutes at 121°C. After sterilization, the flasks were cooled and inoculated with 10% (5ml) volume of inoculum, then incubated at 30°C and initial pH5 under still culture condition for 7 days.

3.6.3 Enzyme Extraction

Crude enzyme was extracted by mixing 3g of fermented matter with 10ml of distilled water. The content was mixed thoroughly by shaking for 1h at room temperature on a rotary shaker at 180rpm. The suspension was centrifuged at 8000rpm and 4°C for 10min, then filtered using Whatmann filter paper number1 and the solid free supernatant was used as enzyme source to assay for amylase activity (Sumitra *et al*., 2004).

3.6.4 Biomass Estimation

The biomass of *Streptomyces* spp was estimated after filtering the suspension through preweighed Whatmann number 1 filter paper and dried to a constant weight at 80°C and reweighed. The difference in weight denotes the biomass (Ragunathan and Padhmadas, 2013).

3.6.5 Amylase Activity Assay

Amylase activity was determined according to the method of Okolo *et al*. (1995). Thereaction mixture contains: 1% soluble starch; 1.25ml, 0.1M acetate buffer(pH5); 0.75ml and appropriately diluted crude enzyme extract; 0.25ml. After 10min of incubation at 50°C the liberated reducing sugar (glucose equivalent) was estimated bythe addition of 3,5-dinitrosalicylic acid(DNS) followed by boiling for10 minutes and optical density measured at 540nm using a spectrophotometer. Glucose was used as standard and blank contains: 0.1M acetate buffer (pH5); 0.75 and 1% starch solution; 1.25ml. One unit (IU) of amylase activity is defined as the amount of enzyme releasing 1µ mol glucose equivalent per minute under the assay conditions.

3.7 Optimization of the Production Process

The solid state fermentation of the substrate wheat bran was optimized by varying parameters ranges. The various parameters arepH(4,5,6, 7 and 8), temperature (30, 35, 40, 45 and 50°C), moisture content(30, 40, 50, 60 and 70%), inoculum size (1, 2, 3, 4 and 5ml) containing 1.0 x10^6spores/ml and period of fermentation (24 to 240h) (Ragunathan and Padhmadas, 2013). The traditional classical method involved varying parameters at a time by maintaining preoptimizedsolid state fermentation (Maheswariand Soundariya, 2012).

3.8 Analysis of Data

All experiments were conducted in duplicates and values were averaged. Statistical significance between means was tested by analysis of variance and independent t-test using Instat-ANOVA software. The differences between means were considered statistically significant when the test yielded a value $P<0.05$ (Yassien and Asfour, 2011).

CHAPTER FOUR

4.0 RESULTS

4.1Isolation and Identification of *Streptomyces* spp. from Soil

Five (5) strains of Streptomyces species were isolated from the soil samples. The isolates were white and chalky in appearance on starch casein agar. The colonies were hard, not gummy and not easily lifted from the agar.

Morphological characteristics of the *Streptomyces* spp are indicated in Table 4.1, with colour of substrate mycelia, aerial mycelia and their ability to produce melanin pigment. Microscopic examination of cultures of the *Streptomyces* spp showed that they all exhibited a rectiflexible spore chain and retained the primary dye after gram staining by appearing purple under the light microscope

Physiological characteristics of the *Streptomyces* spp. was based on their ability to utilize the sugars stated in Table 4.2. *Streptomyces* spp. S1 was able to utilize the sugars for growth with the exception of inositol and manitol while S2 was unable to utilize rhamnose, sucrose and arabinose. S3 grew on all the carbon sources while S4 was unable to utilize arabinose and inositol. S5 was only able to utilize xylose for growth.

4.2 Screening of Streptmyces spp. Isolates for Amylase Production

The five *Streptomyces* spp. isolated from the soil sample werescreened for amylase production on starch agar medium. Out of the five Streptomyces spp., only *Streptomyces michiganensis* gave a positive result with diameter of clear zone of starch hydrolysis of 38.3 ± 0.45mm as shown in Table 4.3

Table 4.1: Morphological Characterization of *Streptomyces* spp. Isolates from Soil Samples

Isolate code	Spore chain morphology	Colour of aerial mycelia	Colour of substrate mycelia	Melanin pigment	Gram reaction	Probable Identity
S1	Rectiflexible	Gray	Yellow	-ve	+ve	*Streptomyces narbonensis*
S2	Rectiflexible	Yellow	Brown	Yellow	+ve	*Streptomyces michiganensis*
S3	Rectiflexible	Gray	White	Green	+ve	*Streptomyces orientalis*
S4	Rectiflexible	Gray	Orange	+ve	+ve	*Streptomyces* spp.
S5	Rectiflexible	White	Green	+ve	+ve	*Streptomyces* spp

Keys:

+ve	Presence of greenish brown to brown to black pigment	-ve	Absence of brown to black pigment

Table 4.2: Physiological Characterization of *Streptomyces* spp. Isolates from Soil Samples

Isolate code	Glucose	Arabinose	Sucrose	Xylose	Inositol	Mannitol	Fructose	Rhamnose	Raffinose	Identity
S1	++	++	++	+	-	-	++	+	+	*Streptomyces narbonensis*
S2	++	-	-	++	++	+	+	-	+	*Streptomyces michiganensis*
S3	++	++	±	++	++	++	++	+	±	*Streptomyces orientalis*
S4	++	-	++	++	-	++	++	++	++	*Streptomyces*spp.
S5	++	-	-	++	-	-	-	-	-	*Streptomyces* spp.

Keys:

++ Strongly positive + Positive - Negative ± Utilization doubtful

Table 4.3: Screening of *Streptomyces* spp for Amylase Production

***Streptomyces* spp.**	**Amylase activities**	**Zone of Starch Hydrolysis (mm)**
Streptomyces narbonensis	-ve	0.00
Streptomyces michiganensis	+ve	38.3 ± 0.45
Streptomyces orientalis	-ve	0.00
Streptomyces spp.(S4)	-ve	0.00
Streptomyces spp.(S5)	-ve	0.00

Keys

+vePresence of clear zone of hydrolysis -ve Absence of clear zone of hydrolysis

4.3 Production of Amylase by *Streptomyces michiganensis*

Table 4.4 illustrates the production of amylase at different fermentation periods under solid state fermentation. The ability of *Streptomyces michiganensis* to utilize wheat bran as substrate for the production of amylase was studied in comparison with soluble starch which served as a standard. The highest amount of amylase produced was observed at 72h incubation time (8.5 ± 0.21 U/ml) while the least amount was obtained at 168h of incubation (2.9 ± 0.28 U/ml) using wheat bran as substrates. The amount of amylase produced as observed at 24h, 48h, 96h, 120h and 144h were 3.2 ± 0.14 U/ml, 4.9 ± 0.35 U/ml, 6.6 ± 0.42 U/ml, 6.9 ± 0.35 U/ml and 4.4 ± 0.57 U/ml respectively.

The highest amount of amylase produced was observed at 72h incubation time (12.1 ± 0.35 U/ml) while the least amount of amylase produced was obtained at 168h incubation (3.9 ± 0.35 U/ml) using soluble starch as substrate, which serves as positive control. The amount of amylase produced as observed at 24h, 48h, 96h, 120h and 144h were 5.0 ± 0.28 U/ml, 4.6 ± 0.28 U/ml, 8.0 ± 0.42 U/ml, 6.7 ± 0.35 U/ml and 5.7 ± 0.21U/ml respectively. Table 4.4 also showed that amylase production was growth related. Amylase production increased with an increase in biomass and decreased with a decrease in biomass. Also comparison of means showed that there was significant difference ($P<0.05$) between the amylase yield of culture with wheat bran and that with starch.

Table 4.4: Amylase Production by *Streptomyces michiganensis* at different Fermentation Periods

Fermentation Periods (Hours)	**Amylase Activities (U/ml)**			**Biomass (mg/ml)**		
	Wheat bran	**Starch**	**p-value**	**Wheat bran**	**Starch**	**p-value**
24	3.2 ± 0.14^{a}	5.0 ± 0.28^{ab}	0.057	0.5 ± 0.35^{a}	2.0 ± 0.71^{a}	0.312
48	4.9 ± 0.35^{ab}	4.6 ± 0.28^{ab}	0.686	2.5 ± 0.35^{b}	5.0 ± 0.71^{b}	0.051
72	8.5 ± 0.21^{bc}	12.1 ± 0.35^{d}	0.020	5.0 ± 0.71^{c}	9.0 ± 1.41^{c}	0.216
96	6.6 ± 0.42^{c}	8.0 ± 0.42^{c}	0.534	3.0 ± 0.71^{bc}	6.0 ± 0.71^{b}	0.300
120	6.9 ± 0.35^{bc}	6.7 ± 0.35^{bc}	0.804	2.0 ± 0.71^{ab}	4.0 ± 0.71^{b}	0.155
144	3.4 ± 0.57^{ab}	5.6 ± 0.57^{b}	0.268	2.0 ± 0.71^{ab}	3.5 ± 0.35^{ab}	0.312
168	2.9 ± 0.28^{a}	3.9 ± 0.35^{a}	0.388	1.5 ± 0.35^{ab}	2.5 ± 0.35^{ab}	0.333

Values are Mean of two replicates ± SEM; Values with different superscript within the column are significantly different ($P<0.05$) by Independent Sample *t*-Test.

4.4 Effects of Fermentation Conditions on Amylase Production

Figure 4.1 shows the effect of various temperatureson amylase production. The highest amount of amylase produced was obtained at 35°C (11.2 ± 1.13 U/ml), while the least amount of amylase produced was observed at 50°C (4.5±0.49U/ml). The quantity of amylase produced at 30°C, 40°C and 45°C were 8.7±0.35U/ml, 7.5±0.21U/ml and 5.2±0.42U/ml respectively. The result shows that 35°C which is a mesophylic temperature is the optimum temperature that favors amylase production.

Figure 4.2 shows the effect of various moisture contents on amylase production. The highest quantity of amylase produced was obtained at 30% (7.9±0.21U/ml) while the least amount of amylase production was observed at 60% (4.8±0.42U/ml). The amount of amylase produced and recorded at 20%, 40% and 50% moisture content were 7.2±0.14U/ml, 6.2±0.28U/ml and 6.9±0.21U/ml, respectively. This shows that at low moisture content, the production of amylase was enhanced.

Figure 4.3 shows the effect of various pH on amylase production. The highest quantity of amylase produced was obtained at pH 6 (12.2±0.28U/ml). On the other hand, the least amount of amylase produced was observed at pH 4 (6.5±0.35U/ml). The quantity of amylase produced at pH 5, 7 and 8 were 8.2±0.28U/ml, 11.3±0.64U/ml and 7.1±0.35U/ml respectively. From the result obtained, it can be seen that amylase production was favored at pH close to neutrality, while extreme pH values did not favor amylase production.

Figure 4.4 shows the effect of various inoculum size on amylase production. The highest amount of amylase produced (7.9±0.21U/ml) was obtained at 2ml. On the other hand, the least amount of amylase produced (3.4±0.18U/ml) was observed at 5ml. Amylase activities observed at 1ml, 3ml and 4ml were 4.8±0.28U/ml, 6.9±0.35U/ml and 3.7±0.35U/ml respectively.

Figure 4.5 shows the production of amylase at optimum fermentation conditions. The period of fermentation was extended to 240h; a high enzyme production of 17.7±0.41U/ml was recorded at 48h while the least amount of 1.9±0.25U/ml was recorded at 240h.

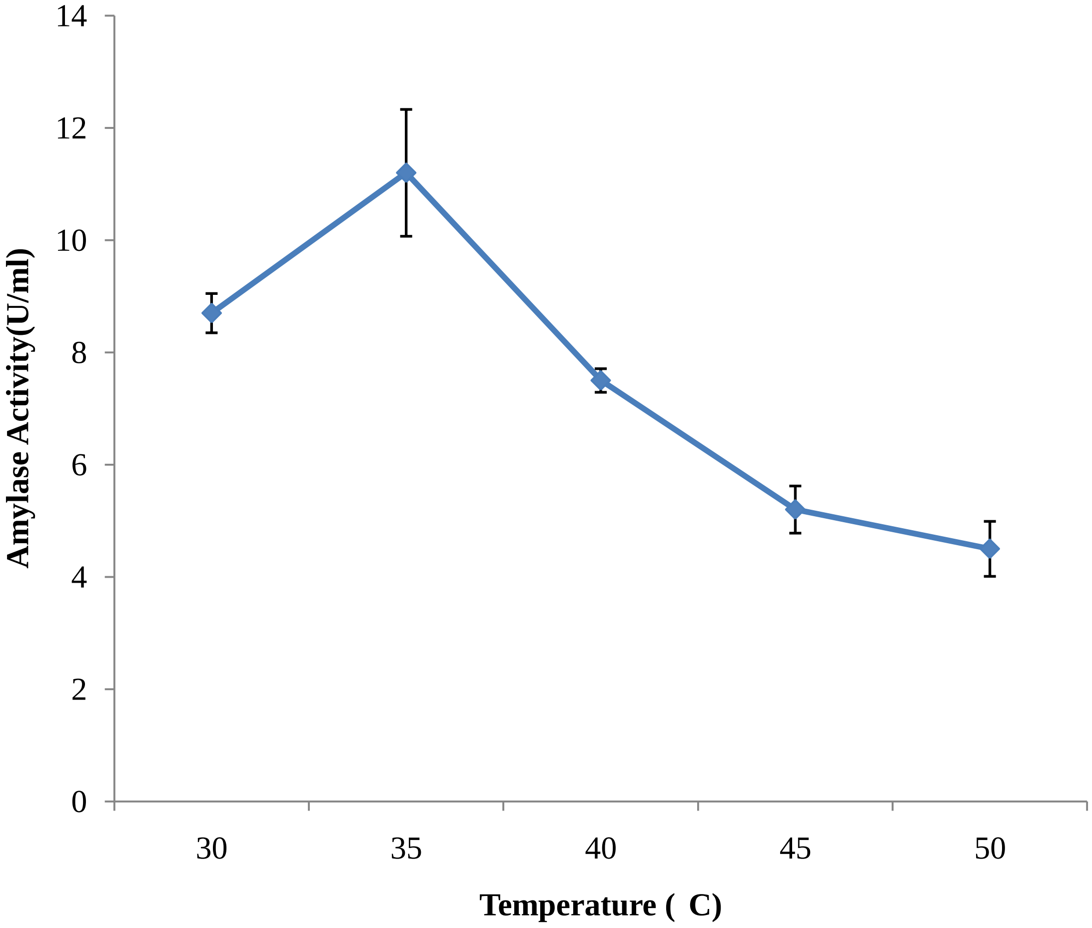

Figure 4.1: Effect of Temperature on Amylase Production by *Streptomyces michiganensis*

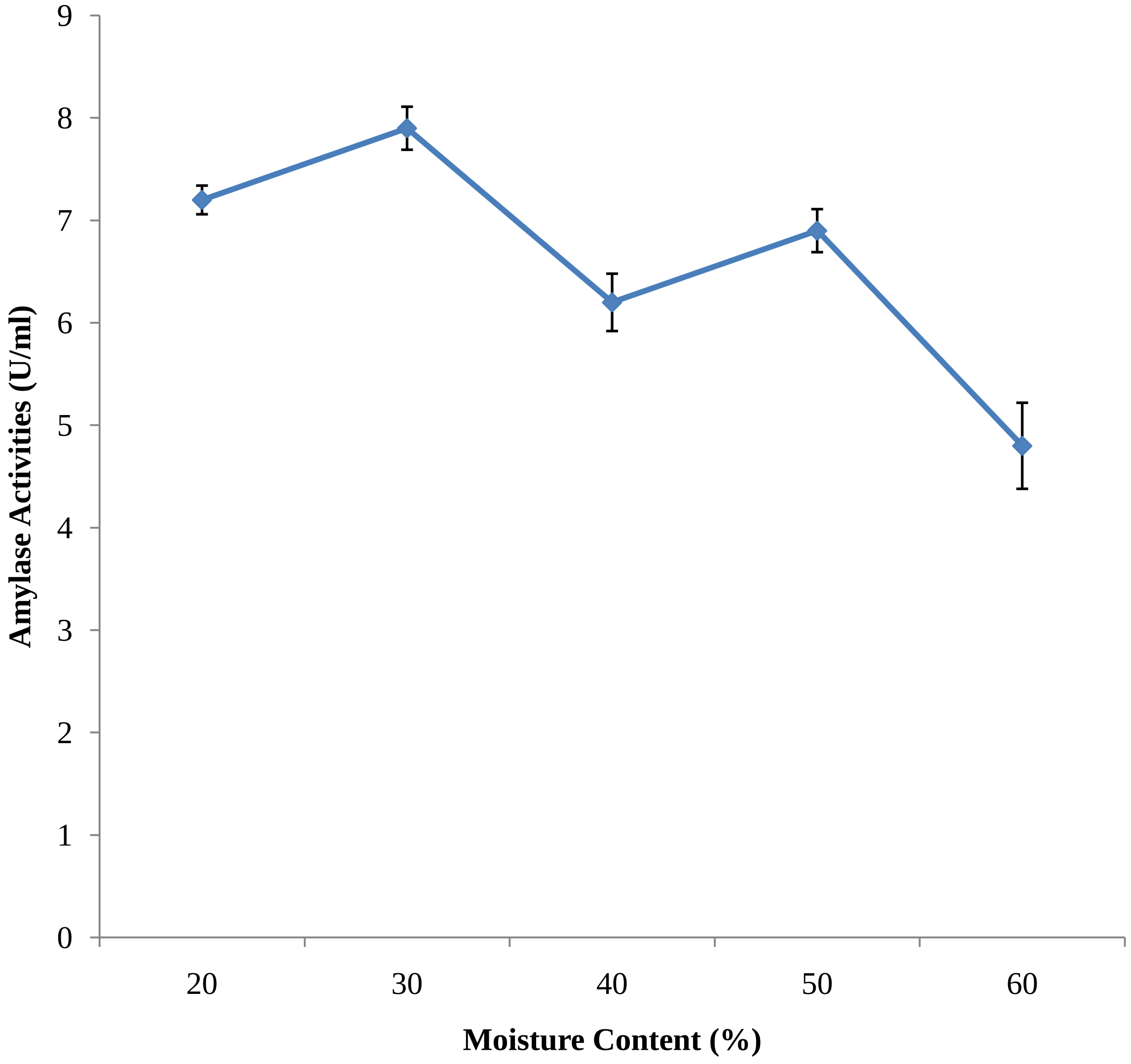

Figure 4.2: Effect of Moisture Content on Amylase Production by *Streptomyces michiganensis*

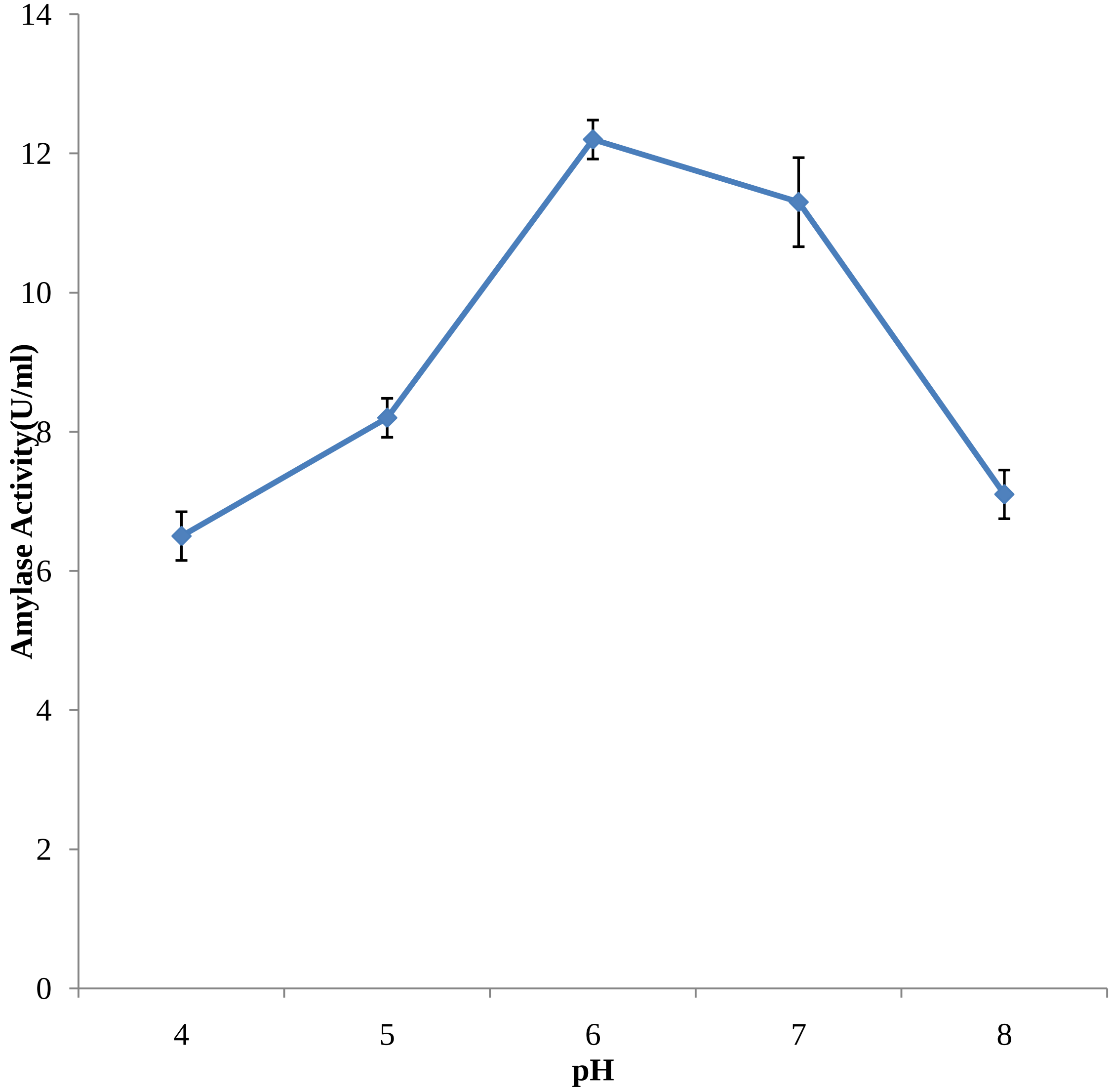

Figure 4.3 Effect of pH on Amylase Production by *Streptomyces michiganensis*

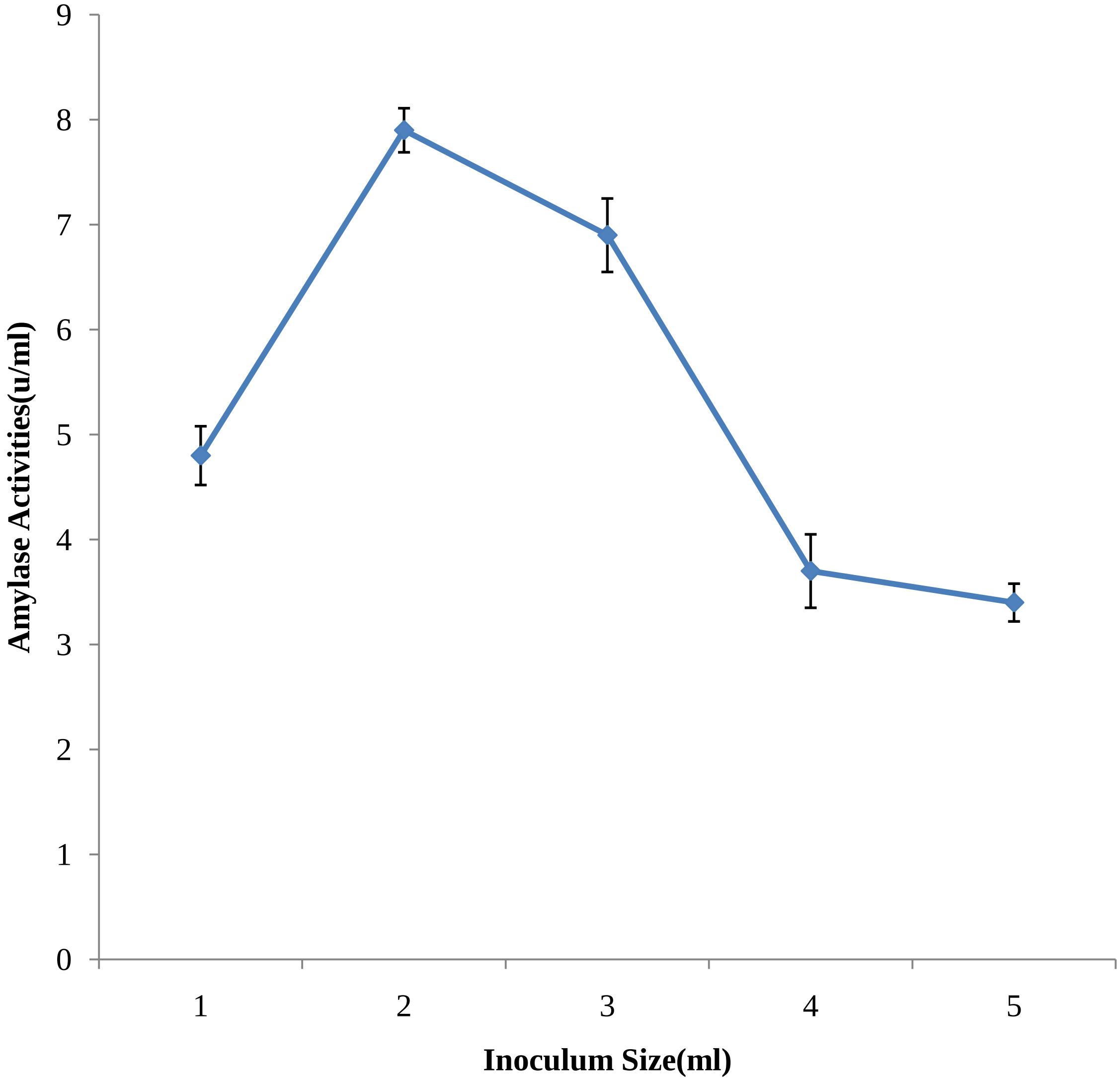

Figure 4.4 Effect of Inoculum Size on Amylase Production by *Streptomyces michiganensis*

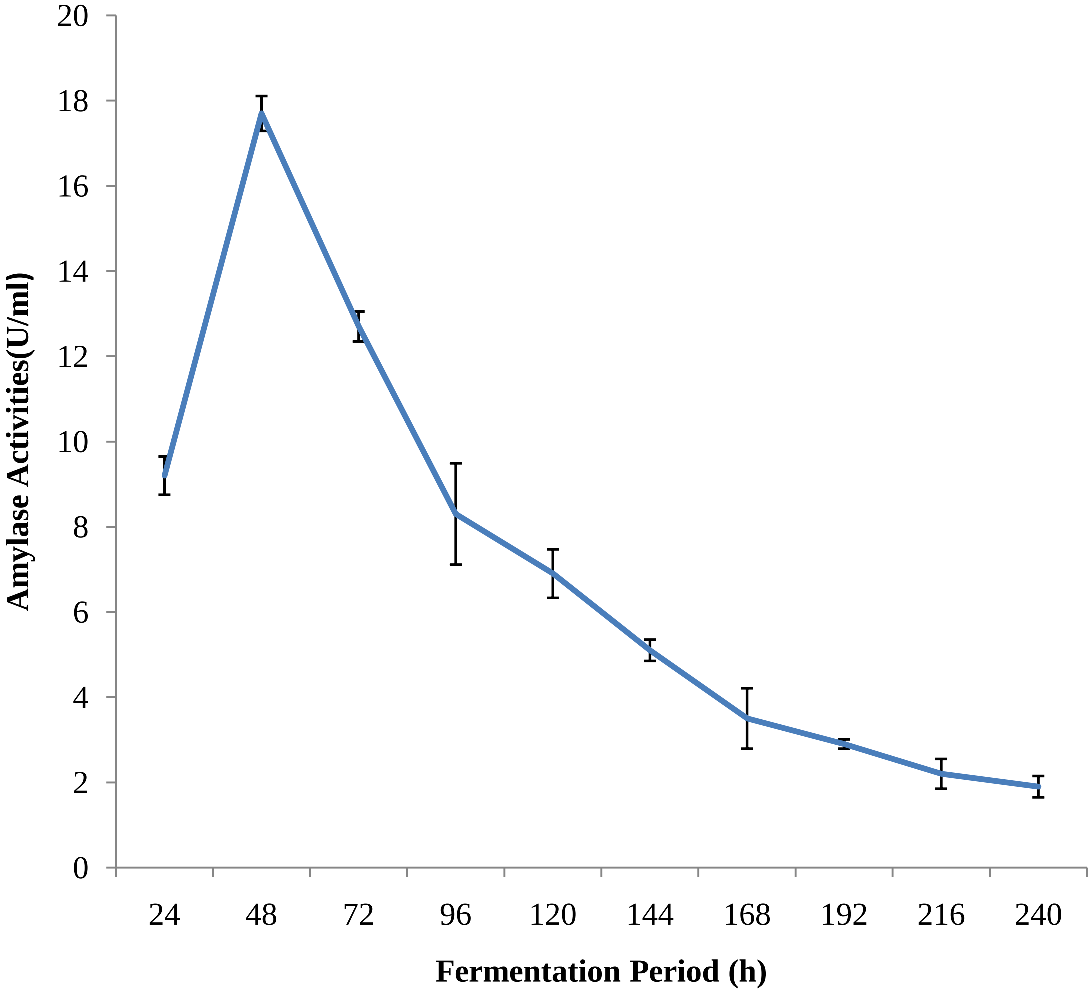

Figure 4.5: Amylase Production at Optimum Fermentation Conditions by *Streptomyces michiganensis*

CHAPTER FIVE

5.0 DISCUSSION

Five different species of *Streptomyces* were isolated from the soil and they were identified as *Streptomyces narbonensis*, *Streptomyces michiganensis*, *Streptomyces orientalis*, *Streptomyces* spp. (S4) and *Streptomyces* spp. (S5) as shown in Table 4.2. This is an indication that *Streptomyces* spp. are natural inhabitants of the soil as reported previously by Sathya and Ushadevi (2014).

The screening result of amylase production by different species of *Streptomyces*(Table 4.3), showed that only *Streptomyces michiganensis* hada clear zone of starch hydrolysis of 38.3±0.45mm. The amylolytic variation observed among the different species may be due to the influence of environmental factors such as pH and temperature on the organisms, which affects the expression of gene that codes for amylase. Similar observations were made by Sathya and Ushadevi (2014) from amylolytic haloes produced by different species of *Streptomyces*after screening, with *Streptomyces* spp RSU2 having the highest zone of 35.0mm.

In this study, the production of amylase increased with an increase in fermentation period and reached its highest at 72h while the least amount produced was recorded at 168h using wheat bran and soluble starch as substrate (Table 4.1). Further increase in fermentation period beyond the optimum time didnot show any significant increase in enzyme productionrather it was decreased. This could be attributed to the decrease in the growth rate of organisms which could have been due to the depletion of available substrate or nutrients and accumulation of other by-products of metabolism which might be toxic to the organism as reported by Riaz *et al*. (2010). This finding is also similar with the result obtained by Krishna *et al*. (2012), who reported a high amylase yield after 72h of incubation using *Aspergilus niger* in solid state fermentation.

Comparison of means showed that there was a significant difference between the amylase yield of wheat bran and soluble starch. This suggests that wheat bran performed below soluble starch.

The optimum temperature for amylase production was found to be 35°C (Figure 4.1). This is an indication that the growth of the organism and enzyme production was stimulated by mesophylic temperature. Further increase in temperature beyond the optimum resulted to a decrease in amylase production which was statistically significant ($P<0.05$). This decrease could be due to the fact that at high temperature the growth of the organism was inhibited therefore, the enzyme production was prohibited as also reported by Pandey *et al*. (2000a). A similar result was reported by Vidyalakshmi *et al*. (2009) using *Bacillus* spp.

The optimum moisture content in this study was 30% (Figure 4.2). The low enzyme yield observed at moisture contents lower than the optimum may be due to high water tension and low solubility of nutrient. Also, low enzyme yield at a moisture content higher than the optimum may be due to reduction in the inter particle distance which resulted in agglomeration of substrate particle, there is also reduction in gas volume and gas diffusion which may lead to impaired oxygen transfer as reported by Prakasham *et al*.(2007).Krishna *et al*. (2012) reported that under moisture condition of 30% the swelling of substrate was found more appropriate with their utilization facilitated by microorganisms. However, this finding is in disagreement with the work of Sivaramakrishnan *et al*. (2007) who recorded the highest enzyme yield at 60% moisture content.

The optimum pH for amylase production in this study was observed to be at pH of 6 (Figure 4.3). Further increase in the pH beyond the optimum resulted in a decrease in the production of amylase which was significant statistically. This could be due to the fact that at high pH, the metabolic action of the organism may be suppressed and thus itinhibits the enzyme productionas stated by Ellaiah *et al*. (2002). This finding is in agreement with the study of Yassien and Asfour (2012) but disagrees with the work of Deb *et al*. (2013) who recorded the highest enzyme yield at pH 9.

The optimum inoculum size in this study was 2ml (Figure 4.4). Further increase in the inoculum size beyond the optimum resulted to a decrease in enzyme production. It may bedue to the fact that an inoculum size higherthan the optimum value may produce a highamount of biomass which rapidly depletes thenutrients necessary for growth and product synthesis. On the other hand, lower inoculum level may give insufficient biomass and allow thegrowth of undesirable organisms in the production medium as stated by Raju and Divakar(2013). Ramachandran *etal.*(2004) also reported that *A. oryzae* showed increasedenzyme production with an increase in inoculum size from the lowest value of 0.5ml and showedmaximum enzyme activity at 2ml inoculum.

There was a significant increase in amylase yield when it was produced under optimum fermentation condition from 8.5±0.21U/ml to 17.7±U/ml. Also it was found that the highest enzyme produced was achieved at a shorter period of time, 48h compared to 72h recorded outside optimum fermentation condition (Table 4.4; Figure 4.5) respectively. Deb *et al*. (2013) also reported 2.43 times increase in amylase yield after optimizing the production condition.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

The isolation of *Streptomyces michiganensis* from the soil is an indication that amylolytic *Streptomyces* spp are inhabitants of the soil.

Streptomyces michiganensis is a potential producer of amylase due to its ability to hydrolyse soluble starch with diameter of clear zone of hydrolysis of 38± 0.45mm as observed in this study. Hence, it can serve as an alternative to other amylolytic fungi and bacteria such as *Aspergillus niger* and *Bacillus spp*. respectively. This is because it has a more efficient secretion mechanism which simplifies subsequent purification step and may increase yield.

The production of amylase using wheat bran as substrate yielded 8.5±0.21U/ml of the enzyme which is less than 12.1±0.35U/ml that was obtained using soluble starch. However the use of wheat bran as substrate in place of soluble starch can still be encouraged because it is cheap and readily available locally.

The optimum fermentation conditions for amylase production was also developed as follows; temperature (35°C), pH 6, inoculum size (2ml containing 1.0 x10^6spores/ml), moisture content (30%) and 48h period of fermentation. The high amylase production observed suggests that for commercials scale production, there is need for the optimum fermentation conditions to be determined.

6.2 Recommendations

i Due to the high importance of amylase in many manufacturing industries, further studies need to be carried out to develop a cost effective amylase process for commercial purpose.

iiThe government should help to facilitate amylase research by sponsoring the process. This will also serve as a source of motivation to interested researchers.

iii The use of wheat bran for production of value added products should be encourage to reduce cost of production and solve pollution problem

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APPENDICES

APPENDIX I: Pictures of Plates

Plate 4.1: Colonies of *Streptomyces* spp.on Starch Casein Agar Plate

Plate 4.2: Micrograph of Gram Positive Rectiflexible *Streptomyces* spp

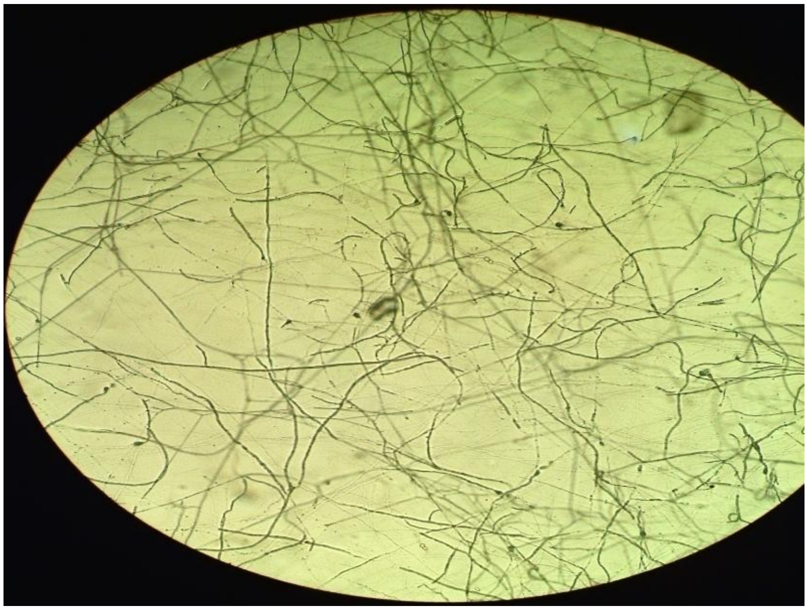

Plate 4.3: Micrograph of *Streptomyces machiganensis*. X 40

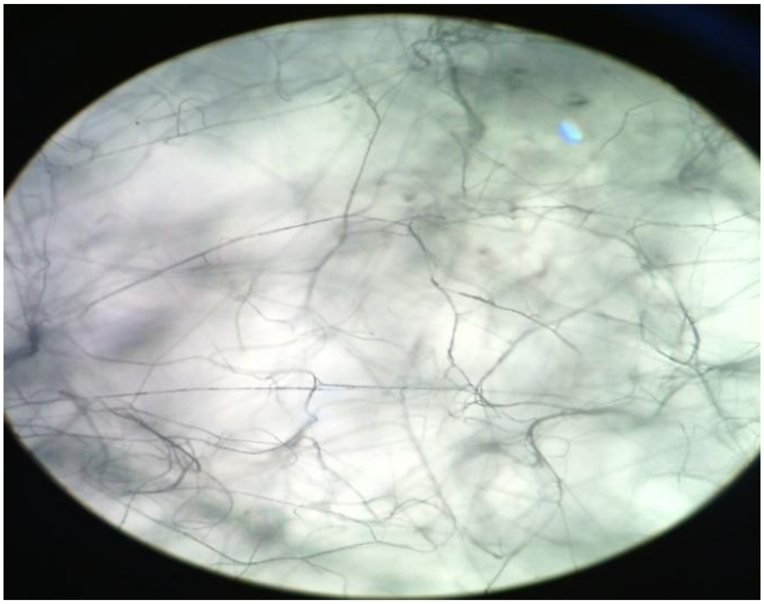

Plate 4.4: Micrograph of *Streptomyces narbonensis*. X 40

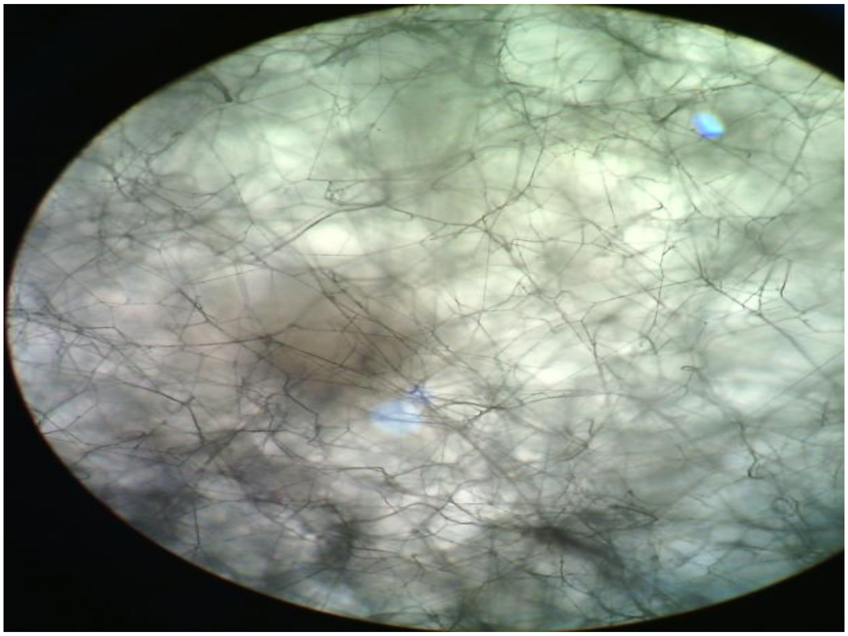

Plate 4.5: Micrograph of *Streptomyces orientalis* X 40

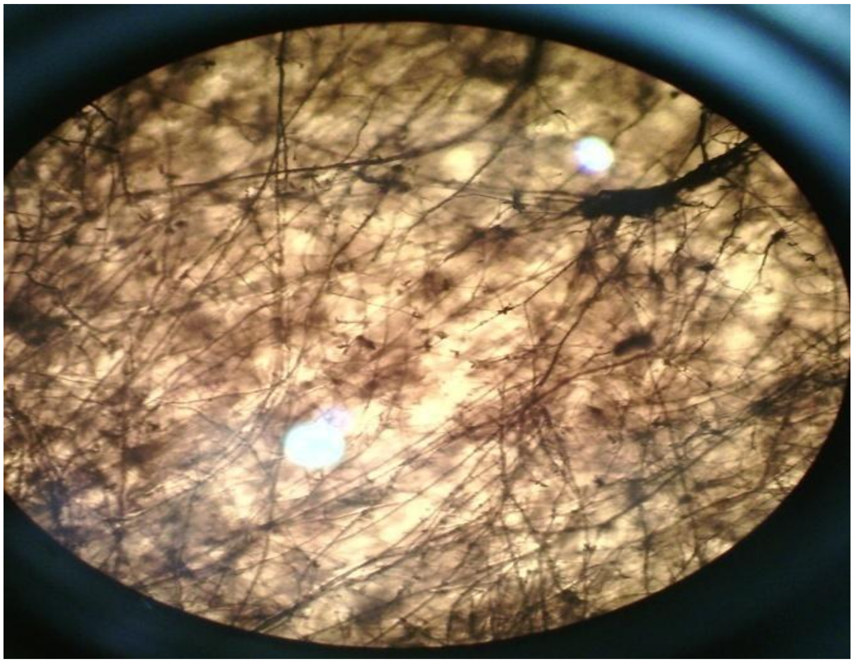

Plate 4.6: Micrograph of *Streptomyces*spp (S4)X 40

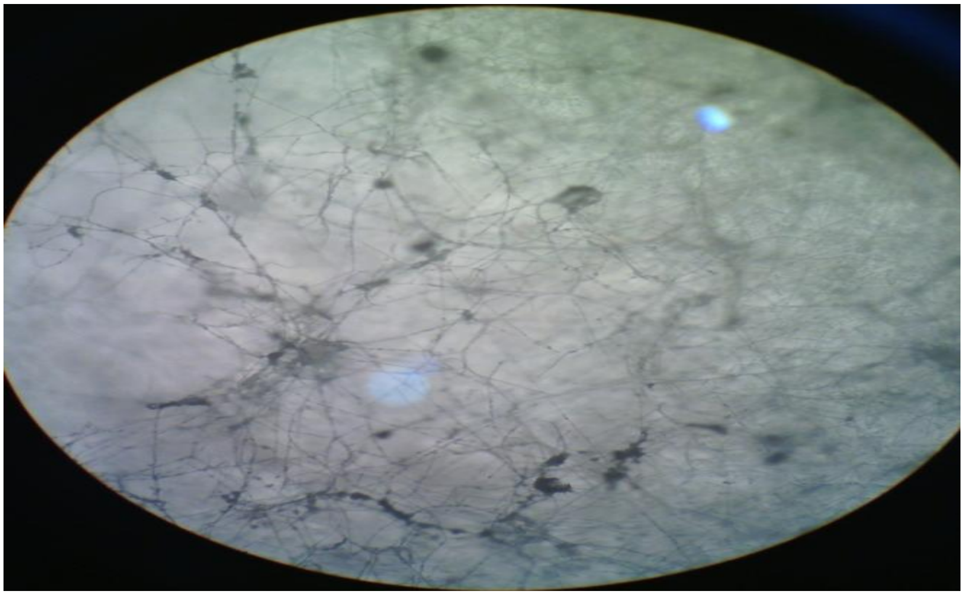

Plate 4.7: Micrograph of *Streptomyces*spp (S5) X 40

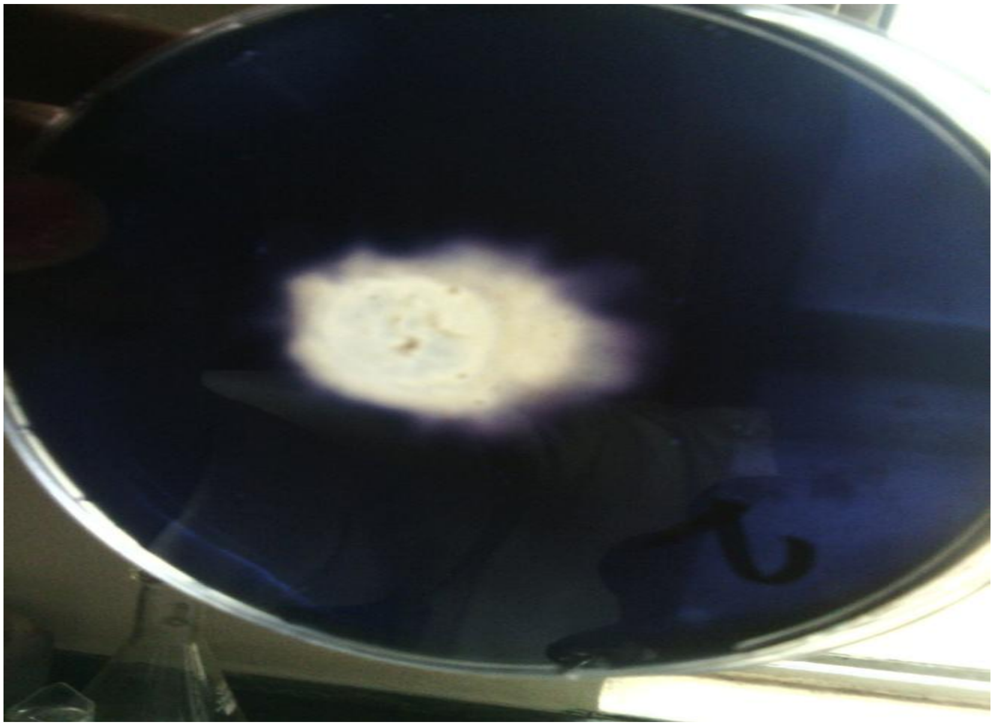

Plate 4.8: Absence of Clear Zone of Hydrolysis by *Streptomyces narbonensis*

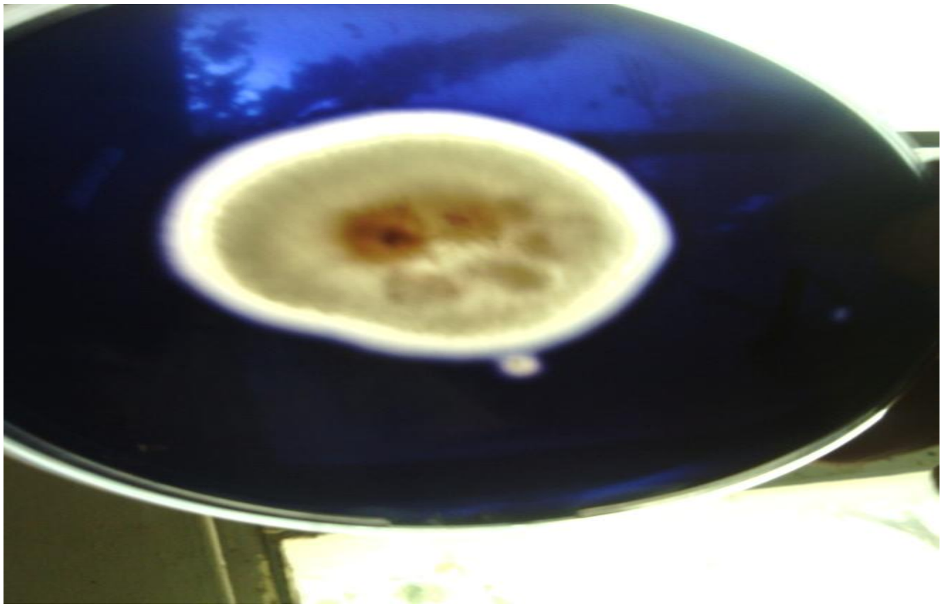

Plate 4.9: Presence of Clear Zone of Hydrolysis by *Streptomyces machiganensis*

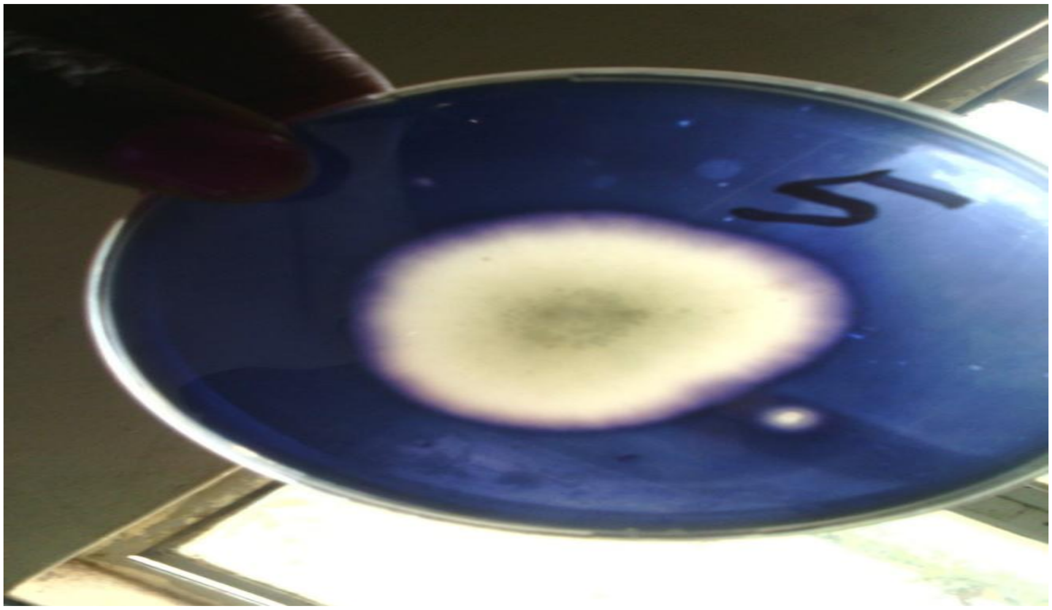

Plate 4.10: Absence of Clear Zone of Hydrolysis by *Streptomyces orientalis*

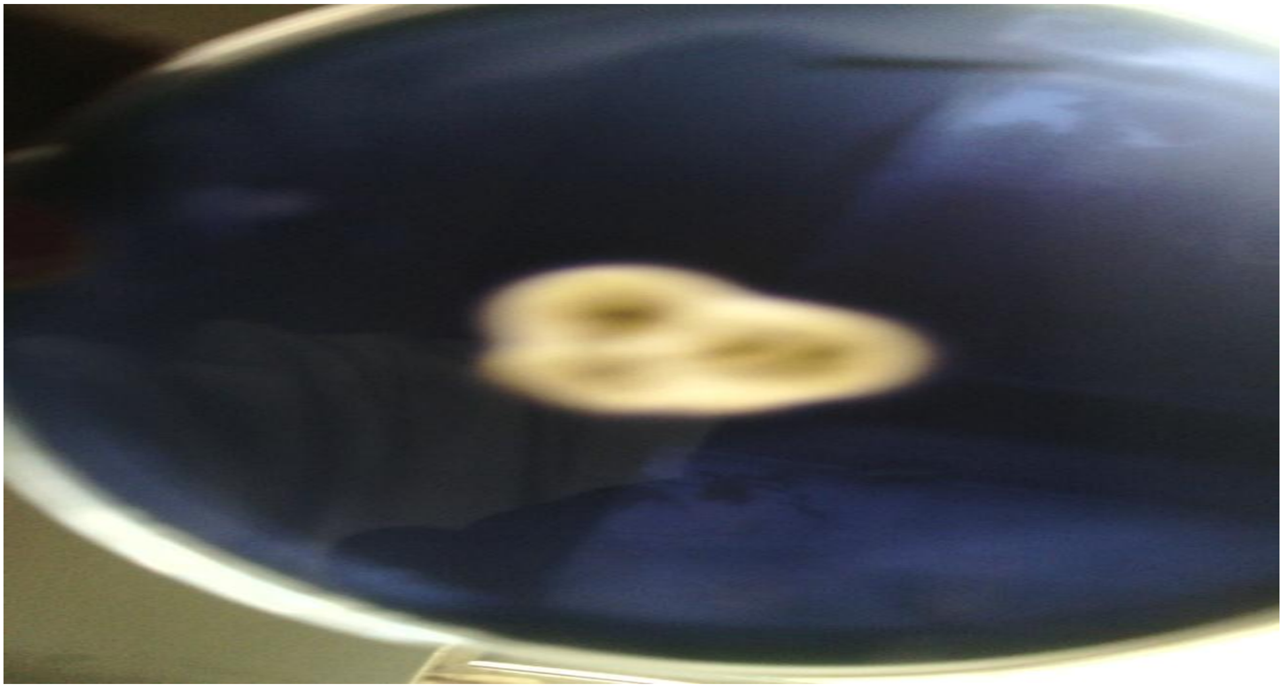

Plate 4.11: Absence of Clear Zone of Hydrolysis by *Streptomyces* spp (S4)

Plate 4.12: Absence of Clear Zone of Hydrolysis by *Streptomyces* spp (S5)

Plate 4.13: Colour of Colony of *Streptomyces machiganensis* on Yeast-extract Malt-extract Agar

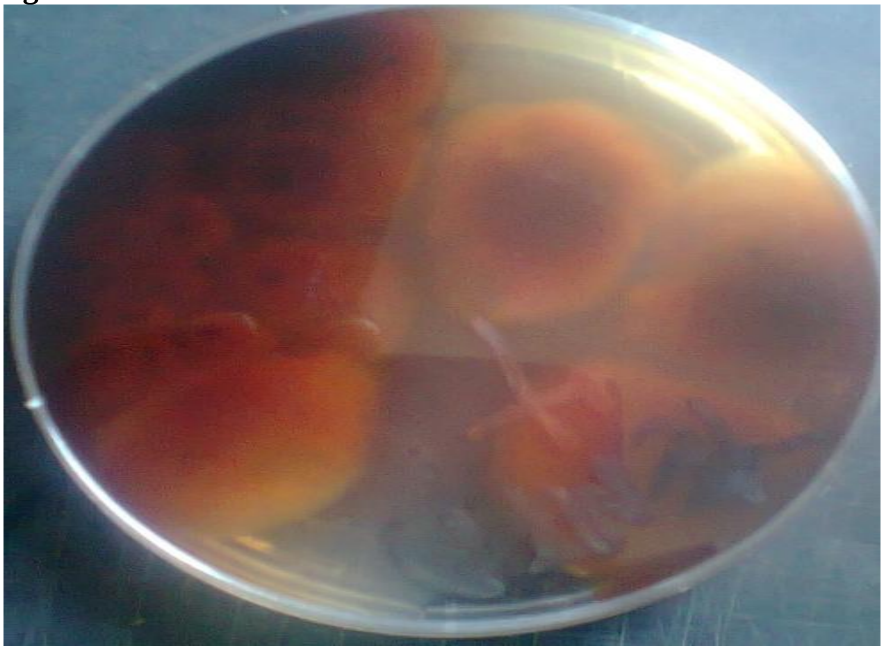

Plate 4.14: Reverse Side of Colony of *Streptomyces machiganensis* on Yeast-extract Malt-extract Agar

Plate 4.15: Fermentation Media for Amylase Production

Plate 4.16: Amylase Assay Showing Colour Change from Yellow to Red

Appendix II: Amylase Production by *Streptomyces michiganensis* using Wheat Bran and Starch Substrate

	Amylase Activity(U/ml)		
Fermentation period(h)	**Wheat Bran**	**Starch**	**P-Value**
24	3.20 ± 0.28^{a}	5.00 ± 0.57^{ab}	0.057
48	4.90 ± 0.71^{ab}	4.60 ± 0.57^{ab}	0.686
72	6.60 ± 0.85^{bc}	12.10 ± 0.71^{d}	0.020
96	8.50 ± 0.42^{c}	8.00 ± 0.85^{c}	0.534
120	6.90 ± 0.71^{bc}	6.70 ± 0.71^{bc}	0.804
144	4.40 ± 1.13^{ab}	5.70 ± 0.42^{b}	0.268
168	3.20 ± 0.57^{a}	3.90 ± 0.71^{a}	0.388

Values are Mean ± SD; Values with different superscript within the column are significantly different ($P<0.05$) by Independent Sample *t*- Test.

Appendix III: Biomass of *Streptomyces michiganensis* using Wheat Bran and Starch Substrate

	Biomas(mg/ml)		
Fermentation period(h)	**Wheat Bran**	**Starch**	**P-Value**
24	0.50 ± 0.71^{a}	2.00 ± 1.41^{a}	0.312
48	2.50 ± 0.71^{b}	5.50 ± 0.71^{b}	0.051
72	5.00 ± 1.41^{c}	9.00 ± 2.83^{c}	0.216
96	3.00 ± 1.41^{bc}	5.50 ± 2.12^{b}	0.300
120	2.00 ± 1.41^{ab}	4.50 ± 0.71^{b}	0.155
144	2.00 ± 1.41^{ab}	3.50 ± 0.71^{ab}	0.312
168	1.50 ± 0.71^{ab}	$3.50 \pm 2.12a^{ab}$	0.333

Values are Mean ± SD; Values with different superscript within the column are significantly different ($P<0.05$) by Independent Sample *t*- Test.

Appendix IV: Effect of Temperature on Amylase Production by *Streptomyces michiganensis*

Temperature(^{o}C)	Amylase Activity(U/ml)
30	8.70 ± 0.71^{bc}
35	11.20 ± 2.26^{c}
40	7.50 ± 0.42^{ab}
45	5.20 ± 0.85^{a}
50	4.50 ± 0.99^{a}

Values are Mean ± SD; Values with different superscript within the column are significantly different ($P<0.05$) by Duncan multiple range test.

Appendix V: Effect of Moisture Content on Amylase Production by *Streptomyces michiganensis*

Moisture content (%)	Amylase Activity(U/ml)
20	7.20 ± 0.28^{bc}
30	7.90 ± 0.42^{c}
40	6.20 ± 0.57^{b}
50	6.90 ± 0.42^{bc}
60	4.80 ± 0.85^{a}

Values are Mean ± SD; Values with different superscript within the column are significantly different ($P<0.05$) by Duncan multiple range test.

Appendix VI: Effect of pH on Amylase Production by *Streptomyces michiganensis*

pH	Amylase Activity
4	6.50 ± 0.71^{a}
5	8.20 ± 0.57^{a}
6	12.20 ± 0.57^{b}
7	11.30 ± 1.27^{b}
8	7.10 ± 0.71^{a}

Values are Mean ± SD; Values with different superscript within the column are significantly different ($P<0.05$) by Duncan multiple range test.

Appendix VII: Effect of Inoculum Size on Amylase Production by *Streptomyces michiganensis*

Inoculum Size(ml)	Amylase Activity(U/ml)
1	4.80 ± 0.57^a
2	7.90 ± 0.42^b
3	6.90 ± 0.71^b
4	3.70 ± 0.71^a
5	3.40 ± 0.57^a

Values are Mean ± SD; Values with different superscript within the column are significantly different ($P<0.05$) by Duncan multiple range test.

Appendix VIII: Amylase Production at Optimum Fermentation Conditions by *Streptomyces machiganensis*

Fermentation period(h)	Amylase Activity(U/ml)
24	9.2±0.45^{cd}
48	17.7±0.41^{e}
72	12.7±0.35^{d}
96	8.3±0.06^{cd}
120	6.0±0.57^{c}
144	5.1±0.25^{c}
168	3.5±0.71^{ab}
192	2.9±0.11^{a}
216	2.2±0.35^{a}
240	1.9±0.25^{a}

Values are Mean ± SD; Values with different superscript within the column are significantly different ($P<0.05$) by Duncan multiple range test.