ISOLATION OF BACTERIA WITH POTENTIAL FOR PRODUCTION OF THERMOSTABLE EXTRACELLULAR CELLULASE FROM SAWDUST

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ABSTRACT

Cellulolytic enzyme is a major renewable source of production of cellulase with high potential for degradation of lignocellulosic waste materials into useful end-products. Industrial processes involved in recycling lignocellulosic wastes generate heat which reduce efficiency or denature cellulase used in degradation of such wastes. There is an urgent need to manage bulk wastes effectively and economically by the use of thermostable cellulase. The aim of this study was to isolate thermophilic bacteria with potential for producing thermostable cellulase useful in degradation of lignocellulosic wastes.

Sawdust samples were purposely collected from sawmills in Ede, Osun State. Bacteria were isolated from the samples, identified and screened for cellulase production using standard microbiological procedures. Isolates with cellulase activity were selected, studied for thermostable cellulase production and their stability were determined. Optimisation of cellulase production was carried out using different carbon and nitrogen sources, temperature, pH and metal ions in pre-treated and untreated sawdust, corncob and sugarcane bagasse as substrates. Isolates with significantly high cellulase production were selected and identified by PCR amplification of 16SrDNA genes. Cellulase was purified using ammonium sulphate precipitation, dialysis and affinity chromatography, and used to hydrolyse lignocellulosic materials. Data were analysed using descriptive statistic and ANOVA at p= 0.05.

Out of 79 bacteria isolated from sawdust samples, 20 were cellulase producers and identified as *Actinomyces nueslundii* (4), *Pseudomonas aeruginosa* (12), *Thermoactinomyces vulgaris* (1), *Roseomonas* sp. A1 (1), and *Anoxybacilus rupiensis* (2). *Roseomonas* sp. (A1), *Anoxybacilus rupiensis* E1 and *Anoxybacilus rupiensis* 5H were the best three thermostable cellulase producing bacteria. Their extracellular enzymes were stable at 60^0C to 65^0C for 1hour, and 50^0C to 55^0C for 2hour. These isolates exhibited significant differences in cellulase production in the presence of carbon and nitrogen sources at 0.5% to 2.5% (w/v) concentration. *Anoxybacillus rupiensis* (5H) in medium supplemented with 1% carboxymethylcellulose produced 9.22 U/mL of cellulase while *Anoxybacillus rupiensis* (E1) produced 3.0 U/mL of cellulase in medium supplemented 1% (w/v)tryptone. Optimum production of cellulase was at 50^oC and pH of 7, while medium supplemented with magnesium sulphate at 1% (w/v) concentration produced 7.78 U/mL of cellulase. Significant difference was observed in the production of cellulase from corncob, sugarcane bagasse and sawdust at varying concentrations of 1.0-3.0 % (w/v) by *Roseomonas* sp. (A1), *Anoxybacillus rupiensis* (E1) and *Anoxybacillus rupiensis* (5H). Purified cellulase obtained using Sephadex G-75 gave the best activity (26.7 U/mg). Highest percentage lignocellulose hydrolysis by enzymes from *Roseomonas sp.* A1 and *Anoxybacillus rupiensis* E1 (21.0 and 29.2 respectively) was obtained in sugarcane bagasse; while 38.9 hydrolysis in the corncob was recorded for *Anoxybacillus rupiensis* 5H.

Roseomonas species *and Anoxybacillus rupiensis* demonstrated high hydrolytic action on lignocellulosic materials at high temperature. The cellulolytic enzymes produced by these organisms will be of potential application in lignocellulosic industrial processes that require elevated temperature.

Keywords: Thermostable-cellulase, Lignocellulosic waste, *Roseomonas* and *Anoxybacillus*

Word count: 479.

CERTIFICATION

I certify that this research work was carried out by HAFIZ AWOFE AKINYELE under my supervision in the Department of Microbiology, University of Ibadan, Ibadan, Nigeria;

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DEDICATION

This research work is dedicated to Allah (SWT) and to the service of humanity.

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The foundation laid by my late parents will not go unremembered, I am sincerely grateful to you and pray that the Almighty Allah forgive and accept you to HIS paradise.

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Hafiz Akinyele

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Abbreviations

CMC-	Carboxymethylcellulose
DNA-	Deoxyribonucleic Acid
SEM-	Scanning Electron Microscope
TEM-	Trasmission Electron Microscope
M-	Molarity
DNSA-	Di-ntrosalicylic Acid
EDTA-	Ethylenediaminetetraacetic Acid
O.D-	Optical Density
Tris-HCl-	Hydroxymethyl amino methane
GRAS-	Generally Regarded as Safe
CMCase-	Carboxyl methyl cellulase
IU-	International Unit
kDa-	KiloDalton
K_{m-}	Michaelis constant
V_{mas-}	Maximum Velocity
Lat.-	Latitude
Long. -	Longitude
TSI-	Triple Sugar Iron
SIM -	Sulphide Indole Motility
µL -	Litre
SDS-PAGE-	Sodium dodecyl sulphate –polyacrylamide gel Electrophoresis
EEO-	Electroendosmosis

CHAPTER ONE

1.0 INTRODUCTION

1.1 General considerations

Nigeria is an agrarian country where a lot of plantations exist including the plantations of woody plants. Majority of these wood materials waste away at the end of the day. These woods could be very useful economically if serious considerations are given to them since in them are locked up energy yielding organic molecules in forms of sugars (Roberto *et al*., 2005). Moreover, the disposal of residue from forest industry, including foliage and wood residue can pose a problem. Yet these residues have significant potential to be used economically and ecologically in the production of energy of numerous products for which there is high demand.

The accumulation of residue in the Nation's sawmills can become an obstacle to the production process, so they need to be removed quickly. Some sawmills process and sell them to enterprises that exploit them for a variety of uses, but often they are discarded as rubbish or burnt indiscriminately, which is a waste of organic matter that is rich in nutrients. Furthermore, the accumulation of sawdust can have negative environmental effects arising from the following:

As it decomposes, the carbon-dioxide contained in organic matter is dispersed in the atmosphere. Sun and high temperature can provoke a low temperature pyrolysis in large piles of sawdust, causing them to emit polluting gases. This combustion also increases the temperature in the area, causing a hot house effect. The residue can become an ideal medium for propagation of plagues and illness.

Vast quantities of cellulosic, lignocellulosic agricultural and agro-industrial wastes including sawdust that can be beneficial are accumulating in the country. Greater use of such wastes might be possible through their enzymatic degradation for production of chemicals, energy and microbial proteins (El –Naghy *et al*., 1991).

Cellulose, the most abundant renewable resource, has received much attention as potential energy and carbon source for production of useful products

such as glucose, alcohol, and protein for food and feed. However, due to their resistance to microbial degradation, most native celluloses are yet to be effectively utilized (Singh *et al*., 1998).

For the efficient and complete hydrolysis of lignocellulosic residues, it would be desirable to have thermophilic and thermostable cellulases from thermophilic microorganisms. By operating at elevated temperature, it would be possible to limit the incidence of contamination while the thermostable enzymes would maintain their activities and could be recycled for addition to fresh substrate.

Again when compared with the intense work that had been carried out on mesophilic microorganisms, the search for highly cellulolytic and thermophilic microbes have been relatively limited. Very little information is available on the cellulase productivities and temperature stabilities of thermophilic microorganisms especially bacteria.

Thermophiles represent obvious source of thermostable enzymes, being reasonable to assume that such character will confer a high thermal stability to some of their proteins. This is so as can be appreciated in the case of several biotechnologically relevant enzymes from the hyperthermophilic archaebacteria *Pyrococcus furiosus* and *Thermotogs* spp. (Adams *et al*., 1995: Fischer *et al*., 1996 and Adams and Kelly, 1998) as reported by Andrew lllanes (http/www.ejbiotechnology.info/content/vol2/issue/full /1bip)

1.2 **Statement of problem**

Cellulase enzymes are very important in food, poultry, pulp and paper industries. Majority of these are produced from mesophiles. The use of these enzymes in industries lead to their denaturation due to the heat generated during industrial process. This leads to constant replenishment of the enzyme if production is to continue. Therefore, there is need for production of thermostable cellulase from thermophilic organisms that will be able to withstand such heat when generated in the course of industrial process.

However, the technological use of thermophiles still faces several challenges because knowledge on their physiology is poor, they are fastidious, grow slowly and yet to be recognized as safe. Hence, thermostable enzymes used by industries are still produced from mesophiles and commercial enzymes from thermophiles are still scarce.

1.3 **Justification**

Thermostable cellulases that are capable of withstanding the high temperature of industrial process are being advocated as alternative to the one produced by mesophilic organisms. The ability to isolate thermophilic cellulase-producing bacteria that will synthesize thermostable cellulase will go a long way in reducing the cost of constant procurement of fresh cellulase after the denaturation of the used one.

1.4 **Aims and objectives**

The objectives of this study are:

(i) To isolate and identify different thermophilic bacteria from sawmill dump sites using standard procedures.

(ii) To further characterize the isolates using molecular identification techniques

(iii) To study the growth and physiological characteristics of the different isolates

(iv) To purify and characterize the extracellular thermophilic enzymes from the isolates.

(v) To subject the enzymes to degradation of pulp and pulping materials.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Enzymes

Enzymes can be defined as biological catalysts which are proteinous in nature and capable of affecting the rate of biochemical reactions without themselves undergoing any overall change (White *et al.*, 1978). Only small quantities of the enzymes, relative to the concentration of their substrates, are required to catalyze a chemical transformation in a reaction system. Enzymes are extremely important because they are highly specific for their substrates and highly active under mild environmental conditions and biodegradable (Stryer, 1981*;* Wojtezak *et al.*, 1987). The specificity of enzymes at different pH and temperature ranges have also been reported (Manning *et al*., 1961). All enzymes have their optimum pH and temperature at which they perform maximally. However, these will also depend on the type of organisms from which the enzymes are derived. Most enzymes isolated from bacteria are slightly acidic to neutral while those from fungi are acidic (2- 6) pH (Khasin *et al*., 1993; Kulkarni *et al*., 1999). Enzyme activities are also influenced by enzyme amount, type of substrates, modulator inhibitors and amount of product (Dashed and Micales, 1997). Some of the factors that can affect the level of expression of enzymes using different substrates include substrate availability, accessibility, rate and amount of release of the oligosaccharides, and their chemical nature and quantity of reducing sugar released which act as carbon source and as an inhibitor of enzyme synthesis (Kulkarni *et al*., 1999).

Depending on the reaction catalyzed, enzymes are called protease, amylase, cellulase and lipase when they are capable of hydrolyzing proteins, starch, cellulose and lipids or oil respectively (Sani *et al*., 1992). Lokendra *et al*. (1994) reported that the need for improved efficiency of industrial processes catalyzed by enzymes has necessitated the use of microbial enzymes as opposed to whole microbes, because

there will be higher catalytic activity, avoidance of undesirable side reactions and reproducibility of results are achieved with such enzymes. The use of enzymes in various industries including breweries, bakery, leather and textile and mining industries is well known (Sani *et al*., 1992; Kwan *et al*., 1993; Patel *et al*., 1993).

Enzymes are found in all living systems where they activate and regulate chemical reactions essential to the continue existence of the individual organism. Though various enzymes have been isolated from different types of organisms (plants, animals and microorganisms), the production of enzyme from microorganism is desirable because it is easy, economical, require short production time and their production can be improved by genetic and environmental manipulations. Moreover, the number of enzymes from microbes is limitless while their mode of production and amount to be produced can be manipulated by the producers since the genetic mechanism of microbes and environmental conditions are within human manipulation (Jones and Warner, 1990; Kwan *et al*., 1993).

Enzymes are produced in microbes in two ways. These are either intracellular or extracellular in which case enzymes are secreted into the cells or into the immediate environment of the cell respectively (Lealem and Gashe 1994).

For some time now, microorganisms form the major source of enzymes due to the fact that obtaining even a small quantity of plant enzyme require the use of large amount of plant materials which makes large scale production of plant enzymes uneconomical especially if the plant has some other economic uses (Oguntimehin and Safarik, 1993). For animals, enzymes obtained from them are usually by-products for meat industry and hence, the supply becomes limiting. Furthermore, other valuable products may be needed from the same organs used for enzymes production. Such competition will further cut down the amount of materials from which these enzymes can be extracted. By contrast, microbial enzymes are not subjected to any of these problems. Unlike plants and animals, microbes can be cultivated in the laboratory on synthetic media and the growth rate of microbes are very rapid and so the laboratory study of microbial enzymes production is possible within a short period of time (Onyeocha and Ogbonna, 1983)

Considerable research had been undertaken with extracellular enzymes produced by a wide variety of microorganisms (Fogarty and Kelly, 1979). Unlike intracellular enzymes of plants and animals, extracellular enzymes leak into the culture medium of microbes producing them and they can be recovered by either filtration or

centrifugation (Oso, 1979; Lealem and Gashe, 1994; Immanuel *et al*., 2006). Due to the advantage of microbial enzymes, researchers have shifted attention to the exploitation of microbial production of various enzymes of industrial importance. Microbial enzymes could degrade complex substrate such as cellulose, hemicelluloses, lignin and starch (EL-Naghy *et al.,* 1991; Kwan *et al*., 1993; Patel *et al*., 1993).

2.2 **Celluloses**

Cellulose is an organic compound with the formular $(C_6\ H_{10}\ O_5)_n$, a polysaccharide derived from β–1,4 linked D-glucose units. It is the structural component of the primary cell wall of green plants, acetic acid bacteria, many forms of algae and fungi of the family oomycetes. Cellulose is the major polysaccharide constituent of plant cell wall and one of the most abundant available organic compounds in the biosphere and its estimated synthesis rate of 10^{10} tones per year (Schlesinger, 1991; Singh and Hayashi, 1995; Lynd *et al*., 2002; Stefan *et al*., 2006)). Cellulose-rich plant biomass is one of the foreseeable and sustainable sources of fuel, animal feed and feed stock for chemical synthesis (Bhat, 2000).

The utilization of cellulosic biomass continues to be a subject of worldwide interest in view of fast depletion of oil reserves and food shortages (Kuhad *et al*., 1997b; Gong *et al*., 1999a). Cellulose serves as a vast reservoir of glucose residues linked by β-1,4 glycosidic bonds. The conversion of cellulosic mass to fermentable sugars by saccharification through biocatalyst cellulase derived from cellulolytic organisms has been suggested as a feasible process and offers potential to reduce use of fossil fuels and reduce environmental pollution (Dale, 1999; Lynd *et al*., 1999). Cellulose is a complex carbohydrate polymer and is one of the major constituents found in wood. Its complete degradation requires a set of enzymes that acts synergistically (Martin, 1983). Cellulose is deceptively simple chemical , a polymer consisting only of glucose linked only by beta-1-4 bonds. But cellulose samples of different origin vary widely in chain length and the degree of interaction between the chains. Waste cellulose usually consists of only 40-60% cellulose with the balance consisting of hemicellulose, lignin and other materials (Mandel *et al*., 1976). Several different crystalline structures of cellulose are known, corresponding to the location of hydrogen bonds and within strands.

Cellulose is a polymer of glucose in which the glucose molecules are joined by β - 1 – 4 linkages. The chains of cellulose are unbranched and may be several

thousand units. Cellulolysis is the process of breaking down cellulose into smaller polysaccharides called cellodexrins or completely into glucose units through a hydrolysis reaction. Because cellulose molecules bind strongly to each other, cellulolysis is relatively difficult compared to the breakdown of other polysaccharides (Hatami *et al*., 2008).

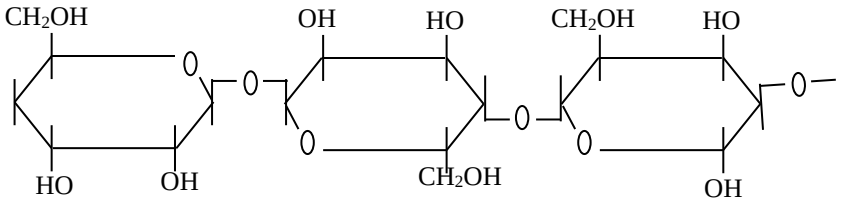
Currently, there two major ways of converting cellulose to glucose: chemical versus enzymatic. The research on both methods has for decades occupied the attention of many investigators world wide. Because each cellulose molecule is unbranched polymer of 1000 to 1million D-glucose units, linked together with beta-1,4 glycosidic bonds, cellulose from various sources are all the same at the molecular level. However, they differ in the crystalline structures. It is this difference that makes possible a persistent research on cellulose. The model chemical compound commonly used in today`s research are carboxymethyl cellulose (CMC), which has a generally amorphous structure, and avicel, which has a highly crystalline structure. There are two types of hydrogen bonds in cellulose molecules: those that form between the C3 hydroxyl group and the oxygen in the pyrenose ring within the same molecule and those that form between the C6 hydroxyl group of one molecule and the oxygen of the glycosidic bond of another molecule (Bertram and Dale, 1985).

Ordinarily, the beta-1, 4 glycosidic bonds themselves are not too difficult to break. However, because of these hydrogen bonds, cellulose can form very tightly packed crystallites. These crystals are sometimes so tight that neither water nor enzymes can penetrate them; only exoglucanase, a subgroup of cellulase that attacks the terminal glycosidic bond, is effective in degrading it. The inability of water to penetrate cellulose also explains why crystalline cellulose is insoluble (Linko, 1977; Bertran and Dale, 1985). On the other hand, amorphous cellulose allows the penetration of endoglucanase, another subgroup of cellulase that catalyzes the hydrolysis of internal bonds. The natural consequence of this difference in the crystalline structure is that the hydrolysis rate is much faster for amorphous cellulose than crystalline cellulose (Linko, 1977; Bertran and Dale, 1985).

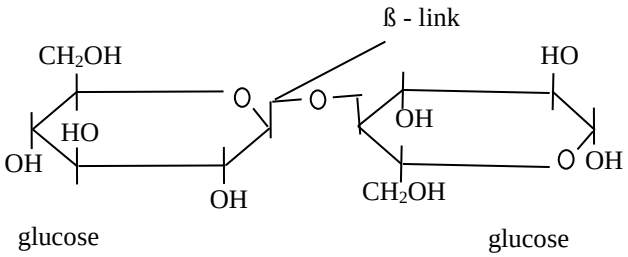
The process of breaking the glycosidic bonds that hold the glucose basic units together to form a large cellulose molecule is called hydrolysis because a water molecule most be supplied to render each broken bond inactive. In addition to crystallinity, the chemical compounds surrounding the cellulose in plants, for example, lignin, also limit the diffusion of the enzyme into the reaction sites and play an

important role in determining the rate of hydrolysis. To be able to convert cellulose and hemicellulose in biomass to fuel and chemicals, lignin must be degraded and modified.

Structure of Cellulose



Structure of Cellobiose



Cellulose chain (http://www.greenspirit.org.uk/resources/cellulose.gif) Retrieved, September, 2008

There is a hydrogen bond that occurs within and between cellulose molecules, resulting in a crystalline state, which contributes to resistance of cellulose digestion.

Some animals, particularly ruminants and termites can digest cellulose with the help of symbiotic microorganisms. Cellulose is not digestible by humans and is often referred to as dietary fiber acting as a hydrophilic bulking agent for faeces (Updegraff, 1969). Mammals do not have the ability to break down cellulose directly, a characteristic possessed only by certain bacteria (which have specific enzymes) like *Cellulomonas, Pseudomonas* e.t.c and which are often the flora on the gut walls of ruminants like cows and sheep or by fungi, which in nature are responsible for cycling of nutrients (Wenzel *et al*., 2002).

Natural cellulose is cellulose I, with cellulose Iα and $I_{\beta.}$ Cellulose in regenerated cellulose fibres is cellulose II. The conversion of cellulose I to cellulose II is not reversible, suggesting that cellulose I is metastable and cellulose II is stable. With various chemical treatments it is possible to produce the structures of cellulose III and cellulose IV (Perez and Mackie, 2001).The natural crystal is made up from metastable cellulose I with all the cellulose strands parallel and no inter-sheet hydrogen bonding. Cellulose I contains two coexisting phase cellulose I_α (triclinic) and cellulose I_β (monoclinic) in varying proportions dependent on its origin; I_α being found more in algae and bacterial whilst I_β is the major form in higher plants.

Cellulose I_α and cellulose I_β have the same fibre repeat distance (1.043nm) for the repeat dimmer interior to the crystal, (1.029nm) on the surface (Davidson *et al*., 2004) but differing displacements of the sheets relative to one another. The neighbouring sheets of cellulose I_α (consisting of identical chains with two alternating glucose monomers) are regularly displaced from each other in the same direction whereas sheets of cellulose I_β (consisting of two conformationally distinct alternating sheets, each made up of crystallographically identical glucose conformers) are staggered (Jarvis, 2000). It has been found that cellulose I_β significantly alters the water structuring at its surface out to about 10 Å which may affect its enzymatic digestion (Matthew *et al*., 2005). Cellulose I_α and cellulose I_β are interconverted by bending during microfibril formation (Jarvis, 2000) and metastable cellulose I_α convert to cellulose I_β on annealing.

Cellulose-rich plant biomass is one of the foreseeable and sustainable sources of fuel, animal feed and feedstock for chemical synthesis (Dickman, 1931). The bioconversion of cellulosic materials has been considered largely as a contribution to

the development of a large-scale conversion process beneficial to mankind (Kumakura 1997).

Plant residues contain 15-60 percent cellulose, 10-30 per cent hemicellulose, 5-30 per cent lignin, 2-15 per cent protein and 10 per cent sugars, amino acids and organic acids (Mandel *et al*., 1976). Cellulose occurs in a semicrystalline form with a molecular weight of 106. Cellulase enzyme complex decomposes cellulose into disaccharide cellobiose which is hydrolyzed by the enzyme cellobiase to glucose. Hemicelluloses are various polymers of hexoses, pentoses and sometimes uronic acids with commonly occurring monomers such as xylose and mannose. Pectin is an example of hemicellulose and is an important constituent of the middle lamella of cell walls (Sylvia *et al*., 2005). Pectin is degraded by the enzyme pectinase which is a complex of several enzymes.

2.3 **Agro industrial waste and cellulase production**

Wheat bran is extensively produced as by-product of the flour mill industries. Bran, the outer layer of the grain, consist of combined aleurone and pericarp (Bing-qin *et al*., 1987). After removal of bran, the grains lose some of the nutritional components, as bran is particularly rich in dietary fibre and contains significant quantities of protein, vitamins and dietary minerals. The effect of wheat bran on the production of CMCase by *Trichoderma reesei* Rut C30 was investigated employing submerged fermentation (Yu *et al*., 1998). The addition of wheat bran resulted in enhanced CMCase activity i.e. 125.78U/ml from growth medium containing 2% Avicel and 3% wheat bran after 6days. In case of FPase 12.85U/ml was obtained from medium including 1%Avicel and 5% wheat bran (Yu *et al*., 1998).

One of largest cellulosic agro-industrial by-product is sugar cane bagasse, a fibrous residue of cane stalk leftb over after crushing and extraction of juice. It is a lignocellulosic residue of the sugar cane industry and is used by the sugar mill as fuel for boilers. Presently there is an increase in trend for more efficient utilization of agro-industrial by-products including sugar cane bagasse and one of the important uses is the production of enzymes. However, the economy of such process is affected by the high cost of the product; the utilization of cheaper and indigenous substrates has contributed to the economical recovery (Pandey *et al*., 2000).

Sugar cane bagasse may be chemically treated to generate different bagasse samples with varying quantity of lignin and hemicelluloses, keeping cellulose fraction intact. Adsul *et al*. (2004) treated bagasse samples and evaluated for the production of

cellulase and xylanase enzymes by *P. janthinellum* NCIM1171 and *T. viride* NCIM 1051 in the production medium. The bagasse treatment with $NaClO_2$ (Bagasse: $NaClO_2$ 1:0.25) resulted in increased level of xylanase and β-glucosidase production by both organisms. The productivity of all enzymes except cellulase were higher in the media with this bagasse sample than that of pure cellulose powder.

Mishera *et al*. (1984) had previously reported *P. funiculosum* as a good producer of cellulase system having CMCase (15-20IU/ml), exo-glucanase (1.5-2.0IU/ml) and β-glucosidase (8-10IU/ml). The saccharificafion of alkali-treated cotton and sugar cane bagasse by *P. funiculosum* enzyme were 70 and 63%, respectively. A glucose concentration of 30% was obtained using 50% bagasse.

Later, Correa and Tengerdy (1997) co-cultured *T. reesei* LM-UC4 and its mutant LMUC4E1 with *A. phoenicis* QM329 for cellulase production on bagasse by mixed culture solid substrate fermentation. The milled bagasse was treated with 1.2% NaOH. A mutual synergism was observed between the parent *Trichoderma* strain and *Aspergillus*. It was observed that *A. phoenicis* boosted the cellulase production of *Trichoderma* and which in turn enhanced biomass and β-glucosidase production of *Aspergillus* resulting in increased activities of cellulase (38.3 IU/g DW of biomass), CMCase (210.9 IU/g DW of biomass) and β-glucosidase (93.0 IU/g DW of biomass) This kind of synergism was absent in the case of mutant *Trichoderma* strain that may be attributed to loss of ability for cooperative interaction with other microbes as a consequence of hypermutation. In another study, ammonia-treated bagasse (5%) was subjected to mixed culture fermentation using *Trichoderma reesei* and *Aspergillus phonenicis* at 30^{o}C with ammonium sulphate as nitrogen source for cellulases production (Duenas et *al*., 1995). Significantly higher activities of all enzymes of cellulase complex were achieved in 4 days of mixed culture fermentation than the single (*T. reesei*) fermentation.

Similarly cellulase production using corn cobs as substrate was carried out by *Trichoderma reesei* ZU-02. Under batch process, the optimum concentration of substrate was 40g/l with optimum C/N ratio of 8.0. The CMCase activity 5.25IU/ml (213.4IU/g cellulose) was observed after seven days (Beldman *et al*., 1985). For large scale production, after four days the CMCase and cellobiose activities were reported to be 5.48IU/ml (222.8IU/g CMCase) and 0.25IU/ml (10.2IU/ g cellulose) respectively.

2.4 **Lignocellulosic materials**

Lignocellulosics are abundant sources of carbohydrate, continually replenished by photosynthetic reduction of carbondioxde by sunlight energy. Thus, they are the most promising feedstock for the production of energy, food and chemicals (Wuz and Lee, 1997; Solomon *et al.* 1990). Lignocellulose has been described as a collective name for three polymeric materials, cellulose, hemicellulose and lignin which occur in woody plants and grasses (Nottipong and Alissara, 2006). Lignocellulosic substrates have enormous biotechnological value due to their chemical properties. Large amount of lignocellulosic biomass are generated through forestry and agricultural practices. These huge amounts of residual plant biomass can be converted into various value added products including biofuels, chemicals, cheap energy sources for fermentation, improved animal-feed and nutrients (Howard *et al*., 2003).

2.5 **Lignin**

Lignin is much more complex than cellulose and is formed by chemical reaction involving phenols and free radicals without any specific order. Lignin gets encrusted on the cellulose and hemicellulose matrix. Compounds like caffeic acid and ferulic acid have structures similar to lignin and they have been used 'in studies on degradation of lignin(Chloe *et al*., 2002). The lignin molecule has only three elements carbon, hydrogen and oxygen. The molecule is a polymer of aromatic nuclei with either a single repeating unit or several similar units as building blocks. The repeating units range from about 200 to 1000 depending upon the origin of lignin and the methods used to determine the molecular weight.

Degradation of lignin is brought about by fungi mainly belonging to Basidiomycetes. The genera of fungi which degrade lignin as well as cellulose are *Clitocybe*, *Collybia*, *Mycena*, *Marasmius*, *Polystictus*, *Armillaria*, *Polyporus*, *Stereum*, *Ganoderma*, *Pleurotus*, *Trametes*, *Fomes* and *Ustulina*.

Lignin protects cellulose and hemicellulose from enzymatic attack by some microorganisms, thereby limiting the recycling of carbon. Although many actinomycetes are reported to modify lignin, they cannot mineralize it. White rot fungi are said to be capable of mineralizing substantial fraction of lignin (Ahmed *et al*., 2001). To be able to convert cellulose and hemicellulose in biomass to fuel and chemicals, lignin must be degraded and modified. Lignin is a complex chemical compound most commonly derived from wood, and an integral part of secondary cell

walls of plants (Lebo *et al*., 2001). Lignin is the second most abundant organic polymer in nature next to cellulose and it is of about 30% non-fossil organic carbon (Boerjan *et al*., 2003) and constituting from a quarter to a third of the dry mass of the wood. The lignin content in plant varies from about 18%-35% of the total wood content. 5-36% of biomass of both softwood and hardwood is lignin (Boerjan *et al*., 2003). Lignin confers mechanical strength to the cell wall and by extension to the plant as whole (Chabannes, 2001). Lignin plays a crucial role in conducting water in plant stems. The reason being that the polysaccharide components of plant cell walls are highly hydrophilic and permeable to water, whereas lignin is more hydrophobic. Again, lignin being generally associated with reduced digestibility of the overall plant biomass, helps defend against pathogens and pests (Harkin, 1967; Sarkanen and Ludwig, 1971).

2.6 **Pretreatment of Lignocellulosic Materials**

In order to make cellulose and hemicelluloses in plant material accessible to enzymatic treatment, the plant materials need to be treated usually either by acid or alkali(Singh *et al*., 1998). In the use of acid for degradation of plant-derived carbohydrates (PDFCs), materials are treated with usually sulphuric acid to obtain mono-sugars. In the process, acid first breaks the matrix structure of the fibre into more accessible cellulose, hemicelluloses and lignin and then further reduces these polysaccharides to mono-sugars (Fengel and Wegener, 1984). Either concentrated acid at low temperature or dilute acid at a high temperature are commonly used (Sun and Cheng, 2002). However, the degradation by concentrated acid seems to be the efficient (Grahmann *et al*., 1985) with a reported glucose yield of 72-82% being achieved from mixed wood chips using such a concentrated acid hydrolysis process (Iranmahboob *et al*., 2002). In the same vain the use of auto-hydrolysis approach was adopted for the production of glucose from lignocelluloses under extremely low acid and high acid condition in the pretreatment of sawdust (Ojumu and Ogunkunle, 2005). Pretreatment of sawdust at 210^0C for 18minutes yielded the maximum glucose of 70%. The value was 1.4 times of that obtained from untreated sawdust under the same condition.

However, the use of acid for the degradation of PDFCs is not suitable because of the stringent pH conditions that must be adhere to in order to prevent the degradation of the monosaccharides at high pH (Sharma *et al*., 2006)

Some bases can also be used to breakdown lignocellulosic materials (Fan *et al*., 1987). The mechanism of alkaline hydrolysis is believed to be saponification of intermolecular ester bonds crosslinking xylan hemicelluloses and other components, like lignin and other hemicellulose. The porosity of lignocellulosic materials increases with the removal of the crosslinks (Tarkow and Feist, 1969). Dilute sodium hydroxide (NaOH) treatment of lignocellulosic materials cause swelling, leading to an increase in internal surface area, a decrease in the degree of polymerization, a decrease in crystallinity, separation of structural linkage between lignin and carbohydrates, and disruption of the lignin structure (Fan *et al*., 1987). The digestibility of NaOH-treated hardwood increased from 14% to 55% with decreased of lignin content from 24-55% to 20%. However, no effect of dilute NaOH breakdown was observed for softwoods with lignin content greater than 26% (Millet *et al*., 1976) Dilute NaOH pretreatment was also effective for hydrolysis of straws with relatively low lignin content of 10-18% (Bjerre *et al*., 1996).

In order to improve animal feed shortage and disposal problem of waste, attempts have been made to produce microbial proteins from lignocellulosic wastes such as rice straw, banana, potato and cassava wastes, sugar cane bagasse, groundnut hulls and water hyacinth (Ibrahim and Antai, 1986; Antai and Mgbomo, 1993; Deabald and Crawford, 2002; Nwodo-Chinedu *et al*., 2007).

In nature, a variety of microorganisms and enzymatic mechanisms are involved in the complete degradation of lignocellulose. The structure of lignocellulose sets barriers for chemical and enzymatic degradation. Even in the enzymatic hydrolysis of pure cellulose, a gradual drop in the reaction rate is observed. Reasons for this decrease in the rate of hydrolysis of pure cellulose are generally attributed to end production inhibition (Holtzapple *et al*., 1990), depletion of easily degradable parts, enzyme inactivation and unproductive binding or entrapment of the enzymes in the small pores of the lignocellulose (Tanaka *et al*., 1977; Converse *et al*., 1988) Factors affecting the rate of hydrolysis of lignocellulosic materials can be divide into two parts: Those related to the structure of the substrates (degree of polymerization, crystallinity, structural composition, particle size, available surface area, degree of fibre swelling, pore structure and distribution) and those related to the mechanisms and interaction of the lignocellulolytic enzymes (Converse, 1993; Mansfield *et al*., 1999)

2.7 **Hemicellulose**

Hemicellulose is polysaccharide related to cellulose that comprise of 20% of the biomass of most plants. In contrast to cellulose, hemicellulose is derived from several sugars in addition to glucose especially xylose but also include mannose , galactose, rhamnose and arabinose. Hemicellulose consists of shorter chain, around 500-3000 sugar units(Watanabe *et al*., 1989). Furthermore, hemicellulose is branched, whereas cellulose is unbranched. Hemicellulose can be any of the several heteropolymers such as arabinoxylans, present in almost all plant cell walls along with cellulose. While cellulose is crystalline, strong and resistant to hydrolysis, hemicellulose has random amorphous structure with little strength. It is hydrolyzed by dilute acid or base as well as hemicellulase enzymes.

Life on earth depends on photosynthesis, which results in production of plants biomass having cellulose as the major component as well as starch. The carbon cycles closed primarily as a result of the action of cellulose-utilizing microorganisms present in the soil and the gut of animals .Thus microbial cellulose utilization is responsible for one of the largest materials flow at both local and global scales .The importance of microbial cellulose utilization is also integral component of widely used processes such as anaerobic digestion and composting (Lee *et al.*, 2002).

Starch and cellulose are two major polysaccharides of plants. While starch is the nutritional reservoir in plant cellulose serves a structural role. Starch is of two forms: amylose and amylopectin. Amylose, the unbranched type of starch consists of glucose residues in α-1, 4 linkage. Amylopectin, the branched form has about one α-1, 6 linkages per thirty α-1, 4 linkages. Both amylopectin and amylose are hydrolysed by α-amylase. Cellulose on the other hands has unbranched polymer of glucose residue joined by β -1, 4 linkage. The β - configuration allows cellulose to form very long straight chain (Mandels *et al*., 1976; Stryer, 1981). Cellulose samples of different origin vary widely in chain length and the degree of interaction between the chains. Waste cellulose is reported to usually consist of only 40-60% cellulose with the balance being hemicelluloses, lignin and other materials (Mandel *et al*., 1976). Many cellulase preparations also contain hemicellulase. If they are present, the hemicelluloses are rapidly hydrolyzed because they are less resistant to enzyme action than is cellulose (Mandel *et al*., 1976).

2.8 **Amylase**

Amylase or amylolytic enzyme is the one involved in the breaking down of large starch molecules (amylose and amylopectin) to smaller sugars. Amylase enzymes work by breaking the glycosidic bonds which bind the starch monomers (glucose units). Two types of amylases have been identified due to the mechanisms amylase enzymes carry out their hydrolytic reaction. These are the saccharogenic and dextrinogenic amylases which are referred to as β-and α-amylases respectively. Both the alpha and beta –amylases are capable of hydrolyzing 1, 4 - glycosidic linkages of both amylose and amylopectin (Oguntimehin and Safarik, 1993*).* The two types of amylases can be distinguished by their modes of action on amylopectin. β-amylase is capable of partial hydrolysis of amylopectin (not more than 55%). It cleaves α - 1, 4 linkage of amylose and amylopectin chain from the non- reducing end of a chain to release maltose (Stryer, 1981). The cleavage continues in maltose until the whole chain is degraded to release glucose. However, in amylopectin, the branching points constitute barriers thereby stopping the reaction automatically at the glucose unit before the branch point. Even though the two amylases cannot hydrolyze α-1, 6 linkage, α- amylase can by - pass this branch point. It therefore, disrupts the whole amylopectin and limit α- dextrin molecule (Lehninger, 1972). Hence, for complete saccharification of starch to yield maltose and other disaccharides, the combined actions of α-and β- amylases are required.

Amylase enzymes have been directly applied in those industries where sugar forms the basic raw materials. It has been used to breakdown starches to sugars. Apart from brewing, baking, distilling and pharmaceutical industries also use amylase extensively (Klingerberg *et al.,* 1990; Kwan *et al.,* 1993;). In pharmaceutical industries, the production of glucose and high fructose syrup are mediated by α- and β-amylases.

2.9 **Cellulase**

Cellulase is a complex enzyme containing chiefly endo and exo β - glucanases plus cellobiase. For complete hydrolysis of insoluble cellulose, synergistic action between the components is required. Since different cellulase preparations vary widely in the proportion of the different components depending on source, growing conditions of the organisms, harvesting and handling procedures. The rate and extent of their

hydrolysis of cellulose substrates also varies widely (Mandels *et al.,* 1976). The synergistic activity of the components of cellulase has also been reported by several workers (Lee *et al*., 2002). Endoglucanase is the most abundant and best characterized components and it randomly cleaves internal β-1, 4-glycosidic bonds, cellobiohydrolases, which attack cellulose from the chain ends, releasing cellobiose and β-glucosidase which hydrolyses cellobiose to release glucose (Begun and Aubert, 1994) cited *by* Fontes *et al.* (1998). The three major components of cellulase are also reported to be made up of (i) endoglucanases or 1, 4 - β- D - glucan – 4 - glucano - hydrolases (EC3.2.1.4). (ii) Exoglucanases which include 1, 4, β - D – glucan- glucano hydrolases (also known as cellodextronases) (*EC3. 2.1.74*) and 1, 4, - β-D - glucan cellobiohydrolases (cellobiohydrolases) (*EC3.2.1.91*) (iii) β - glucosidases or β - glucohydrolases (EC3.2.1.21) (Hoh *et al*., 1993; Lee *et al*., 2002).

Cellulase production from bacteria can be an advantage as the enzyme production rate is normally higher due to bacterial high growth rate. Screening of bacteria, optimisation of fermentation conditions and selection of substrates are important for the successful production of cellulase.

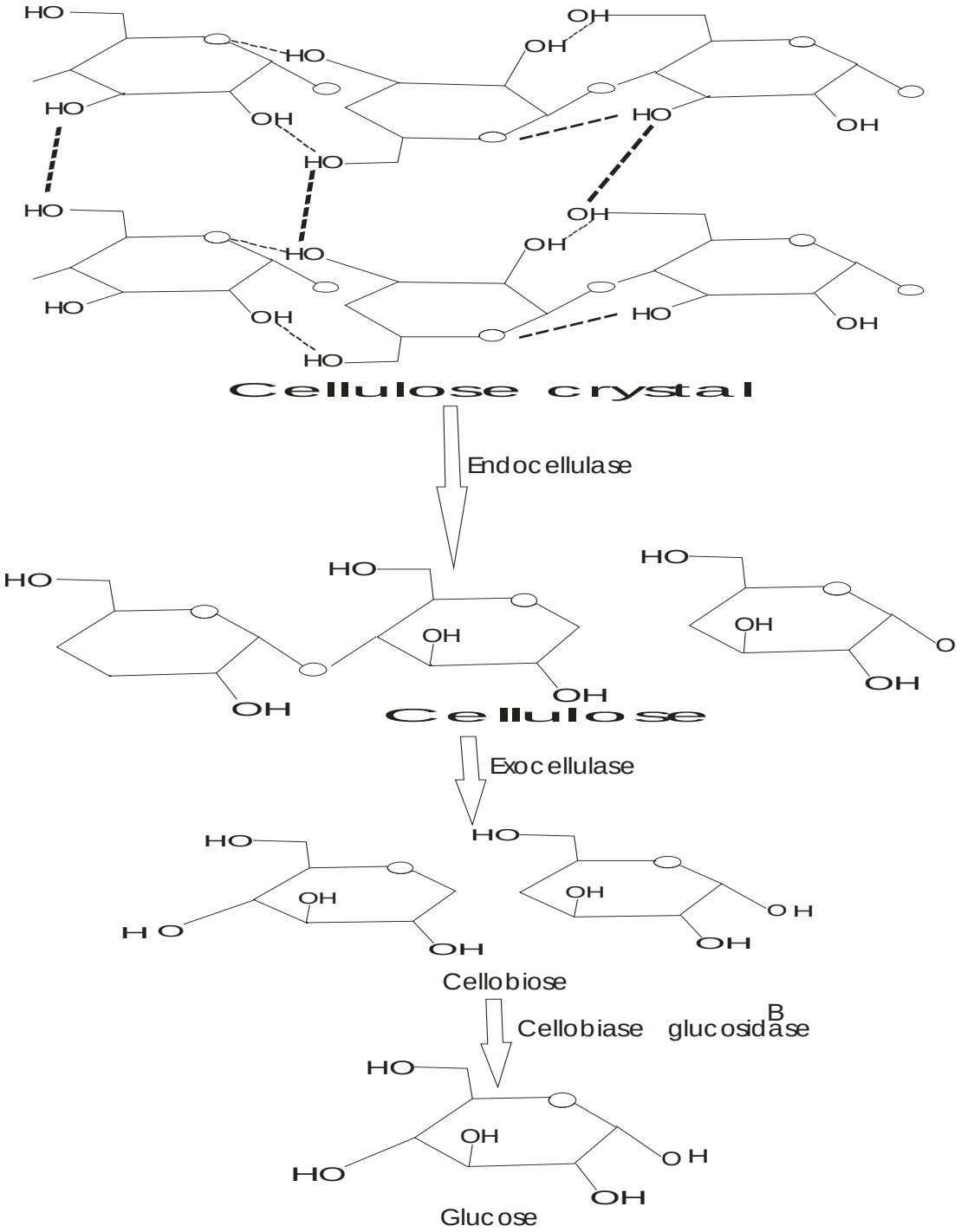
Study on cellulase production confirmed that fermentation parameters such as initial pH, temperature, carbon source and nitrogen source affected cellulase production. Cellulase from B. *pumilus* EB3 was found to be secreted the most at temperature 37^0C, initial pH 7.0, 1% CMC as carbon source and 2 g/L of yeast extract as organic nitrogen source. The activity recorded during the fermentation was 0.006 U/mL, 0.076 U/mL and 0.032 U/mL respectively for FPase, CMCase and β-glucosidase.

Induction of cellulase appears to be affected by soluble products generated from cellulose by cellulolytic enzymes synthesized constitutively at a low level. (Linko *et al.*, 1980). Majority of industrial enzymes produced by large scale submerged fermentation, agro industrial wastes can eventually be used as substrate and act as good sources of carbon and nitrogen. This involves growing selected microorganisms in closed vessel in which all the conditions critical for the growth are carefully controlled. Selected microorganisms are either bacterial (*Bacillus* and *Micrococcus* species) or fungal (*Aspergillus* and *Trichoderma* species) that have been carefully selected and optimized. Cellulase has a wide application in various fields; it is used in the textile industry, in laundry detergents; in the pulp and paper industry for different

purposes; facilitate fermentation of biomass into biofuels. It also has great potential for utilization in food industry; for coffee processing, extraction and clarification of juices (Grassin and Fauguembergue, 1996a; Galante *et al*.,1998a; Uhlig, 1998), extraction of oil from oilseeds and olive oil plant (Sosulski and Sosulski, 1993; Dominguez *et al*., 1995) bread production, brewery and wine biotechnology (Gunata *et al*., 1990; Caldini *et al.*, 1994;Hadara *et al*., 2005) production of fruit nectars and purees and to alter the sensory properties of fruits and vegetables (Hump and Scarier, 1991; Pabst *et al*.,1991; Marlatt *et al*., 1992).

CELLULASE TYPES AND ACTION

Reaction pathway from cellulose to glucose (Enzymeindia, 2008)



Different methods have been used in investigating enzyme production. A lot of works have been done on enzyme production by mesophilic and thermophilic bacterial (Klingerberg *et al*., 1990; Koch *et al*., 1991; Oguntimehin 1993; Kwan *et al*., 1993; Lealem and Gashe 1994).

An inspection of the phylogenetic tree reveals that the ability to digest cellulose is widely distributed among many genera in the domain Bacteria and the fungal groups within the domain Eucarya, although no cellulolytic members of domain Archaea have yet been identified within the eubacteria there is considerable concentration of cellulolytic capabilities among the predominantly aerobic order *Actinomycetales (*Phylum *Actinobacteria)* and the anaerobic order clostridiales (Phylum Fermicutes). Fungal cellulose utilization is distributed across the entire kingdom, from primitive, protilst like chytridomycetes to the advanced Basidiomycetes (Shewale and Sadana, 1978; Lee *et al*., 2002).

2.10 **Fungal Enzymes**

Many investigations have been carried out in the area of fungal enzymes and so various fungal species have been implicated in the production of various enzymes. Cellulase enzymes have been reported produces by *Talaromyces emersonii* (Oso, 1978), *Sporotricum thermophile* (El- Naghy *et al*., 1991), *Penicillium purpurogenum* (Steiner *et al*., 1994) *Arthrographis* sp (Okeke and Obi 1993) and *Basidiomycetes* sp (Shewale and Sadana, 1978). Fungi have also been reported as producers of various types of amylase enzymes (Oso, 1979; Sani *et al.*, 1992; Carolina *et al.*, 1999).

Production of cellulase by mixed culture of *Aspergillus niger* and *Saccharomyces cerevisae* using sorghum pomace as nutrient source was investigated. Sorghum pomace was further supplemented with mineral elements to evaluate its effect on cellulase production. All the sorghum pomace media recorded significantly ($p<0.05$) higher level of cellulase enzyme (2.06-4.06unit/ml) than that of carboxylmethylcellulose medium (1.72unit/ml). Mixed culture fermentation significantly ($p<0.05$) enhanced higher cellulase production than mono culture media. However, mineral supplementation significantly ($p<0.05$) suppressed cellulase production. Cellulosic substrates were more susceptible to crude enzyme from sorghum pomace than that of carboxylmethylcellulose (Abu, 2004). Roberto *et al*. (2005) also reported the production of cellulase and xylanase by *Thermoascus aurantiacus* in solid state fermentation using different agricultural residues (wheat

bran, sugarcane bagasse, orange bagasse, corncob, green grass, dried grass, sawdust and corn straw) as substrates without enrichment of the medium and characterized the enzymes.

T. aurantiacus was more xylanolytic than cellulolytic. The highest levels of enzymes were produced in media containing corncob, grasses and corn straw. The production of cellulase by mutant strain of *Trichoderma reesei* M-7 using batch and continuous cultivation and in the presence of lactobionic acid, gluconic acid, delta-gluconolactone, lactulose and sorbitol was investigated. Lactobionic and lactulose produced positive and better result as compared to lactose because of low utilization of the two cellulase inducers (Janas *et al*., 2002). While *Arthrographis* sp. F4 produced cellulolytic and xylanolytic enzymes at 30^0C and pH 5.0 to 6.0 (Okeke and Obi, 1993), *Penicillium purpurogenum* preferred 28^0C for growth to produce cellulase maximally (Steiner *et al*., 1994). Highest endoglucanase activity was observed using Sigmacell as carbon source and corn steep liquor as nitrogen source. The crude enzymes were stable up to 50^0C and between pH 4.4 to 5.6 for 48h.

2.11 **Bacteria**

Similarly, the production of cellulase and amylase enzymes by different bacteria species have been investigated and reported by various researchers. Bacteria constitute the most abundant group of microorganisms. In normal fertile soils, 10-100 million bacteria are present per g of soil(Vollmen *et al*.,1977; Kennedy and Papendick, 1995). This figure may increase depending on the organic matter content of any particular soil. The bulk of soil bacteria are heterotrophic and utilize readily available source of organic energy from sugars, starch, cellulose and protein. On the other hand, autotrophic bacteria which occupy a smaller portion of the biomass in soil and use inorganic sources such as iron (*Ferrobacillus*) and sulphur (*Thiobacillus*) are not directly involved in organic matter decomposition.

Actinomycetes are one of the diverse groups of soil bacteria possessing commercially useful enzymes and therapeutically useful bioactive molecules, Actinomycetes are aerobic, Gram-positive bacteria, which may form branching filaments or hyphae that may persist as a stable mycelium or may break up into rod-shaped or coccoid elements. Actinomycetes are widely distributed in terrestrial environment (Agrawal, 2003). For much of this time they were regarded as an exotic group of organisms with affinities to both bacteria and fungi. The actinomycetes

comprise a ubiquitous order of bacteria, which exhibit wide physiological and morphological diversity. Actinomycetes have long been a source of commercially useful enzymes and therapeutically useful bioactive molecules. Actinomycetes biodiversity is vast frontier and potential goldmine for the biotechnology industry because it offers countless new genes and biochemical pathways to probe for enzymes, antibiotics and other useful molecules (Agrawal, 2003).

The number of actinomycetes may be as high as 200 million per g of soil and may increase in manured soils. Thermophilic (tolerating 50 to 65^0C) forms are not uncommon in compost piles. Actinomycetes grow on complex substances such as keratin, chitin and other complex polysaccharides and thus play-an active role in humus formation. Soil fungi are mostly heterotrophs and use organic residues easily but their numbers vary in soil depending on whether a species has a dominant vegetative or reproductive phase in the soil environment. Sporulating fungi such as *Mucor, Penicillium* and *Aspergillus* appear on agar plates rather profusely than non-sporulating ones. Soil algae in cultivated soils vary greatly in numbers and may contribute a small amount of organic matter through their biomass but they do not have any active role in organic matter decomposition. The end products of decomposition are CO_2, H_2O, NO_3, SO_4, CH_4, NH_4, and H_2S depending on the availability of air.

Over the past 60 years, products derived from microbial secondary metabolites have been used to meet medical, industrial and agricultural needs, e.g., antibiotics, anticancer drugs, antifungal compounds, immunosuppressive agents, enzyme inhibitors, anti parasitic agents, herbicides, insecticides and growth promoters (Busti *et al*., 2006). In a study to use of microorganisms for conversion of plant biomass into many commercially valuable products two of the isolates, identified as *Streptomyces* sp. and *Pseudomonades* sp.were able to degrade wheat stover and saw dust lignin and polysaccharides. The growth rate of *Streptomyces* sp. and Pseudomonades sp. was higher in wheat stover fed cultures than the saw dust fed cultures. Results also showed that bacteria treatment of lignocelluloses and nitrogen supplementation of culture media had considerable effects on chemical composition of wheat stover and saw dust. *Streptomyces* showed more degradation ability than *Pseudomonades*, especially in culture media containing wheat stover. The utilization of yeast extract (as nitrogen source) improved degradation abilities of bacteria (Borjil, 2006). Pseudomonades have been identified to be of importance in bioremediation as a result of their high capacity for biodegradation. *Pseudomonas* species have been used by Premalatha and

Rajakumar (1994), Kiyohara *et al*. (1992) and Farrell and Quilty (2002) for the biodegradation of a variety of chlorophenolic compounds.

Production of amylase by different microorganisms has been reported *Bacillus cirulans* (Kwan *et al.*, 1993) *Halobacterium halobium* (Patel *et al.,* 1993). *Clostridium* spp. (Kingerberg, 1990) *Aeromonas hydrophila* (*Lokendra et al., 1994*) and (Carolina *et al*., 1999) and *Fervidobacterium* sp. (Cangenella *et al*., 1994). Similarly *Thermoactinomyces thalpophilus* isolated from flour mill wastes was found to produce extracellular amylase in shake flask cultures using sorghum as carbon source. Sorghum in mineral salts medium significantly supported a higher rate of amylase synthesis by the organism than soluble starch, giving peak amylase activity at the stationary phase. The optimum temperature and pH of the enzyme was 90^0C and 5·0, respectively, with more than 50% enzyme activity retained at 100^0C (30min) (Uguru *et al*., 1997)

Cellulase enzymes have also been documented to be produced by Rumen bacteria (Ronald and Peter, 1982) *Celivibrio mixtus* (Fontes *et al.,* 1997*)* and *Thermoproteus tenax* (Carolina *et al.,* 1999). A bacterium identified as *Bacillus brevis* has been isolated from soil. It has been found to secrete cellulase extracellularly whose production increased almost five times on addition of galactose in the culture medium (Singh and Kumar, 1998). Production of cellulase has been found at optimal at pH 5.5, 37^0C and 175rpm speed using environmental orbital shaker. The cellulase was purified using ultrafiltration and Sephadex G-200 column chromatography. The native molecular weight of the enzyme was found to be 33,000 ± 2000 using Sephadex G-200 gel filtration chromatography. The subunit molecular weight (33,000 ± 2000) indicate monomeric nature of the enzyme, the enzyme showed Michaelis Menten kinetics exhibiting Km~1.7 ± 0.1 mg/ml for CMC. The enzyme activity was inhibited by heavy metals like Hg^{+2} and Ag^{+2} (Singh and Kumar, 1998).

Another bacterium, strain CB4, which produced an extracellular enzyme with strong hydrolyzing activity on crystalline cellulose, was isolated from brewery sewage. Strain CB4 was identified as *Cellulomonas uda* from its taxonomical characteristics. However, it differed greatly from other Cellulomonas strains, including the type strain of *Cellulomonas uda*, in production of a cellulolytic enzyme (Nakaruma and Kitamura, 1982). The maximal activity for hydrolyzing crystalline cellulose was obtained by cultivation of CB4 in medium containing crystalline cellulose and Polypepton at 30^0C for 2 days. Activities for hydrolyzing crystalline cellulose, CM-cellulose and cellobiose were detected in the culture filtrate. The optimum pH and temperature for

the crystalline celulose hydrolyzing activity were pH 5.5 and 45-50^{0}C, respectively, and the enzyme was activated by Mn<2+>, Co<2+> and Ca<2+>.(Nakamura and Kitamura, 1982).

An endoglucanase was purified from halophilic alkaline *Bacillus licheniformis* isolated from soil (Aygan *et al*., 2011). The optimal pH and temperature of the endoglucanase produced by *B. licheniformis* C108 were 10.0 30^{0}C, respectively. The enzyme was highly stable up to100^{0}C at pH 10.0 and the enzyme exhibited its complete activity for 6h in 7 to 10% NaCl. The activity of the enzyme was significantly inhibited by sodium dodecyl sulphate (SDS), Triton X-100, zinc chloride ($ZnCl_2$), Phenylmethanesulfonylfluoride (PMSF) and Urea. The partially purified enzyme revealed that, products of carboxylmethylcellulosic hydrolysis were glucose, cellobiose and other longer cellooligosaccharides. Thermostability, alkalinity, halostability and highly hydrolytic capability make enzyme a potential candidate for environmental bioremediation and bioethanol production processes from cellulosic biomasses as well as waste treatment processes (Aygan *et al.*, 2011).

In another study to determine the production of hydrolytic enzymes in rice (*Oryza sativa* L.) root by N_2-fixing bacteria, nine out of twelve bacterial strains were positive for carboxylmethylcellulose (CMC) and pectin reactions (Asilah *et al*., 2009). The bacterial population and the production of hydrolytic enzymes were monitored for 45 days of plant growth. The scanning (SEM) and transmission electron microscopy (TEM) were used to observed bacterial colonization on plant roots. Asilah *et al*. (2009) observed that the population of inoculated diazotrophs were higher in the rhizosphere than the endosphere. There were significant effects of different diazotrophs inoculations on the rice rhizosphere and endosphere populations. Plants inoculated with diazotrophs showed significantly higher specific enzyme activities and soluble proteins compared to non-inoculated control.

Abdelnasser *et al*. (2007) also isolated three thermophilic cellulase producing bacteria, *Geobacillus thermodenitrificans, Geobacillus stearothermophilus* and *Anoxybacillus flavithermus* from soil. Maximal cellulases production by *Anoxybacillus* was detected at the end of the stationary phase (36 h). The crude cellulase had activity towards avicel, CMC, cellobiose, and xylan. It was also reported that the rate of CMC degradation was higher than any other substrates used. The optimum temperature and pH for the crude enzyme activity was 75^{0}C and 7.5 respectively. A thermophilic *Clostridium* species was also isolated from manure. The organism degraded filter

paper in 1 to 2 days at 60^0C in a minimal cellulose medium but was stimulated by yeast extract. The organism fermented a wide range of sugars but produced cellulase only in cellulose or carboxylmethylcellulose media. The cellulase synthesis and activity were repressed and inhibited respectively by 0.4% glucose and 0.3 % cellobiose (Lee and Blackburn, 1975).

In an attempt to optimize the growth and cellulase production by *Bacillus alcalophilus* S39 and *Bacillus amyloliquefaciens* C2, Abou-Taleb *et al*. (2009) discovered that 1% carboxylmethylcellulose (CMC) and 0.7% yeast extract were most effective as the carbon and nitrogen sources respectively for the two organisms. Initial pH 7 and 3% inoculums size were found to be optimal for growth and cellulase production. However, the incubation temperature to achieve the highest activity of the enzyme was 30^0C and 45^0C for *Bacillus alcalophilus* S39 and *Bacillus amyloliquefaciens* respectively.

In studying microbial enzymes production in the laboratory, reports have it that submerged culture in shake flasks have been a recent and reliable technique. The method of separation of the microbial cells and the crude enzymes depends on the method of enzyme production by the microorganisms involved. If the enzyme is formed extracellularly, the separation method is by filtration in the case of a fungus and centrifugation when a bacterium (Oso, 1979; Lealem and Gashe 1994). Again, if the enzyme production is intracellular, the cells of organisms are washed in a suitable buffer and ground with a suitable abrasive material. This helps to break the cells - bound enzymes free into the surrounding. This is then followed by filtration or centrifugation (Lealem and Gashe, 1994).

2.12 **Purification**

The methods of extraction and purification form the major steps in the large scale production of microbial enzymes in general. Then the degree to which the enzyme is purified depends both on the economic factor and on the intended application (Sen and Charkrabarty, 1986). Purification of enzymes is done in various ways which include ammonium sulphate precipitation, gel filtration and ion ex-change chromatography.

Generally, organisms secrete the enzyme directly into the growing medium from where they can be purified by filtration or centrifugation (Ali *et al*., 2002; Immanuel *et al*., 2006; Skowronek and Fiedurek, 2006)

The growth medium, may have other undesirable products of the microorganisms during metabolism in addition to the desired enzyme fraction. The culture may contain a long list of different enzymes and some toxic chemicals produced by the organisms. It then becomes important to purify the enzyme to serve various purposes. The purified enzyme will have higher activity, less risk of hazardous substances and ultimately better application in the specific product. The process of purification is more obligatory in case when the enzyme is to be applied in the products meant for human and animal consumption. Moreover it is important to conduct safety trial on animals modeling prior to the utilization of such enzyme treated products (Bhardwaj and Grag, 2010).

Purification of cellulase from *B. pumilus* EB3 using ion exchange chromatography showed that 98.7% of total CMCase was recovered (Ariffin *et al*., 2006). Protein separation was, however, based on subtractive separation where the contaminants were bound to the column instead of CMCase. Characterisation of the enzyme showed that CMCase from *B. pumilus* EB3 has a molecular weight range from 30-65 kDa and was optimally active at pH 6.0 and temperature 60^0C. The CMCase also retained its activity over a wide pH range (pH 5.0–9.0) and temperature range (30-70^0C).

Partial purification to increase cellulase activity was performed using anion exchange and gel filtration chromatography. One major and one minor band of activity were identified subsequently by SDS-PAGE and zymography. The molecular weight of the major band was estimated at 40 kDa while the minor band was estimated at 30 kDa. Redclaw cellulase enzymes demonstrated broad substrate specificity, hydrolysing polysaccharides containing β-1,4 and mixed β-1,4 and β-1,3 glycosidic bonds but showed a preference for soluble substrates. Hydrolysis products of cellodextrins of various lengths also showed that the enzymes liberated free glucose(Xiao *et al*., 1999).

The purified enzyme of *Bacillus subtilis* ASH revealed a single band on SDS-PAGE gel with a molecular mass of 23kDa. It showed an optimum pH of 7.0 and was stable over a range of 6.0-9.0. The optimum temperature for activity was 55^0C. The purified xylanase did not lose any activity up to 45^0C, however, it retained 80% and 51% of its activity after pre-incubation at 55^0C and 60^0C respectively. The enzyme obeyed Michaelis-Menton kinetics towards birch wood xylan with apparent K_m3.33mg/ml and V_{max}100IU/ml. The enzyme was strongly inhibited by Hg^{2+} and

Cu^{2+} while enhanced by Co^{2+} and Mn^{2+}. The purified enzyme could be stored at 4^0C for six weeks without any loss of catalytic activity (Ashwani *et al.,* 2010).

For the sake of purification and characterization, Chen *et al*. (2004) produced CMCase employing *Sinorhizobium fredii* CCRC15769 and optimized the growth condition of the cultural medium. Maximum cell growth and specific activity of the CMCase was observed using BIII medium with 0.3% myo-inositol. The purification was carried out by using DEAE Sepharose anion-exchange column, and Phenyl-Sepharose column. CMCase activity at the fraction of 94kDa was observed by SDS-PAGE. The characterization of CMCase demonstrated the optimal condition of temperature 35^0C and pH 7.0. They reported about 9.08 fold purification, with specific activity 3.822U/mg and 26.4 recovery yield.

For cellulase production, Eriksen and Goksqyr (1977) cultivated *Chaetomium thermophile* var. dissitum in a liquid medium with cellulose. Three pure components were obtained after concentration of culture filtrate and then subjecting to ion-exchange chromatography and gel filtration. One of these was endoglucanase (CMCase) that caused rapid decrease in the viscosity of CMC solutions but had low effect on native cellulose. When mixed, the three components showed synergistic effect on highly-ordered cellulose. The workers characterized endo- and exoglucanases for molecular size and iso-electric point (pl). The cellulases had pl near 4.5, but had different molecular weights: 67kDa (exoglucanase) and 41kDa (endoglucanase).

Enzyme-producing microorganisms (fungi and bacteria) have been found and isolated from various types of habitats, including soil (Milward - Sadler *et al.,* 1995; Fontes *et al.,* 1998), hot spring (Moreland *et al.,* 1995), decomposed substrates (Singh *et al*., 1998) and rumen (Teather and Wood 1982*;* Gowda *et al.*, 1997*;* Thierry and Rachel., 2000*).*Thermophilic and extremely thermophilic (hyperthermophilic) microorganisms are involved in the production of various enzymes- amylase, pullulanase, Xylanase, cellulase and so on and the sources of these microorganisms are diverse in nature (Klingerberg *et al.,* 1990*;* Koch *et al.,*1991*;* Jin *et al,* 1992*;* Canganella *et al.,* 1994). The enzymes documented to have been produced include thermostable ∝- amylase by *Bacillus* sp-JK Strain (Jin *et al.,* 1992*),* hyperthermoactive ∝- amylase by *Pyrococcus woesei* (Koch *et al.,* 1991) amylolytic and pullulytic by thermophilic archaea and *Fervidobacterium sp* (Cangenella *et al.,* 1994), cellulase by *Sporotrichum thermophile* (El- Naghy *et al.,* 1991*).*

2.13 **Thermophilic microorganisms**

Thermophiles are microorganisms that live and grow at the extreme hot environments that would kill most other microorganisms. Thermophiles are grouped into either prokaryote or eukaryotes, and these two groups of extremophiles are classified in the groups of archaea. Thermophiles either live in geothermal habitat or in environments that create heat themselves. Piles of compost and garbage landfill are two examples of environments that produce heat on their own.

Thermophilic organisms grow optimally between 50^0C and 80^0C. They will not grow if the temperature reaches 20^0C. Their enzymes (thermophilic enzymes) show thermostability properties which fall between those of hyperthermophilic and mesophilic enzymes. These thermophilic enzymes are usually optimally active between 60^0C and 80^0C. Active at high temperatures, thermophilic and hyperthermophilic enzymes typically do not function well below 40^0C (Claire and Gregory, 2001).

2.14 **Thermoenzymes**

Enzymes isolated from thermophiles or hyperthermophiles are called thermoenzymes. They found applications in biotechnology where their unusual thermostability makes them uniquely qualified for tasks under conditions that would easily denature mesophilic enzymes. Extremely high thermostability can be achieved in some proteins through a combination of van der waals, H-bond and electrostatics interaction.

Enzymes synthesized by thermophiles and hyperthemophiles are known as thermoenzymes. These enzymes are typically thermostable, or resistant to irreversible inactivation at high temperature, and thermophilic, i.e. optimally active at elevated temperature between 60^0C and 125^0C (Li *et al*., 2005). Enzyme thermostability encompasses thermodynamics and kinetic stability. Thermoezymes offer the following major biotechnological advantages over mesophilic enzymes: 1. They are easier to purify by heat treatment; 2. Have a higher resistance to chemical denaturants like solvent and guanidinium hydrochloride; and 3. withstand

higher substrate concentration. Because of their stability at elevated temperature, thermoenzyme reactions are less susceptible to microbial contamination and often display higher reaction rate than mesoenzymes catalyzed reactions, hence for these advantages thermoenzymes are attracting much industrial interest (Becker *et al*., 1997).

Reseachers have found no new amino acids, covalent modification and structural motifs that explain the ability of thermoenzymes to function at elevated temperature (Fields, 2001). Thermostability seems to be a property acquired by a protein through a combination of many small structural modifications that are achieved with the exchange of some amino acids for others and the modulation of the canonical forces (e.g.) hydrogen bonds, ion-pair interactions, hydrophobic interactions) found in all proteins (Scandurra *et al*., 1998).

Significant increase attentions are being drawn to cellulases because of their uses in production of biofuel from cellulosic materials than corn kernels (Potera, 2006). Addition of cellulase in the poultry feed had positive effects on the body performance of the experimental birds. Treatments had significant effect on the body weight, carcass weight and chest muscles. The increased body weight after the enzyme addition was due to high feed intake and ultimately better feed conversion ratio. The addition of enzyme leads to better utilization of feed and nutrients resulting in more weight gain and ultimately the carcass weight (von Wettstein *et al*., 2000) Addition of cellulase reduced dough development time (Haros *et al*., (2002a). Diaconescu, (2006) also reported increased in bread volume after enzymatic treatment of the flour. The results are also in harmony to the findings of similar result observed by Harada *et al*., (2005) who prepared breads with volume up to 1050 cm3 by CMCase treatment of flour. Laurikainen *et al*., (1998) recorded an increase of 18-19% in the bread specific volume by treating the flour with CMCase and hemicellulase. Correa *et al*., (1997) also found pronounced effect of the CMCase on the volume of bread made from flour with 10% wheat bran addition.

Enzymatic treatment of pulp significantly reduced the pulp viscosity, this was reported earlier by Carneiro *et al*., (2002); they observed reduced viscosity and the suspended solids (pulp) in the hydrolyzed juice. Conversion of cellulose to glucose by enzymatic hydrolysis helps in production of sugars, single cell proteins, fuels or raw materials for the chemical industry (Narasimha *et al*., 2006). Cellulolytic enzymes are equally used in the treatment of municipal solid wastes to useful products (Mandels *et al*., 1974; Vasudeo *et al*., 2011; Shabnam *et al*., 2011).

Recently cellulase has come to attract serious attention from the standpoint of efficient utilization of biomass resources and consequently become a subject of study. The cellulase produced by the strains of genus *Trichoderma* and genus *Aspergillus* which have won widespread recognition still suffer from various drawbacks such as

exhibiting insufficient hydrolyzing power upon native cellulose, falling to produce complete hydrolysis of cellulose to glucose, and giving rise to large amount of cellobiose and cello-oligosaccharides. Similarly since virtually most types of cellulase known have deficient in thermal stability, they can barely withstand prolong saccharification at temperature of 45^0C to 50^0C. The reacting mixture being saccharified with such cellulase is likely to be contaminated during the course of the reaction. Hence, there is a need to search for thermophilc bacteria with ability to produce thermostable cellulase with higher thermal stability.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Collection of samples

Sawdust samples were collected from sawmill dumpsites at Oke-Gada, Ede, Osun State Nigeria. Lat.(N 07.44.027) Long.(E 004.25.441). The sites were dug to depths of 5cm, 10cm, 15cm, 20cm, 25cm, 30cm, 35cm, 40cm, 45cm, 50cm, 55cm, 60cm, 65 and 70cm with soil auger. The samples were collected from the different depths into different clean 250ml conical flasks and properly labelled. The mouth of the conical flasks were sealed with cotton wool and transported to the laboratory.

3.2 Isolation and culture methods

3.2.1 Preparation of medium for the isolation of cellulase-producing bacteria

The medium used was compounded according to Natalie (2002). The composition of the broth medium include: 10g of Carboxyl Methyl Cellulose, 10g of Tryptone, 10g of Agar- Agar, 1g of yeast extract 0.5g of Di-potassium hydrogen phosphate (K_2HPO_4) and 1000ml of distilled water.

Each of the components was weighed into 1000ml conical flask except Agar-Agar for broth medium and 1000ml of distilled water was added with the aid of measuring cylinder. The flask was placed on magnetic stirrer / hot plate to form homogenous solution. 100ml of the Natalie's broth was dispensed into each of four 250ml conical flasks, sealed with cotton wool and wrapped with aluminum foil. The media were sterilized at 121^0C for15minutes.

3.2.2 Inoculation of Samples

Half gram of the sawdust sample from each of the depth was weighed into each of the conical flasks containing 100ml Natalie's broth and was incubated at 50^0C for 48hours.

Thereafter serial dilution was carried out from this sawdust culture (Harrigan and McCance,1966). 0.1ml of higher dilutions were withdrawn with the aid of sterile pipette and spread on the solid Natalie medium aseptically. The plates were incubated at 50^0C for 5 days.

3.2.3 **Characterization of Isolates**

3.2.3.1 **Morphological characterization**

After the period of incubation, the plates were observed for growth. The colonies on those plates having different morphological properties including colour, form, elevation and margin were selected for sub-culturing to get pure cultures.

They were subcultured on solid Natalie's agar plate until pure isolates were obtained. The pure cultures were then put on slant and stored at 4^0C in the refrigerator for further studies. The isolated bacterial colonies were further characterized for their morphology using Gram`s staining.

Micrscopic examination

Gram Staining Techniques

A sterile wire loop was used to make a smear of the culture on a clean glass slide labelled with each isolate code and heat – fixed.

The smear was flooded with crystal violet for 60 seconds and rinsed off with the gram iodine solution and allowed the iodine was allowed to act for 1 minute. Then the smear was decolourized with 95% alcohol (ethanol) for only 30 seconds and immediately rinsed off in running tap water to remove the alcohol effect.

The slide was counterstained with carbol-fulchin for 30 seconds after which it was rinsed with water and then blotted dried with Whatman filter paper by Whatman. Gram positive cells were purple colour, since they were not decolourized by alcohol and retain the purple colour. Gram-negative cells were pink. This is because alcohol treatment removed the crystal violet complex.

3.2.3.2 **Biochemical characterization**

1. Triple Sugar Iron (TSI) Medium

Triple sugar iron (TSI) agar tube slants were inoculated by stabbing the butts and streaking the slopes of the agar with an inoculating needle and loop respectively. Then the culture tubes were incubated at 50^0C for 48hours. Acid and/or gas in the butts

and slants and blackening of the medium, which is an indication of Hydrogen sulphide production observed were recorded for each of the isolates.

2.Sulphide – Indole – Motility Tests

SIM agar is a semi – solid medium prepared to examine the motility, indole and hydrogen sulphide productions. Agar deep in test - tubes was aseptically stab and inoculated using sterile inoculating needle already charged with the isolate broth culture. The culture tubes were then incubated at 50^0C for 48 hrs. After incubation period, the culture tubes were examined for motility, which was indicated by diffuse growth away from the point of stab in the medium and hydrogen sulphide production.

For indole production, 1 ml of chloroform was first added to each test tube and left to act for 15 minutes, thereafter 1ml of Kovac's reagent was added and then examined for red / rose colour development within 10 minutes as an indication of indole production.

3. Catalase Test

The 0.5ml of the broth culture was aseptically distributed to each of the small test - tubes. Serological pipette was then used to add 0.5ml hydrogen peroxide (10 volume concentration) with varying effervescence of gas, which is the indicative of the presence of catalase. This was therefore examined and recorded.

4. Citrate Utilization Test

The slant and butt portion of the citrate medium was streaked and stab inoculated with each of the organisms broth culture in Bijou bottles respectively. This was incubated at 50^0C for 7 days and then observed for colour change from green to blue as citrate positive. There was no change of colour in negative test.

5. Methyl Red And Voges – Proskauer (MRVP) Test

This test helps to differentiate between the coliforms and the aerogenes bacteria. The VP test is a test for the production of acetoin and this may be tested for in the same as used for MR test. Each broth culture in the test tubes containing the inoculum was aseptically divided into two portions and labelled M and V respectively.

Five drops of methyl red solution was added into each of the test – tubes labelled M and examined for colour change. A red colour showed positive reaction and yellow colour indicated negative reaction. Also, 0.5ml of 6% alpha – naphtol solution was added into each of the test – tubes labelled V. Then 0.5ml of 16% potassium

hydroxide (KOH) was also added and the test – tubes were properly agitated. The development of a red and yellow colouration indicated a positive and negative reaction respectively.

6. Nitrate Reduction Test

Many microorganisms are capable of reducing nitrate to nitrite or even further to hydroxylamine, ammonia or nitrogen. The organism for amino acid synthesis uses these end products. A loopful of the peptone broth culture was inoculated into tubes containing sterile nitrate medium with inverted Durham tubes and incubated at 50^0C for 5 days.

After this, 1ml each of Gries's reagent was added to the culture medium. Pink, red or maroon colour showed the presence of nitrite and recorded as positive. There was no change of colour in negative test. The presence of gas in the Durham tube indicated the formation of gaseous nitrogen and therefore completes reduction of nitrate.

7. Oxidation – Fermentation Test

Bacteria, which attack carbohydrates either, do so aerobically (i.e. oxidatively) or anaerobically (i.e. fermentatively). The basal medium (Hugh and Leifson's medium) was employed and sterilized by autoclaving with an inverted Durham tube. Then the sterile dextrose solution was added aseptically to the sterile molten basal medium to give a final concentration of 1% and the tubes were inoculated with a straight wire previously charged with the inoculum.

Sterile liquid paraffin was added to a thickness of 1cm to one set of test – tubes meant for each isolate, while the other set was left uncovered with sterile liquid paraffin. The culture tubes were incubated at 50^0C for 5 – 7 days and then examined for gas production and colour change in each culture medium. Fermentative organisms produced acid in both tubes, while oxidative organisms produced acid in the open tube only. Acid production was shown by a change in the colour of the medium from blue to yellow.

8. Sugar Fermentation

This test was used to show the ability of the bacteria to ferment some sugar, which include glucose (monosaccharide), lactose (disaccharide), sucrose (disaccharide), maltose (disaccharide) and mannitol (an alcoholic sugar).

The basal medium of peptone water containing 1.0% of fermentable substrate was prepared and 1% Andrade's indicator was added.

The dissolved medium was dispensed into 5 test tubes containing Durham tube in inverted position before autoclaving. The sterile medium was inoculated with broth culture with desired bacteria and the tubes were incubated at 50^0C for 5 days. The culture tubes were observed for gas production, acid production or both.

3.2.3.3 **Identification of bacterial Isolates**

The isolated bacteria were biochemically characterized using sugar fermentation tests, oxidase, catalase and obtained results were used for identification with reference to Bergey`s Manual of Systematic Bacteriology (Sneath, 1986). The isolates were then identified based on the results of Gram`s reaction and biochemical tests.

3.2.3.4 **DNA extraction from the bacterial isolates for molecular identification**

Isolates were molecularly identified by 16S rDNA gene sequence analysis. 1.5ml of broth culture of the isolates was spinned at 13,000rpm for 30min and the supernatant was decanted. 400µl of CTAB buffer was added, 75µl of 10% SDS was also added to the residue. The mixture was put into a water bath at 65^0C for 30min. It was then left for some time to cool. 10µl proteinase K was added and incubated for 30inm at 37^0C. After the incubation, 500µl chloroform was added and mixed for 5min. It was spinned at 16,000rpm for 10min. The supernatant was collected in eppendorf tube. 500µl isopropanol added to the supernatant, kept at -20^0C for 1hr and spinned at 10,000rpm for 10 min. Supernatant was then decanted gently and residue washed with 70 % ethanol, dried for 1hr and re-suspended in 200µl sterile water.

Amplification of the Extracted DNA

The purified DNAs of the pure cultures of *Roseomonas* sp. A1, *Anoxybacillus rupiensis* E1 and 5H were amplified with primers 518f (5`-CCAGCAGCCGCGGTAATACG-3` and 800r (5`-TACCAGGGTATCTAATCC-3` spanning V3 region of the 16S rDNA (Ǿvreas *et al*., 1997). Ampilification of the 16S rDNA was carried out in Eppendorf thermal cycler (Mastercycler Personal Model, Fisher Scientific, UK) using the PCR method described by Ampe *et al*. (1999). Each mixture (final volume, 25 µl) contained 1µl of template DNA, each primer at a concentration of 0.25 µM, each deoxynucleotide triphosphate at concentration of 200

µM, 1.5mM $MgCl_2$, 2.5 µl of 10 x PCR buffer, and 1.25U of *Taq* (Eurogentic). Template DNA was denatured for 5min at 94^0C. To increase the specificity of amplification and to reduce the formation of spurious by-products, a "touchdown" PCR was performed. The initial annealing temperature used was 10^0C above the expected annealing temperature (65^0C), and the temperature was decreased by 1^0C every second cycle until the touchdown temperature, 55^0C was reached; then 10 additional cycles were carried out at 55^0C. Primer extension was carried out at 72^0C for 3min. The tubes were then incubated for 10min at 72^0C (final extension). Aliquots (2µl) of the amplification products were analyzed first by electrophoresis in agarose gels.

The PCR products were then analyzed by denaturing gradient gel electrophoresis (DGGE) by using Bio-Rad DCode apparatus. Samples were applied to 8% (wt/vol) polyacrylamide gel in 1x TAE. The optimal denaturation gradient was determined by electrophoresing a perpendicular gel. Optimal separation was achieved with a 30 to 60% urea-formamide denaturing gradient (100% corresponded to 7M urea and 40% [vol/vol] formamide). All parallel electrophresis experiments were performed at 60^0C by using gels containing a 30 to 60% urea-formamide gradient increasing in the direction of electrophoresis. The gels were elecrophoresed for 10min at 20V and for 3h at 200V, stained with ethidium bromide for 10 to 15min, and rinsed for 20 to 30min in distilled water.

Sequencing Analysis

Sequencing was performed using the primers for PCR products. Sequencing was determined by the dideoxy chain termination method. The sequencing reaction mixture (20 µl) contained 6 µl of 2.5 × sequencing buffer, 2 µl of Terminator reaction mix (Big dye version 1.0), 1 µl of primers, 5 µl Template DNA and 6 µl of Nuclease-free water. The amplification program was 25 cycles of 96 ^{0}C for 15s, 55 ^{0}C for 15s and 60 ^{0}C for 4min.The DNA molecules were precipitated from the sequencing mix and re-suspended in 25 µl deionized formamide. The closest relatives of 16S rDNA sequences were determined by a search of the GenBank DNA database using the BLAST algorithm (Altschul *et al.,* 1990). Identities of isolates were determined on the basis of the highest score.

3.2.3.5 **Maintenance of stock cultures**

The cultures of the isolates were maintained on the Natalie medium by subculturing of organisms every two weeks and storage in refrigerator at 4^0C.

3.3 **Screening for Cellulase Activity**

. The pure colonies were screened for their cellulase activity using modified method of Teather and Wood (1982). A discrete colony from the pure culture was transferred individually by stabbing on the set prepared Natalie's agar plate and incubated at 50^oC for 48 hours. After incubation, there were colonies on the plates, the plates were flooded with 1% Congo – red solution and were allowed to stand for 20 minutes. The Congo – red solution was then poured off, the plates flooded with 1M Sodium chloride (NaCl) solution and allowed to stand for 20 minutes at room temperature and the sodium chloride solution was poured off. A clear zone was shown around the growing colonies of cellulase positive cultures against the dark red background, the unreacted cellulase taking up the stain while hydrolyzed portion remained colourless. The plates were further flooded with 1M Hydrochloric Acid solution for 20 minutes to enhance the contrast. The bacteria that were of good clearance beyond the growth were selected and stored on slant for further studies as potential cellulase secretors.

3.4 **Physiological Studies of the Isolates**

3.4.1 **Effect of pH on the Growth of Isolates**

The growth medium consisted of 1g of yeast extract, 10g of tryptone, 10g of carboxyl methyl cellulose (CMC) and 0.5g of K_2HPO_4. Each component was weighed into a sterile conical flask and 1000ml of distilled water was added.

Five milliliters of the medium was dispensed into each of sixty McCartney bottles. Triplicates were adjusted to each of the following pH (4, 5, 6, 7, 8 and 9) using 0.1M dilute hydrochloric acid for pH 4, 5 and 6 while 0.1M dilute sodium hydroxide was used for pH 7, 8 and 9 (Okeke and Obi, 1993). The bottles and the content were sterilized at 121^0C and 1.05Kgcm^2 for 15minutes. After cooling the bottles were inoculated with 0.5ml of 48hrs broth cultures of the isolates, adjusted to O.D. 0.3 at 540nm and incubated at 50^0C for 48hrs. Uninoculated bottles served as control. Growth measurement was determined at 540nm using Lab-Tech digital colorimeter.

3.4.2 **Effect of Temperature on the Growth of Isolates**

Natalie`s medium was prepared as previously described. Five milliliters of the medium was dispensed into each of the sixty McCartney bottles, autoclaved and inoculated with 0.5ml of 48hrs broth cultures of the isolates adjusted to O.D. 0.3 at 540nm. Inoculated bottles in triplicates with same content were incubated at temperatures of 45^0C, 50, 55 and 60^0C for 48hrs. Uninoculated bottles served as control. The growth measurement was determined at optical density 540nm using Lab – Tech digital colorimeter.

3.4.3 **Effect of Carbon Source on the Growth of the Isolates**

Natalie's broth was prepared by substituting the sugars (galactose, Lactose, sorbitol and glucose,) for carboxyl methyl cellulose (CMC). Five milliliter of the broth of this carbon source was dispensed into different McCartney bottles sterilized at 121^0C for 15 minutes and allowed to cool to 45^0C in a water bath. After cooling, they were inoculated with 0.5ml of the isolates adjusted to O.D. 0.3 at 540nm and incubated at 50^0C for 48 hours. The growth measurement was determined at optical density of 540nm using Lab – Tech colorimeter.

3.4.4 **Effect of different Carbon sources on cellulase production**

Different carbon sources were investigated for their effect on cellulase induction by replacing the carboxymethylcellulose in the medium for cellulase production with galactose, lactose, sorbitol and glucose. The carbon sources were added at 1.0% concentration. Ten milliliters of the medium was dispensed in McCartney bottle in duplicate, autoclaved and inoculated with 1ml of 48hours culture of the isolates adjusted to O.D. 0.3 at 540nm. The bottles were incubated at 50^0C for 48hours. The uninoculated served as control

3.4.5 **Effect of Different Nitrogen Sources on Growth of the Isolates from Sawdust**

Natalie`s broth was prepared by substituting its nitrogen source with 1% (weight per volume) of ammonium nitrate, sodium nitrate, potassium nitrate, yeast extract and tryptone, with the pH adjusted appropriately.

Five milliliters of the basal medium was dispensed into McCartney bottles in triplicate. The bottles were autoclaved at 121^0 C and 1.05Kg/cm ^{2} for 15minutes. They were inoculated with 0.5ml of a 48hrs bacterial inoculums, adjusted to O.D. 0.3 at

540nm, using a sterile pipette. The bottles were incubated at 50^0 C for 48hrs. The growth measurement was estimated at 540nm using a Lab-Tech Digital colorimeter.

3.4.6 **Effect of different nitrogen sources on cellulase production**

Different nitrogen sources were investigated for their effect on cellulase induction by replacing the nitrogen source (tryptone and yeast extract) in the medium for cellulase production with tryptone and yeast extract separately as well as NH_4Cl, $NaNO_3$ and KNO_3. The nitrogen sources were added at 1.0% concentration. Ten milliliters of culture medium was dispensed in McCartney bottle in duplicate, autoclaved and inoculated with 1ml of 48hours culture of the isolates adjusted to O.D. 0.3 at 540nm. The bottles were incubated at 50^0C for 48hours. The uninoculated served as control. The supernatant was assayed for cellulase using the DNSA reagent.

3.4.7 **Effect of varying temperature on the cellulase production**

McCartney bottles containing 10ml basal medium with carbon source for cellulase production were inoculated with 1ml of the isolates adjusted to O.D. 0.3 at 540nm. The bottles were incubated at temperatures of 45, 50, 55 60^0C. For each temperature, the cell free culture supernatant fluid obtained after centrifugation at 5000rpm for 20min served as the source of crude enzyme. The supernatant was assayed for cellulase using the DNSA reagent.

3.4.8 **Effect of different pH values on cellulase production**

Sodium citrate buffer (pH 4.0 to 6.0), phosphate buffer (pH 7.0 and 8.0) and borate buffer (pH 9.0) were used to prepare the basal medium to desire pH values of 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0. The effect of pH on cellulase production was studied in McCartney bottles containing 10ml of the basal medium with the carbon source for cellulase production set at the desired pH value by appropriate buffer . The sterile pH-adjusted media were inoculated with 1ml of 48hr old isolates adjusted to O.D. 0.3 at 540nm and incubated for 48hr at 50^0C. The culture supernatant obtained was used for cellulase assay.

3.5 **Characterization of the obtained enzyme**

3.5.1 **Cellulase Assay**

Cellulase was assayed as described by Wood and Bhat (1988). The culture broth was centrifuged at 5000rpm for 20min at 4^oC and the supernatant served as the enzyme

source. 0.5ml of enzyme solution was added to 0.5ml of 1% Carbohydrate (CMC) substrate taken in 0.2M phosphate buffer (pH 7). After incubation at 50^0C for 30min, the reaction was stopped by the addition of 1ml dinitrosalicylic (DNSA) reagent. The reaction mixture was put in boiling water in water bath for 5min and then quickly cooled to room temperature, the degree of enzymatic hydrolysis of the cellulose was determined by measuring the absorbance at 540nm using Lab-Tech Digital colorimeter. Glucose was used as standard. Cellulase activity was expressed as 1µmol of reducing sugar (glucose equivalent) per milliliter of enzyme solution.

Glucose standards were made by preparing different concentrations of the sugar containing 0.2 to 5.0 mg/ml of glucose. The absorbance of the sugar concentrations were determined by the DNSA method using sugar as test sample. The absorbance read at 540nm was plotted against the concentration to get the standard curve.

Filter paper activity was determined by incubating 0.5ml of culture supernatant with 1.0ml of 0.05M of phosphate buffer pH 7.0 containing 1cm× 6cm strip (50mg) of Whatman No.1 filter paper (Mandels *et a*l., 1976). After incubation for 1hr at 50^0C, the reaction was stopped by adding 3ml of dinitrosalcylic acid reagent. The reaction mixture was placed in a boiling water bath at 100^0C for 5min and thereafter cooled to room temperature (Miller, 1959). Absorbance was read at 540nm using Lab-Tech colourimeter. Glucose (Sigma Chemical Co., St. Louis, Mo.) was used as standard. One filter paper (U) of activity was expressed as 1µmol of reducing sugar (glucose equivalent) released per min per milliliter of enzyme solution.

3.5.2 **Protein Estimation**

Protein estimation was carried out by the method of Lowry *et al*., (1951). The stock solutions were prepared as follows: Lowry solution A was 1% $CuSO_4.5H_2O$ in deionised water; Lowry solution B was 2% sodium potassium tartrate ($NaKC_4H_4O_6.4H_2O$); and Lowry solution C contained 2% $NaCO_3$ in 0.1M NaOH. Lowry alkaline cupper reagent was prepared fresh, within 1hr of use. 0.5ml of Lowry solution A was mixed with 0.5ml of Lowry solution B and 49ml of Lowry solution C. 2N Folin-Ciocalteau regent was diluted 1:1 in deionised water before use. Bovine serum albumin (1mg/ml) was used as standard.

1.2ml of the test sample was pipetted into a test tube and 6ml of Lowry alkaline copper reagent added to it. This was incubated at room temperature for 10minutes and then 0.3ml of diluted Folin reageng was added and mixed immediately. The tubes were

incubated at room temperature for 30min and the absorbance read at 490nm against water blank using colorimeter.

3.5.3 **Effect of temperature on the cellulase activity**

The influence of temperature on cellulase activity of the isolates was monitored in a reaction mixture containing 0.5ml of 1% CMC in 0.2M phosphate buffer pH 7.0 and 0.5ml of crude enzyme , incubated at different temperature of 45^0C, 50^0C, 55^0C, 60^0C, 65^0C and 70^0C for 30min. The reaction was terminated by adding 1ml of DNSA reagent and the reaction mixture was placed in a boiling water bath at 100^0C for 5min and there after cooled to room temperature (Miller, 1959). Absorbance was read at 540nm with Lab-Tech colorimeter. Glucose was used as standard.

3.5.4 **Effect of different pH on cellulase activity**

The optimum pH for isolates cellulase activities was determined by carrying out the enzyme assay in medium which had been adjusted to pH values in the range of 4.0 to 9.0 at 50^0C for 30min using sodium citrate buffer (0.2M) for pH 4.0to 6.0 phosphate buffer (0.2M) for pH 7.0 to 8.0 and borate buffer for pH 9.0. Enzyme assays were carried out as earlier described.

3.6 **Purification of the enzyme**

3.6.1 **Ammonium sulphate precipitation**

The crude enzyme supernatants obtained by growing *Roseomonas* sp. A1, *Anoxybacillus rupiensis* E1 and 5H at established optimal (Temperature, pH, Carbon source etc) enzyme production conditions for the isolates were subjected to ammonium sulphate ($(NH_4)_2SO_4$ precipitation within limits of 0 and 100% saturation (Dixon and Webb, 1971). One hundred milliliters (100ml) of the crude enzyme supernatant was sequentially treated with solid $(NH_4)_2SO_4$) to 0-20%, 20-60%, 60- 80% and 80-100% saturation by slowly adding 11.4g, 26.2g, 14.3g and 15.7g of the $(NH_4)_2SO_4$ respectively. The mixture of each batch of saturation was slowly and continuously stirred until the $(NH_4)_2SO_4$ completely dissolved in the medium. After 24hr at 4^0C, the precipitate was recovered by centrifugation at 1500g for 15min using 4^0C. The supernatants were then treated to the next batch of saturation until the required level of saturation was achieved. The precipitates of each batch were then pooled together and re-suspended to the initial volume of the crude enzyme supernatant with 50mM phosphate buffer pH 7.0.

3.6.2 **Dialysis**

3.6.2.1 **Preparation of dialysis tubing**

The dialysis tubes were cut into small pieces and boiled in de-ionised water for 30min and later for 30min in 1 litre of solution containing 0.2M Na_2HCO_3, 0.01M EDTA and 2litre de-ionised water. After thoroughly rinsing twice in de-ionised water, the tubes were again boiled in 0.01M EDTA for 10min. Thereafter, the tubes were thoroughly rinsed in changes of de-ionised water and 25% ethanol and stored in 25% ethanol. The tubes were washed with de-ionised water and tied one end with a clean twine rope before use (www. Bioprotocol.com).

3.6.2.2 **Dialysis procedure**

Ten milliliter of the re-dissolved precipitated protein of the isolates culture supernatant in 50mM phosphate buffer was pored in the dialysis tube which was then tied at the other end and dialyzed against 1 litre of the same buffer in a beaker . The dialysis was allowed to take place for 24hr against several changes of the buffer at 4^0C.

3.6.3 **Chromatography**

3.6.3.1 **Preparation of Sephadex beads**

Ten (10) grams of Sephadex G75 was weighed out into a clean bowls. Fifty ml of sterile de-ionized water was measured, added to the weighed beads and mixed to form a paste. Two hundred milliliter of 50mM phosphate buffer pH 7.0 was added to the mixture to obtain a fine suspension. This was kept in the refrigerator for 3 days with constant mixing.

3.6.3.2 **Loading of column**

The soaked and refrigerated Sephadex was vigorously shaken to mix after the third day and dispensed into a clean chromatographic column (1×50cm) with the use of a clean funnel. The Sephadex was allowed to compact in the column with the occasional removal of excess buffer and refilling of the column with Sephadex to the zero point of the column. This was done for 48hrs before the previously dialyzed samples were applied to the column.

3.6.3.3 **Fractionation on Sephadex G-75**

Two milliliter of the dialyzed enzyme of the isolates were applied separately to the Sephadex G75 column and eluted in 5ml fractions with 50mM phosphate buffer pH 7.0. Each fraction was analyzed for protein and cellulase enzyme. The active enzyme fraction was pooled.

3.6.3.4 **Sodium dodecyl sulphate- polyacrylamide gel electrophoresis (SDS-PAGE)**

The molecular weight of the purified enzymes of the isolates was determined using sodium dodecyl sulphate –polyacrylamide gel electrophoresis (SDS-PAGE) by method of Laemmli (1970) using BIO-RAD mini- PROTEAN® 3 Cell electrophoretic unit. The electrophoresis in denaturing conditions was carried out in 10% polyacrylamide gel slabs at pH 7.2 using 25mM Tris-HCl buffer containing 0.1% sodium dodecyl sulphate. The purified enzyme samples were mixed (1:1v/v) with 2 times the sample loading buffer. Ten microliters (10µl) of each sample to be analyzed was loaded on gels and the protein molecular weight marker into lines 1. The electrophoresis was performed with vertical slab unit

After electrophoresis, the gels were stained by laying the gels overnight in a solution containing 0.25w/v Coomassie blue R-250, 50%w/v methanol and 7.5% acetic acid and then de-stained in 7.5% acetic acid and 30% methanol. Silver staining was done to get an enhanced visualization of protein bands.

The mobility of the purified protein was compared with those of the following molecular mass standard: Protein markers used were BIORAD pre-stained SDS-PAGE standards of α-lactalbumin (14.2kDa), lysozyme (20.1 kDa), soya bean trypsin inhibitor (30 kDa), ovalbumin (45kDa), bovine serum albumin (66 kDa), phosphorylase b (97kDa).

3.7 Characterization of purified enzyme

3.7.1 Effect of temperature on enzyme activity

The influence of temperature on enzyme activity of the isolates cellulase were monitored in a reaction mixture containing 0.5 ml of 1% CMC in 0.05M phosphate buffer pH 7.0 and 0.5 ml of $(NH_4)_2SO_4$ precipitated and dialysed enzyme, incubated at different temperature of 45^0C, 50^0C, 55^0C, 60^0C, 65^0C, 70^0C, 75^0C, 80^0C and 85^0C for 30min. The reaction was terminated by adding 1ml of dinitrosalicylic acid reagent and the reaction mixture was placed in a boiling water bath at 100^0C for 5min and thereafter cooled to room temperature (Miller, 1959). Absorbance was read at 540nm using Lab-Tech Digiter colorimeter. Glucose (Sigma Chemical Co., St. Louis, Mo.) was used as standard.

3.7.2 Effect of temperature on enzyme stability

The effect of temperature on the stability of the cellulase produced by the isolates was studied by incubating the enzyme for 30min in the absence of substrates over a

range of 45^0C, 50^0C, 55^0C, 60^0C, 65^0C, 70^0C, 75^0C, 80^0C and 85^0C. Enzyme activities were then measured at 50^0C for 30min using the pre-incubated enzyme solution by cellulase assay methods described earlier

3.7.3 **Thermal stability studies**

Thermal stability studies of the isolates cellulase were carried out by incubating 20ml of the enzyme in the absence of substrate at 45^0C 50^0C, 55^0C, 60^0C and 70^0C. Samples of the pre-incubated enzyme solutions were withdrawn periodically at 0, 10,20,30,40,50,60,70,80,90,100,110 and 120mins. To determine the residual enzyme activity by the standard assay methods as earlier described.

3.7.4 **Effect of pH on enzyme activity**

The optimum pH for the isolates cellulase enzyme activities was determined by carrying out the enzyme assay in medium which has been adjusted to pH values in the range of 3.0 to 9.0 at 50^0C for 30min using sodium citrate buffer (0.1M) for pH 3.0 to 6.0 and phosphate buffer (0.1M) for pH 7.0 to 9.0. Enzyme assays were carried as earlier described.

3.7.5 **Effect of pH on enzyme stability**

The enzymes produced by the isolates were firstly pre-incubated in the 0.1M sodium citrate (pH 3.0 to 6.0) and 0.1M phosphate (pH 7.0 to 9.0) buffers for 2 hr at 50^0C. Subsequently the residual cellulase activity was determined as described earlier.

3.7.6 **Effect of time on enzyme activity**

The influence of time on the isolates cellulase enzyme activity was determined by performing the standard assay procedures (pH 7.0, 50^0C) for cellulase at different time (0-60 min)

3.7.7 **Effect of metal ions, SDS, Tween 80 and EDTA on enzyme activity**

The effect of metal ions (Zn^{2+}, Ca^{2+}, K^{+}, Cu^{2+}, Fe^{2+}, Mg^{2+}, Na^{+} , Mn^{2+} , Hg^{2+}), detergents (sodium dodecylsulphate (SDS); Tween 80 and chelating agent ethylene diamine tetraacetic acid (EDTA) on isolates cellulase was determined by adding them to the reaction mixture to a final concentration of 0.1mM, 1.0mM and 10mM and incubating at 50^0C for 30min. This was followed by determination of relative activity under assay condition as previously described. The enzyme activity without the metal ions served as the control and was considered as 100% activity.

3.7.8 **Effect of substrate concentration on enzyme activity**

Various concentrations (0.1, 0.25, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0%) of substrate, carboxyl methyl cellulose, in the cellulase assay medium were used to study the effect of substrate concentration on enzyme activity of the isolates cellulase. The enzyme assay was carried out as described earlier at 50^0C for 30min.

3.7.9 **Substrate specificity**

The study of cellulase substrate specificity was carried out with carboxyl methyl cellulose, Oat-spelt Xylan, Salicin and starch substrates. The substrates (1.0%) prepared in 0.1M phosphate buffer (pH 7.0) were incubated at 50^0C for 30min with the purified enzyme from the isolates. The reaction was stopped and quantified under the described assay conditions.

3.8 **Determination of kinetic parameters**

3.8.1 **K_m and V_{max} determination**

The kinetic parameters (K_m and V_{max}) were determined from Lineweaver-Burk representation using different concentrations of CMC. Each experimental point was determined in triplicate. Tubes with different concentrations (2-10mg/ml) of substrates were preincubated at 50^0C for a few minutes. A fixed amount of the enzyme from the isolates was added to the tubes and the reaction was stopped by the addition of dinitro salicylic acid (DNSA). The change in absorbance at 540nm at 1 min interval for several minutes was measured. The average change in absorbance (optical density-OD_{540}) per minute (ΔOD_{540}) was recorded. The ΔOD_{540} was used as a measure of velocity of the reaction (V_i). The reciprocals of the rate of reaction ($^1/_{vi}$) were plotted against the reciprocals of the substrate concentrations ($^1/_{[s]}$) and the K_m and V_{max} values determined by fitting the resulting data.

3.8.2 **Inhibition studies**

The inhibition constant (K_i) of glucose and cellobiose were determined from a Lineweaver-Burk plot. Four test tubes with different concentrations of CMC (2, 4, 6, 8 and 10mg/ml) were set up and a fixed amount of glucose of known concentration (1 and 2mg/ml) was added to each tube. A known amount of the purified cellulase from the isolates was added and enzymatic reaction was stopped by the addition of DNSA. The change in the absorbance at 540nm was measured over several minutes. The reciprocals of substrate hydrolysis ($^1/_{vi}$) for each sugar concentration were plotted

against the reciprocals of the substrate concentrations and the K_i was calculated from the equation.

$$V = \frac{V_{max}[S]}{K_m[1+{}^{[I]}/_{K_i}] + [S]} \quad \text{---------- Equation 1}$$

Where K_m = Apparent Michaelis constant due to inhibitor

K_m = Michaelis constant

[I] = Inhibitor concentration

K_i = Inhibitor constant

Vmax= Maximum velocity

[S]= Substrate concentration

This procedure was repeated by substituting glucose with cellobiose (Lin *et al*., 1999).

3.9 **Plasmid DNA extraction from the isolates (A1, E1 and 5H)**

2ml of the culture was suspended in 300µl of solution of (TAE) containing Tris-acetate, pH 8.0 (adjusted with glacial acetic acid) and 0.002M EDTA. 500µl of lysis buffer (0.05MTris, 3% SDS, pH 12.6 (adjusted with 2M NaOH), mixed(×5) by inversion and incubated at 37^0C for 5min. 400µl of ice cold 3M KOAC buffer pH 5.2 was added and mixed by inversion (×5). It was then incubated on ice for 10min, centrifuged at 12,000rpm for 3min. The supernatant was decanted into a new tube. Equal volume of isopropanol was added to the pellet, mixed gently by inversion (×5) and allowed to stand at room temperature (RT) for 20min. It was again centrifuged at 12,000rpm for 10min, isopropanol decanted and the plasmid pellet air dried. The pellet was then dissolved in 50µl of TE buffer (Kado and Liu. 1981).

Gel electrophoresis

Eight microlitres (8µl) of DNA sample was mixed with 2µl of loading buffer (0.05M EDTA, 2% Ficoll, 0.25% bromophenol blue in H_2O). The gel was prepared by pouring 25ml of 1% low electroendosmosis (EEO) agarose in TBE buffer (0.089M Tris, 0.089M boric acid, 0.0025M EDTA) on a 10×7cm glass slide and allowed to solidify. Samples were loaded in lane 1, 2 and 3 while the makers were loaded at lane M. The same TBE buffer was used as electrophoresis buffer. Electrophoretic separation was done at 80voltage overnight. Intercalating dye ethidium bromide was used for visualization on DNA bands and photography. One drop of a 10mg/ml stock

solution was added to the staining tray containing water and the gel. The tray was then covered with a lid. It was left for 30min and observed under shot wave length uv light (Meyers *et al*., 1976).

3.10 **Hydrolysis of lignocellulosic substrates**

3.10.1 **Lignocellulosic substrates**

Agricultural wastes namely sugar cane bagasse, sawdust and corn cob were used as substrates for the work. They are cheap and readily available sources of lignocellulosic materials and were procured locally. Sawdust from timber of *Chlorophora excelsa* (``Iroko``) *Tectona grandis*(``Teak``) *Irvingia* sp.(`Oro`) and *Funtumia africana*(Ire) were obtained from Oke Gada sawmill Ede Osun State and corncob was obtained from Timi market Ede. Sugar cane bagasse was obtained by milling fresh sugar cane and squeezing out the juice. The bagasse obtained was dried. All the three types of substrates were ground, washed several times in hot water to remove free reducing sugars remaining in the materials and dried (Ratanakhanokchai *et al*., 1999).

3.10.2 **Alkali pre-treatment of substrates**

The substrates were alkali-treated by autoclaving the washed and dried lignocellulosic substrates at 121^0C for 30min with 0.25M NaOH (20ml/g substrate). The substrates recovered by filtration through muslin cloth were thoroughly washed with de-ionised water and neutralized with 0.25M HCl. The substrates were finally washed with many changes of de-ionised water and dried at 65^oC to constant weight (Singh *et al*., 1988).

3.10.3 **Hydrolysis of substrates**

A suspension of substrate (10mg/ml) was prepared by adding 100ml of 50mM phosphate buffer (pH 7.0) to 1gram of the substrate. Fifteen milliliter (15ml) each of the substrate suspension was sterilized at 121^0C for 20min in 50ml conical flasks. Five ml of partially purified enzyme obtained as described previously from the isolates was added to the sterilized substrates. Hydrolysis was performed at 50^0C for 48hr and samples were withdrawn at 1,6,24 and 48hr for analysis of reducing sugar. The resultant supernatant following centrifugation (2500g, 15min) was assayed for total reducing sugars using DNSA method (Miller, 1959). The release of sugar is expressed as equivalent to cellulose. The percentage hydrolysis was calculated as given by Baig *et al*., (2004).

$$\% \text{ Hydrolysis} = \frac{\text{Glucose (mg/ml)} \times 100}{\text{Substrate (mg/ml)}} \quad \text{Equation 2}$$

3.10.4 **Effect of pH on hydrolysis**

Hydrolysis of sugarcane bagasse and corncob using isolates cellulases was carried out as described in section 3.9.3. The suspension of substrates (sugatcane bagasse and corncob) were prepared in 50mM phosphate buffer at pH 4, 5, 6, 7, 8, 9 and 10. Total reducing sugar produced was determined by the DNSA method (Miller, 1959).

3.10.5 **Effect of temperature on hydrolysis**

The optimum temperature for hydrolysis of sugarcane bagasse and corncob by isolates cellulases was determined by incubating reaction mixtures at different temperatures rangeing between 45^0C and 80^0C for 48hr as described in section 3.9.3.

3.10.6 **Effect of time on hydrolysis**

The optimum time for hydrolysis was obtained by withdrawing aliquot from the reaction mixture of sugarcane bagasse and cellulase from *Roseomonas* sp. and *Anoxybacillus rupiensis* E1 and corncob with cellulase from *Anoxybacillus rupiensis* 5H after 1 6, 12, 18, 24, 30 and 36hr and determining the level of reducing sugar released as outlined in section 3.9.3.

3.10.7 **Effect of substrate concentration on hydrolysis**

The effect of substrate concentration on the extent of hydrolysis was determined by varying the concentration of the sugarcane bagasse and corncob (1--10%) in the reaction mixtures with cellulase from *Roseomonas* sp. A1, *Anoxybacillus rupiensis* E1 and 5H respectively. The hydrolysis was carried out as described in section 3.8.3.

3.11 **Statistical analysis**

All the data were mean values of at least two replicates. The data, where applicable, were analyzed by one-way ANOVA. Test of significant differences between and within Means were determined by Duncan`s Multiple Range Test at $P \leq 0.05$ using SAS (SAS, 1999).

CHAPTER FOUR

4.0 RESULTS

4.1 Identification of Isolates

Microscopic appearance and Biochemical Characterisation of the Isolates

Table 4.1 shows the cell shapes, Gram`s reaction and biochemical characteristics of the isolates. Only isolates *Anoxybacillus rupiensis* (E1 and 5H), *Thermoactinomyces vulgaris* ($P_3$), *Actinomyces nueslundii* (3B 6A1, 6A2 and 6B) and *Aerococcus viridans* (S16) reacted positively to Gram`s staining, others were negative to the staining. While S16 was observed to have coccal appearance, others were rods but of different length. Twenty isolates (C, E, H, I, J, K, O, P, 3, 8, P3, A4, 2D4, 2D5, S14, A3, 3B, 6A1, 6A2 and 6B) were catalase positive and only three isolates *Roseomonas* sp. (A1), *Proteus retigeris* (B) and *Citrobacter freundii* (C) utilized citrate. Similarly only three isolates A1, C and *Pseudomonas alcaligenes* (A3) did not reduce nitrate. The five sugars used in fermentation test study were fermented differently. While isolates produced both acid and gas others could produce only the acid. In all glucose was the most fermented by majority of the isolates. Isolates later identified as *Pseudomonas* spp. did not ferment the sugar.

4.2 Occurrence of cellulolytic bacteria in sawdust

Distribution of the isolates from the sampled sawdust is represented in Table 4. 2. A total number of sixteen (16) bacteria were isolated at the depth of 5cm. Out of these, ten of them were *Pseudomonas aeruginosa* (E,H,I,J,K,L,O,P,3,8), two (2) *Klebsiella pneumonia* (M,N); One each of *Klebsiella edwardsii* (3D), *Roseomonas sp*.(A1) *Proteus retigeris*(B) and *Citrobacter freundii* (C) was found respectively. Only two *Pseudomonas aeruginosa* (IP3,A4)were isolated from the depth of 10cm. From the total of eleven bacteria isolated from the depth of 15cm, there were five *Klebsiella pneumonia* (B4,C1,D1,3E, 3E5) followed by two *Klebsiella edwardsii* (3D4,3G) and one each of *Thermoactinomyces vulgaris* (P3), *Anoxybacillus rupiensis* (E1), *Pseudomonas alcaligenes* (A_3) and *Campylobacter jejuni* (H5) occurred. Similarlly, five *Klebsiella pneumoniae* (D4, D5, F5, E5, 2E5) two *Campylobacter jejuni* (K5 ,G5)

and one each for *Pseudomonas aeruginosa* (2D4) and *Klebsiella edwardsii* (3A1) at 20cm depth.

At the depth of 30cm, the dominant bacterial isolate was *Klebsiella edwardsii* (3A,3C,3C2,3F,3F6,3H,K3,2A4,CI,5A,5A$_{1,}$5C1) with 12 in number and the other isolate was one *Actinomyces nueslundii* (3B). Only two isolates were identified from the depth of 45cm and they were one each for *Aerococcus viridans* (S16) and *Pseudomonas aeruginosa* (S14). Twelve bacteria were isolated from the depth of 50cm with *Klebsiella edwardsii* (5C1,5D,5B,5E5,5F6,5B1,5G) being nine followed by *Klebsiella pneumonia* (D4,F5) two and one *Anoxybacillus rubiensis* (5H). Five isolates were derived from the depth of 60cm, three of which were *Actinomyces nueslundii* (6A1,6A2,6B) one *Klebsiella edwardsii* (6C) and one *Campylobacter jejuni* (6A). At the depth of 70cm nine bacteria were isolated with *Klebsiella edwardsii* (7B2,7C,7C1,7D4,7F6,7F,7A) being seven and two *Klebsiella pneumonia* (7E,7E1) (Table 4.2)

Table 4. 1. Biochemical and Physiological Characterization of Bacterial Isolates from Saw Dust Dumps

Isolate code	Cell Shape	Gram reaction	Spore production	Catalase	TSI reaction	SIM reaction	Citrate utilization	MR	VP	SUGAR FERMENTATION					O-F or H-L	Nitrate Reaction	Possible organism
										Glucose	Maltose	Mannitol	Sucrose	Lactose			
A_1	SR	-	-	+ + +		- + -	+	+	-	Y	Y	Y	Y	NC	None	-	*Roseomonas sp*
B	SR	-		+ + +	YG NC NC	+ - -	+	+	-	YG	NC	YG	NC	NC	None	+	*. Proteu retigeri*
C	SR	-		-	YGY+	- + +	+	+	-	YG	YG	YG	NC	Y	F	-	*.Citrobacter freundii*
E_1	SR	+	+	+	Y NC NC	- - -	-	+	+	Y	Y	NC	Y	NC	F	+g	*Anoxybacillus rupiensis*
E.. H, I, J, K,O,P,3,8 1P3A4,2D4,2D5, S14	SR	-	ND	-	NC NC NC	- - -	-	-	-	NC	NC	NC	NC	NC	OX	+	*Ps aeruginosa*
M,A4,B4,C1,D1,D4, D5,F5	MLR	-	ND	+ + +	Y NC NC	- - -	-	+	-	Y	NC	NC	NC	NC	F	+ (G)	*Kl. pneumoniae*
N,E5,2E5,3E5, 5C,5E,7E, 7E1	LR	-	ND	+ + +	Y NC NC	- - -	-	+	-	Y	NC	NC	NC	NC	F	+	*Kl. Pneumoniae*
A_3	MLR	-	ND	-	NC NC NC	- - -	-	-	-	NC	NC	NC	NC	NC	None	-	*Ps, alcaligenes*
3A,3A1,3C,3C2,3E,3 E5 3F,3F6,3H,5A,5A_1,5C 1 5D,6C,7B2,7C,7C1,7 AD4,7 7E5,7F6,A	LR	-	ND	+	Y NC NC	- - -	-	+	+	Y	NC	NC	NC	NC	F	+	*Kl, edwardsii*
3B	LR	+	-	-	NG	- - -	-	+	-	Y	Y	NC	NC	NC	F	+g	*Act. naeslundii*
3D,3D4,3G,5B,5B1,5 E5 5F6,5G,7F,K3,2A4	MLR	-	ND	+	YG NC NC	- - -	-	+	+	Y	NC	NC	NC	NC	F	+	*Kl. edwardsii*
P3	LR	+	ND	+	Y NC NC	- - -	-	+	+	Y	Y	NC	Y	NC	F	+g	*Thermoactinomyces vulgaris*
5H	SR	+	+	+	Y NC NC	- - -	-	+	+	Y	Y	NC	Y	NC	F	+g	*Anoxybacillus rupiensis*
H5,K5,G5,6A,	SR	-	ND	+	NG	- + -	-	-	-	Y	Y	NC	NC	NC	F	+G	*Campylobacter jejuni*

Isolate code	Cell shape	Gram reaction	catalase	TSI reaction	SIM reaction	Citrate utilization	MR	VP	SUGAR FERMENTATION					0-For H&L	Nitrate reduction	Possible organism
									Glucose	Maltose	Mannitol	sucrose	lactose			
,6A1,6A2,6B	VLR	+	-	+	NG	- - -	+	-	Y	Y	NC	NC	NC	F	+	*Actinomyces naeslundii*
S16	Cocci	+	-	-	Y Y NC	- - -	-	-	Y	Y	Y	Y	Y	F	+	*Aerococcus viridans*

LEGEND:	
SR – Short rod MLR – Medium long rod LR – Long rod NG – No growth VLR – Very long rod.	Y – Acid production only NC – No Change g – Gas Production F – Fermentative

Table 4. 2. Occurrence of cellulolytic bacteria from sawdust.

Identities of the isolates with the frequency of their occurrence.

Samples(cm)	*Aerococcus viridand*	*Aoxybacillus rupiensis*	*Pseudomonas aeruginosa*	*Actinomyces nueslundii*	*Klebsiella pneumoniae*	*Klebsiella edwardsii*	*Pseudomonas alcaligenes*	*Campylobacter Jejuni*	*Roseomonas sp.*	*Proteus rettgeris*	*Citrobacter freundii*	Total
5			10		2	1			1	1	1	**16**
10			2									**2**
15		1	1		5	2	1	1				**11**
20			1		5	1		2				**9**
30				1		12						**13**
45	1		1									**2**
50		1			2	9						**12**
60				3		1		1				**5**
70					2	7						**9**
Total	**1**	**2**	**15**	**4**	**16**	**33**	**1**	**4**	**1**	**1**	**1**	**79**

4.3 **Effect of different carbon sources on the growth of the isolates**

Generally, there was increase in the growth of the organisms as the concentration of the carbon sources increased except in the case of galactose for *Roseomonas* sp. (Table 4.3). Isolate A1 (*Roseomonas* sp.) grew as the concentration of CMC increased from 0.5% to 1.0% (w/v) after which there was decline in growth. Similar trend was observed for isolate D, E and H (*Pseudomonas aeruginosa*) as well as E1 (*Anoxybacillus rupiensis*). However, except for *Roseomonas* sp. (A1) and *Anoxybacillus rupiensis* (E1), all others achieved maximum growth at 1.5% (w/v) of CMC. There was significant difference in the growth of *Roseomonas* sp. (A1) at 1.0% when compared to other concentrations of CMC. For *Anoxybacillus rupiensis* (E1), the highest value of 1.33±0.04 was observed and is significantly higher than other values.

When glucose was sole carbon source, there was increase in the growth of isolates *Pseudomonas aeruginosa* (D, E, and H) and *Anoxybacillus rupiensis* (E1) as the concentration of the carbon source increased from 0.5 to 1.0% (w/v) after which the growth decreased gradually. However, the increase in the growth of *Roseomonas* sp. (A1) was gradual up 1.5% (w/v) of glucose before a sharp decline in growth was observed. There was significant growth increase of *Pseudomonas aeruginosa* (E and H) as well as *Anoxybacillus rupiensis* (E1) at 1.0% concentration of glucose as compared to other concentrations.

In the case of sorbitol, even though there was decrease in the growth of A1 from 0.5 to 2.5% (w/v) that of between 0.5 to 1.5% was not significant. For isolates E, E1 and H, there were gradual increase in the growth between concentration 0.5 and 1.0% (w/v) and the higher growth at 1.0% was significant for the three isolates. However, the growth in D increased significantly between 0.5 to 1.0% that 1.0 to 1.5% was not significant.

In Table 4.4, there were gradual increase in the growth of isolates I, L and O (*Pseudomonas aeruginosa*) in CMC from 0.5 to 1.5% (w/v) and the increase was significant while the growth decreased, after the optimum, down to 2.5% (w/v) of the carbon gradually. The increase in growth of J and K was between concentration of 0.5 and 1.0%(w/v) beyond which the growth decreased and values were significantly different.

The optimum growth of isolate I was at 1.5% in sorbitol (Table 4.4). The organism growth increased from 0.5 to 1.5% gradually, though the growth increment

was not significant. The growth then decreased down from 1.0 to 2.5% of carbon source. All other isolates in this table and in relation to sorbitol had their growth increase between 0.5 and 1.5% beyond which there was significant decrease in growth up to 2.5%. However, the increase in the growth of I was between 0.5 to 1.5%, but the increment in the growth between 1.0 and 1.5% was not significant.

In the case of glucose on this table, all the isolates` growth increased from 0.5 to1.0% (w/v) and then decreased gradually down to 2.5% (w/v). The increase and decrease in growth were significant. The highest growth was for isolate O 1.40$(O.D)_{540nm}$

In Table 4.5, the utilization of carbon source by isolates P, 3, 8 and S14 (*Pseudomonas aeruginosa*) as well as P3 (*Thermoactinomyces vulgaris*) indicated that there were gradual increase in their growth as the concentration of the carbon sources increased. The increment in growth for P, 3 and S14 were from 0.5 to 1.5% (w/v) where maximum growth was achieved in the presence of CMC and the growth decreased gradually down to 2.5%. Isolates *Pseudomonas aeruginosa* (3) and *Thermoactinomyces vugaris* (P3) growth increment were between 0.5 and 1.0% w/v of CMC beyond which the growth decreased. However, the increase in the growth of isolate *Pseudomonas aeruginosa* (P) between 1.0 and 1.5% was not significant, though the values differ. It was evident from this table (4.5) that *Thermoactinomyces vulgaris* (P3) metabolised sorbitol maximally at concentration of 0.5% (w/v).

The highest growth for *Pseudomonas aeruginoas* (P) was recorded at 1.5% (w/v) of Sorbitol. But the value observed here is not significantly different from that observed for 1.0% (w/v). The same was true of isolate *Pseudomonas aeruginoas* (8). While the increase in the growth of *Pseudomonas aeruginoas* (P, 3, 8 and S14) was gradual, there were fluctuations in that of P3 as there was decrease in the growth between 0.5 and 1.0% followed by increase in growth between 1.0 and 1.5% which later decreased at 2.5% (w/v).

The utilization of glucose by *Thermoactinomyces vulgaris* (P3) was different from other isolates in this experiment as the increase was consistent from 0.5 to 2.5% (w/v) where a maximum was achieved. For the remaining isolates, the increase in growth was between 0.5 and 1.0% after which there was decrease in growth as sugar concentration decreased to 2.5% (w/v).

The utilization of carbon sources for the growth by *Actinomyces nueslundii* (3B, 6A1, 6A2 and 6B) and *Anoxybacillus rupiensis* (5H) is represented in table 4.6.

There was general increase in the growth of the isolates from 0.5 to 1.0% (w/v) with CMC as carbon source and the increment at 1.0% was significant except for 6A1. There was increase in the growth of organisms 3B, 6A1, 6A2 and 6B with sorbitol as carbon source in the growth medium between 0.5 and 1.5% (w/v) and with significant growth difference from 0.5 to 1.0% for 3B, 5H, 6A2 and 6B. However, the growth difference in sorbitol for 3B between 1.0 and 1.5% was not significant.

Glucose was the most utilized by isolates 5H, 6A1, 6A2 and 6B with values 1.48±0.01, 1.10±0.01, 1.035±0.05 and 1.30±0.01 $(O.D)_{540nm}$ respectively. These values were observed at 1.0% (w/v) concentration. For isolate 3B 0.5% of glucose supported the best growth which was significantly different from other concentrations. However, the best utilized carbon for isolate 3B was lactose.

Table 4.3. Effect of Different Concentration of Carbon sources on the Growth of Bacterial Isolates from Sawdust

Carbon source ---------- Type	Isolates ---------- % Concent	/Growth(O.D)$_{540nm}$ ---------- *Roseomonas* sp. (A_1)	*Ps. aeruginosa* (D)	*Ps. aeruginosa* (E)	*Anoxybacillus rupiensis* (E1)	*Ps aeruginosa* (H)
Galactose	0.5	0.73±0.03^{a}	0.56±0.01^{ab}	0.57±0.06^{ab}	1.07±0.01^{a}	0.91±0.04^{a}
	1.0	0.71±0.03^{a}	0.58±0.03^{ab}	0.67±0.04^{ab}	1.26±0.03^{b}	1.01±0.03^{b}
	1.5	0.62±0.03^{b}	0.67±0.08^{bc}	0.56±0.05^{ab}	1.23±0.01^{b}	0.83±0.05^{ab}
	2.0	0.50±0.00^{c}	0.41±0.02^{d}	0.47±0.05^{ab}	1.03±0.04^{a}	0.76±0.05^{b}
	2.5	0.39±0.04^{d}	0.32±0.02^{d}	0.34±0.04^{c}	0.56±0.04^{c}	0.60±0.01^{c}
Lactose	0.5	0.76±0.08^{a}	0.62±0.04^{ab}	0.48±0.03^{a}	0.69±0.04^{a}	0.61±0.03^{a}
	1.0	0.68±0.06^{b}	0.68±0.02^{ab}	0.67±0.06^{b}	0.84±0.05^{b}	0.71±0.02^{b}
	1.5	0.63±0.01^{b}	0.79±0.03^{c}	0.63±0.01^{b}	0.95±0.06^{c}	0.61±0.03^{a}
	2.0	0.4±0.03^{c}	0.55±0.02^{a}	0.48±0.01^{a}	0.63±0.00^{a}	0.54±0.03^{c}
	2.5	0.06±0.04^{d}	0.42±0.03^{d}	0.30±0.09^{c}	0.63±0.02^{a}	0.40±0.02^{d}
CMC	0.5	0.8±0.03^{a}	0.47±0.06^{a}	0.5±0.01d^{ac}	0.51±0.01^{ad}	0.49±0.04^{a}
	1.0	1.14±0.03^{b}	0.7±0.03^{b}	0.68±0.01^{b}	0.8±0.03^{b}	0.59±0.01^{b}
	1.5	0.75±0.03^{a}	0.82±0.02^{c}	0.57±0.04^{ac}	0.81±0.11^{bc}	0.62±0.01^{b}
	2.0	0.19±0.03^{c}	0.64±0.01^{b}	0.46±0.04^{ad}	0.67±0.06^{abd}	0.5±0.01^{a}
	2.5	0.16±0.02^{c}	0.36±0.04^{d}	0.34±0.04^{e}	0.53±0.04^{acd}	0.38±0.01^{c}
Sorbitol	0.5	0.59±0.03^{a}	0.77±0.06^{a}	0.73±0.05^{ac}	0.89±0.03^{a}	0.75±0.03^{a}
	1.0	0.55±0.03^{a}	0.95±0.02^{b}	0.91±0.01^{bc}	1.33±0.04^{b}	0.86±0.05^{b}
	1.5	0.55±0.06^{a}	0.98±0.06^{b}	0.82±0.06^{abc}	0.86±0.02^{a}	0.73±0.04^{a}
	2.0	0.43±0.01^{b}	0.74±0.02^{a}	0.71±0.02^{a}	0.71±0.01^{dc}	0.59±0.04^{c}
	2.5	0.21±0.01^{c}	0.52±0.06^{c}	0.55±0.06^{d}	0.68±0.03^{d}	0.49±0.01^{d}
Glucose	0.5	0.36±0.03^{a}	0.50±0.01^{a}	0.52±0.03^{a}	0.55±0.00^{a}	0.61±0.01^{a}
	1.0	0.43±0.01^{b}	0.97±0.01^{b}	1.01±0.01^{b}	1.28±0.03^{b}	1.05±0.01^{b}
	1.5	0.72±0.03^{c}	0.67±0.06^{c}	0.74±0.04^{c}	0.56±0.01^{a}	0.81±0.02^{c}
	2.0	0.14±0.01^{d}	0.41±0.02^{d}	0.51±0.03^{a}	0.28±0.06^{c}	0.61±0.00^{a}
	2.5	0.14±0.00^{d}	0.24±0.04^{e}	0.30±0.01^{d}	0.13±0.02^{d}	0.27±0.05^{d}

Each value is a mean of two replicates; ± stands for standard deviation among replicates; means with different letters within each column differ significantly ($p \leq 0.05$) CMC- carboxymethyl cellulose

Table 4.4. Effect of Different Concentration of Carbon sources on the Growth of Bacterial Isolates from Sawdust

Carbon source	% Concent	*Ps. aeruginosa* (I)	*Ps. aeruginosa* (J)	*Ps. aeruginosa* (K)	*Ps. aeruginosa* (L)	*Ps. aeruginosa* (O)
Galactose	0.5	0.53±0.04^{a}	0.87±0.06^{a}	0.41±0.12^{a}	0.86±0.02^{a}	1.03±0.04^{a}
	1.0	0.54±0.05^{a}	0.99±0.01^{b}	0.55±0.04^{ab}	0.91±0.01^{a}	1.20±0.01^{b}
	1.5	0.70±0.01^{b}	0.82±0.06^{a}	0.47±0.06^{a}	0.84±0.03^{a}	1.05±0.01^{a}
	2.0	0.49±0.04^{ac}	0.71±0.01^{c}	0.45±0.02^{ad}	0.72±0.04^{b}	0.98±0.02^{a}
	2.5	0.41±0.02^{c}	0.59±0.03^{d}	0.30±0.01^{acd}	0.59±0.01^{c}	0.60±0.01^{c}
Lactose	0.5	0.61±0.04^{a}	0.61±0.05^{a}	0.47±0.06^{a}	0.61±0.04^{a}	0.66±0.05^{a}
	1.0	0.63±0.09^{a}	0.73±0.01^{b}	0.64±0.01^{b}	0.74±0.01^{b}	0.73±0.02^{a}
	1.5	0.80±0.01^{b}	0.65±0.06b^{a}	0.52±0.01^{a}	0.65±0.03^{a}	0.82±0.03^{b}
	2.0	0.56±0.02^{ac}	0.53±0.04^{a}	0.48±0.02^{a}	0.53±0.02^{c}	0.70±0.03^{a}
	2.5	0.47±0.05^{c}	0.40±0.02^{c}	0.28±0.01^{c}	0.40±0.01^{d}	0.57±0.04^{c}
CMC	0.5	0.44±0.05^{a}	0.49±0.04^{a}	0.49±0.01^{a}	0.48±0.01^{ca}	0.59±0.04^{ac}
	1.0	0.64±0.06^{b}	0.57±0.00^{b}	0.69±0.02^{b}	0.57±0.05^{b}	0.71±0.03^{bc}
	1.5	0.74±0.05^{b}	0.52±0.02^{ab}	0.56±0.02^{c}	0.62±0.01^{b}	0.81±0.05^{b}
	2.0	0.64±0.01^{b}	0.50±0.04^{a}	0.46±0.01^{a}	0.50±0.01^{acd}	0.63±0.06^{abc}
	2.5	0.44±0.05^{a}	0.36±0.04^{c}	0.34±0.03^{d}	0.34±0.03^{d}	0.56±0.02^{a}
Sorbitol	0.5	0.79±0.03^{a}	0.73±0.02^{a}	0.67±0.04^{a}	0.71±0.02^{a}	0.81±0.03^{a}
	1.0	0.92±0.02^{b}	0.85±0.02^{b}	0.87±0.05^{b}	0.83±0.03^{b}	1.25±0.03^{b}
	1.5	**0.97±0.01^{b}**	0.73±0.04^{a}	0.73±0.04b^{a}	0.73±0.04^{a}	0.94±0.04^{c}
	2.0	0.77±0.06^{a}	0.59±0.02^{c}	0.61±0.12^{a}	0.59±0.01^{c}	0.79±0.01^{a}
	2.5	0.55±0.06^{c}	0.49±0.01^{d}	0.51±0.04^{a}	0.49±0.02^{d}	0.67±0.05^{d}
Glucose	0.5	0.50±0.02^{a}	0.56±0.05^{a}	0.52±0.03^{a}	0.59±0.01^{a}	0.56±0.02^{a}
	1.0	0.97±0.00^{b}	1.01±0.04^{b}	0.99±0.01^{b}	0.97±0.04^{b}	1.40±0.40^{b}
	1.5	0.67±0.04^{c}	0.79±0.01^{c}	0.71±0.01^{c}	0.78±0.08^{c}	0.81±0.01^{c}
	2.0	0.42±0.04^{d}	0.61±0.01^{a}	0.50±0.03^{a}	0.61±0.04^{a}	0.53±0.05^{a}
	2.5	0.32±0.02^{e}	0.40±0.01^{d}	0.29±0.01^{d}	0.34±0.04^{d}	0.44±0.04^{d}

Each value is a mean of two replicates; ± stands for standard deviation among replicates; means with different letters within each column differ significantly ($p \leq 0.05$) CMC- carboxymethyl cellulose

Table 4.5. Effect of Different Concentration of Carbon sources on the Growth of Bacterial Isolates from Sawdust

Carbon source	%	*Ps. aeruginosa* (P)	*Ps. aeruginosa* (3)	*Ps. aeruginosa* (8)	*Thermoactinomyces vulgaris* (P3)	*Ps. aeruginosa* (S14)
Galactose	0.5	0.39±0.08^{ab}	0.60±0.05^{abc}	0.60±0.05^{a}	0.29±0.06^{a}	0.99±0.01^{a}
	1.0	0.48±0.01^{ab}	0.67±0.04^{ab}	0.69±0.04^{a}	0.35±0.07^{a}	1.11±0.04^{b}
	1.5	0.60±0.01^{bc}	0.55±0.03^{ac}	0.61±0.01^{a}	0.52±0.03^{bc}	0.94±0.04^{a}
	2.0	0.43±0.05^{abc}	0.48±0.03^{c}	0.49±0.03^{b}	0.62±0.04^{bc}	0.82±0.02^{c}
	2.5	0.36±0.06^{ab}	0.34±0.06^{d}	0.36±0.04^{c}	0.69±0.03^{c}	0.60±0.04^{d}
Lactose	0.5	0.54±0.006^{ac}	0.48±0.08^{a}	0.62±0.05^{ac}	0.05±0.00^{a}	0.66±0.01^{a}
	1.0	0.56±0.05^{a}	0.67±0.04^{bc}	0.63±0.11^{ac}	0.25±0.21^{ac}	0.72±0.04^{a}
	1.5	0.72±0.02^{b}	0.58±0.11^{abc}	0.79±0.03^{b}	0.13±0.04^{a}	0.82±0.01^{b}
	2.0	0.53±0.01^{ac}	0.48±0.03^{ac}	0.55±0.01^{ac}	0.55±0.04^{bc}	0.67±0.05^{a}
	2.5	0.44±0.04^{ac}	0.30±0.01^{d}	0.41±0.02^{c}	0.46±0.06^{ab}	0.55±0.05^{c}
CMC	0.5	0.44±0.02^{ac}	0.50±0.00^{a}	0.47±0.04^{a}	0.63±0.04^{a}	0.57±0.03^{a}
	1.0	0.60±0.01^{b}	0.68±0.03^{b}	0.70±0.01^{b}	0.83±0.03^{b}	0.71±0.02^{b}
	1.5	0.70±0.10^{b}	0.57±0.02^{c}	0.82±0.03^{c}	0.16±0.06^{cd}	0.80±0.02^{c}
	2.0	0.60±0.02^{bc}	0.46±0.04^{a}	0.64±0.05^{b}	0.08±0.03^{cde}	0.68±0.01^{b}
	2.5	0.45±0.06^{d}	0.34±0.01^{d}	0.39±0.02^{a}	0.02±0.01^{de}	0.52±0.03^{a}
Sorbitol	0.5	0.76±0.05^{a}	0.68±0.13^{acd}	0.75±0.06^{a}	1.53±0.03^{a}	0.79±0.03^{a}
	1.0	0.89±0.02^{b}	0.90±0.03^{bc}	0.93±0.03^{b}	0.65±0.00^{b}	1.20±0.01^{b}
	1.5	0.92±0.01^{b}	0.81±0.08^{abc}	0.98±0.01^{b}	0.79±0.08^{c}	0.94±0.03^{c}
	2.0	0.77±0.05^{a}	0.69±0.02^{acd}	0.74±0.06^{a}	0.29±0.01^{d}	0.75±0.02^{a}
	2.5	0.57±0.05^{c}	0.55±0.03^{ad}	0.55±0.01^{c}	0.25±0.07^{d}	0.66±0.04^{d}
Glucose	0.5	0.49±0.04^{a}	0.53±0.02^{a}	0.45±0.06^{a}	0.71±0.01^{a}	0.59±0.04^{ad}
	1.0	0.92±0.07^{b}	0.95±0.04^{b}	0.88±0.03^{b}	0.81±0.04^{b}	1.05±0.11^{b}
	1.5	0.67±0.10^{c}	0.73±0.02^{c}	0.62±0.11^{c}	1.23±0.04^{cd}	0.84±0.05^{c}
	2.0	0.49±0.03^{a}	0.53±0.04^{a}	0.43±0.01^{a}	1.29±0.00^{cde}	0.53±0.04^{da}
	2.5	0.32±0.01^{d}	0.33±0.02^{d}	0.64±0.41^{c}	1.30±0.00^{de}	0.43±0.01^{d}

Each value is a mean of two replicates; ± standard deviation among replicates; means with different letters within each column differ significantly ($p \leq 0.05$) CMC- carboxymethyl cellulose

Table4.6. Effect of Different Concentration of Carbon Source on the Growth of Bacterial Isolates from Sawdust

Carbon source Type	% Concent	*Actinomyces nueslundii* (3B)	*Anoxybacillus rupiensis* (5H)	*Actinomyces nueslundii* (6A1)	*Actinomyces nueslundii* (6A2)	*Actinomyces nueslundii* (6B)
Galactose	0.5	0.33 ± 0.04^{ab}	0.23 ± 0.01^{abc}	0.81 ± 0.01^{a}	0.56 ± 0.05^{a}	0.29 ± 0.03^{a}
	1.0	0.74 ± 0.06^{ab}	0.63 ± 0.49^{ac}	0.87 ± 0.01^{a}	0.89 ± 0.04^{a}	1.27 ± 0.07^{b}
	1.5	0.71 ± 0.03^{bc}	0.90 ± 0.11^{ac}	0.645 ± 0.05^{a}	0.55 ± 0.06^{bc}	0.95 ± 0.07^{a}
	2.0	0.55 ± 0.01^{abc}	1.08 ± 0.08^{c}	0.51 ± 0.02^{b}	0.52 ± 0.01^{bc}	0.51 ± 0.03^{c}
	2.5	0.55 ± 0.10^{ab}	0.86 ± 0.04^{d}	0.46 ± 0.07^{c}	0.42 ± 0.01^{c}	0.45 ± 0.07^{d}
Lactose	0.5	0.44 ± 0.00^{ac}	0.92 ± 0.03^{a}	0.51 ± 0.03^{ac}	0.51 ± 0.01^{a}	0.53 ± 0.04^{a}
	1.0	0.59 ± 0.06^{a}	0.38 ± 0.01^{bc}	0.41 ± 0.03^{ac}	0.43 ± 0.01^{ca}	0.57 ± 0.03^{a}
	1.5	0.61 ± 0.04^{b}	0.45 ± 0.00^{abc}	0.51 ± 0.02^{b}	0.50 ± 0.05^{a}	0.60 ± 0.02^{b}
	2.0	0.76 ± 0.03^{ac}	0.59 ± 0.06^{ac}	0.71 ± 0.11^{ac}	0.44 ± 0.01^{bc}	0.72 ± 0.00^{a}
	2.5	1.38 ± 0.08^{ac}	0.50 ± 0.04^{d}	0.50 ± 0.05^{c}	0.40 ± 0.01^{bc}	0.55 ± 0.06^{c}
CMC	0.5	0.98 ± 0.06^{ac}	0.57 ± 0.04^{a}	0.86 ± 0.06^{a}	0.68 ± 0.06^{a}	0.70 ± 0.01^{a}
	1.0	1.09 ± 0.07^{b}	1.19 ± 0.09^{b}	0.96 ± 0.05^{a}	0.79 ± 0.04^{b}	0.73 ± 0.04^{b}
	1.5	0.39 ± 0.03^{b}	0.57 ± 0.03^{c}	0.65 ± 0.01^{b}	0.45 ± 0.02^{cd}	0.30 ± 0.01^{c}
	2.0	0.27 ± 0.04^{bc}	0.45 ± 0.01^{a}	0.55 ± 0.02^{b}	0.34 ± 0.10^{cde}	0.26 ± 0.06^{b}
	2.5	0.22 ± 0.03^{d}	0.34 ± 0.06^{d}	0.14 ± 0.03^{a}	0.16 ± 0.04^{de}	0.04 ± 0.00^{a}
Sorbitol	0.5	0.70 ± 0.01^{a}	1.04 ± 0.03^{acd}	0.54 ± 0.06^{a}	0.47 ± 0.03^{a}	0.46 ± 0.06^{a}
	1.0	0.78 ± 0.08^{b}	1.34 ± 0.01^{bc}	0.51±0.01a	0.55 ± 0.04^{b}	0.58 ± 0.00^{b}
	1.5	0.98 ± 0.01^{b}	0.81 ± 0.01^{abc}	0.81 ± 0.01^{b}	0.67 ± 0.06^{c}	0.64 ± 0.02^{c}
	2.0	0.59 ± 0.03^{a}	0.70±0.10d[a]	0.40 ± 0.01^{a}	0.47 ± 0.06^{d}	0.75 ± 0.07^{a}
	2.5	0.49 ± 0.03^{c}	0.49 ± 0.01^{a}	0.20 ± 0.00^{c}	0.32 ± 0.02^{d}	0.93 ± 0.03^{d}
Glucose	0.5	0.58 ± 0.03^{a}	0.85 ± 0.98^{b}	0.95 ± 0.02^{a}	0.94 ± 0.03^{a}	1.05 ± 0.06^{da}
	1.0	0.48 ± 0.06^{b}	1.48 ± 0.1^{ac}	1.10 ± 0.01^{b}	1.035 ± 0.05^{b}	1.30 ± 0.01^{b}
	1.5	0.47 ± 0.00^{c}	1.13 ± 0.04^{ad}	0.46 ± 0.01^{c}	0.51 ± 0.02^{dc}	0.34 ± 0.06^{c}
	2.0	0.43 ± 0.00^{a}	0.65 ± 0.05^{a}	0.22 ± 0.00^{a}	0.30 ± 0.01^{cde}	0.32±0.01d[a]
	2.5	0.31 ± 0.03^{d}	0.49 ± 0.06^{d}	0.50 ± 0.42^{c}	0.15 ± 0.03^{de}	0.21 ± 0.01^{d}

Each value is a mean of two replicates; ± standard deviation among replicates; means with different letters within each column differ significantly ($p \leq 0.05$) CMC-carboxymethyl cellulose

4.4 **Effect of Nitrogen sources on the growth of the Isolates**

The growth of isolates in relation to different concentrations of various nitrogen sources was reported (tables 4.7 to 4.10).

The highest growth of *Pseudomonas aeruginosa* (H) was observed at 1.0% concentration of yeast extract while its least growth was recorded in 2.0% (w/v) of the yeast extract for the organism. The growth increased from 0.5% to 1.0%. *Roseomonas* sp. (A1) and *Pseudomonas aeruginosa* (D) also had their maximum growth at 1.0% (w/v) concentration whereas *Pseudomonas aeruginosa* (E) and *Anoxybacillus rupiensis* (E1) had their maximum growth at 1.5% (w/v) concentration. The growth increment were gradual and the highest growth recorded for *Roesomonas sp*. (A1), *Pseudomonas aeruginosa* (D, E and H) were significantly different from the growth of other concentrations.

The utilization of NH_4NO_3 showed that there was increase in the growth of all the isolates from 0.5% to 1.0% (w/v) after which the growth decreased gradually and was minimal at 2.0%. (Table 4.7) The highest growth at 1.0% was significant in all cases when compared to other concentrations.

Table 4.8 revealed the growth behaviour of isolates I, J, K, L and O (all *Pseudomonas aeruginosa*) when Tryptone, Yeast extract, $NaNO_3$, KNO_3 and NH_4NO_3 at varied concentrations were incorporated in the basal medium. Yeast extract gave the highest growth at 1.0% (w/v) concentration for isolates J, K and L while maximum growth was achieved at 1.5% for I and O. These maximum growths were significant for all the organisms in this table except for isolate J where the highest growth was observed at concentration of 1.0% but was not significantly higher than the value for 1.5%. There were also increase in the growth of the isolates as the concentration of NH_4NO_3 increased from 0.5% to 1.0% (w/v) in this medium for isolates I, J, K and L while the increase in the growth was observed for isolate O up to 1.5% after which the growth decreased in all cases. The highest growth in table 4.9 was observed with the inclusion of 1.0% yeast extract in the basal medium for *Thermoactinomyces vulgaris* (P3). The amount (1.52±0.06) was significantly higher when compared to other concentrations between 0.5 and 2.0%. With exception *Pseudomonas aeruginosa* (8) that had its maximum growth at 1.5%, the best concentration for maximum growth of others was 1.0% beyond which the growth decreased to 2.0%.

The increase in the growth of *Pseudomonas aeruginosa* (P, 3, 8, and S14) and *Thermoactinomyces vulgaris* (P3) in the presence of NH_4NO_3 (Table 4.9) were gradual

from 0.5 to 1.0% (w/v) after which the growth decreased to 2,0%. The highest value of each of the isolate at 1.0% (w/v) was significantly different from other values at other concentrations.

The growth of isolates 3B, 5H, 6A1, 6A2 and 6B (Table 4.10) in relation to nitrogen sources showed that there was general and gradual increase in the growth of 3B (*Actinomyces nueslundii*) and 5H (*Anoxybacillus rupiensis*) from 0.5 to 1.0% (w/v) while the increase in the growth of 6A1, 6A2 and 6B was from 0.5 to1.5% in medium containing yeast extract. The highest growth of 1.08± 0.03 and 0.86± 0.08 for 3B and 5H respectively were significantly higher than other concentrations for the isolates. Similarly, the maximum growth of 1.13±0.05, 0.90±0.01 and 1.11±0.01 for 6A1, 6A2 and 6B respectively were also significant compared to other concentrations. The least growth of 0.50±0.06 was observed for 3B in the medium containing yeast extract in this table.

The growth of the isolates in the medium with NH_4NO_3 also increased from 0.5 1.0% (w/v) for the five isolates in table 4.10 beyond which there was decreased in the growth. The least growth value of 0.02±0.00 $(O.D)_{540nm}$ was recorded for isolate 3B at 2.0%. However, the highest growth was recorded for *Actinomyces nueslundii* (6A1) in the presesnce of yeast extract while the least growth was observed for *Anoxybacillus rupiensis* (5H) when $NaNO_3$ was used at 2.0% w/v.

4.5 **Effect of pH on the growth of the Isolates**

Isolates H, K and 3B achieved maximum growth at pH 6.0 compared to other pHs (Figures 4.1, 4.2, 4.3 and 4.4) , while isolates A1 and 6A2 produced best growth at pH 8, (Figure 4.1 and Figure 4.4 respectively). Other isolates grew best at pH of 7.0 as shown in Figures. 4.1-4.4. There was gradual increase in the growth of the isolates A1, D and H from pH 4-5 while sharp increases were recorded for the isolates D, E, E1 and H between pH 5.0 and 6.0. Isolates E, E1, D and H increased mildly between pH 6.0 and 7.0. All the isolates decreased sharply between pH7.0 and 8.0 except A1 and H with the same trend but between pH 8.0 and 9.0.

Figure 4.2 showed the sharp increase in the growth of isolates I, J and K between pH 5.0 and 6.0 but mild increase between pH 6.0 and 7.0 for isolates I and J. Similarly, there was mild increment in growth between pH 6.0 and 7.0 for L and pH 5.0 and 7.0 for O respectively. The decrease in the growth of all the isolates in Figure 4.2 after attaining their optimum pH of growth was gradual except isolate J. Apart

from isolate P3 with exceptionally good growth in pH 5.0, 6.0 and 7.0 and with pH 7.0 being the optimum followed by isolate 8 between pH 6.0 and 7.0, the remaining isolates in Figure 4.3 did not produce good growth except at pH of 7.0.The growth of all the isolates except P3 were gradual from pH 4.0 to 7.0 and the growth declined as pH increased to pH 9.0. Similar features were seen for isolates 5H in Figure 4.4 and P3 in Figure 4.7. Isolates 3B, 6A1 and 6A2 did not produce any growth at pH 4.0 and 5.0 but began to grow gradually to reach pH 6.0, 7.0 and 8.0 for 3B, 6A1 and 6A2 respectively as their optimal growth.

Table 4.7. Effect of Different Concentration of Nitrogen sources on the growth of Bacterial Isolates from sawdust

Nitrogen source		Isolates/Growth ($(O.D)_{540nm}$)				
Type	% Concent.	*Roseomonas* sp. (A1)	*Ps. aeruginosa* (D)	*Ps. aeruginosa* (E)	*Anoxybacillus rupiensis.* (E1)	*Ps. aeruginosa* (H)
Tryptone	0.5	0.55 ± 0.07^{a}	0.50 ± 0.05^{a}	0.64 ± 0.01^{b}	0.65 ± 0.03^{a}	0.43 ± 0.12^{ac}
	1.0	0.64 ± 0.03^{a}	0.71 ± 0.03^{b}	0.81 ± 0.03^{c}	0.79 ± 0.06^{b}	0.58 ± 0.06^{b}
	1.5	$0.54+0.03^{a}$	0.58 ± 0.06^{a}	0.57 ± 0.06^{b}	0.57 ± 0.02^{a}	0.50 ± 0.01^{cb}
	2.0	0.46 ± 0.01^{a}	0.47 ± 0.05^{a}	0.41 ± 0.03^{d}	0.48 ± 0.01^{c}	0.36 ± 0.04^{a}
Yeast extract	0.5	0.68 ± 0.01^{a}	0.44 ± 0.01^{a}	0.40 ± 0.01^{a}	0.46 ± 0.00^{b}	0.38 ± 0.01^{a}
	1.0	1.01 ± 0.04^{b}	0.88 ± 0.05^{b}	0.77 ± 0.06^{b}	1.01 ± 0.04^{a}	0.70 ± 0.11^{ce}
	1.5	0.07 ± 0.06^{ac}	0.71 ± 0.02^{c}	0.90 ± 0.02^{c}	1.05 ± 0.03^{a}	0.60 ± 0.02^{cf}
	2.0	0.65 ± 0.01^{a}	0.51 ± 0.01^{ad}	0.59 ± 0.04^{d}	0.70 ± 0.00^{d}	0.56 ± 0.06^{c}
$NaNO_3$	0.5	0.06 ± 0.01^{a}	0.05 ± 0.01^{a}	0.03 ± 0.01^{a}	0.15 ± 0.00^{a}	0.03 ± 0.01^{a}
	1.0	0.02 ± 0.00^{b}	0.07 ± 0.01^{b}	0.06 ± 0.01^{b}	0.18 ± 0.01^{b}	0.07 ± 0.01^{b}
	1.5	0.01 ± 0.00^{b}	0.04 ± 0.01^{a}	0.04 ± 0.02^{a}	0.19 ± 0.01^{b}	0.10 ± 0.03^{b}
	2.0	0.02 ± 0.00^{d}	0.02 ± 0.00^{c}	0.01 ± 0.00^{c}	0.12 ± 0.00^{c}	0.06 ± 0.01^{a}
KNO_3	0.5	0.04 ± 0.01^{a}	0.05 ± 0.00^{a}	0.04 ± 0.00^{a}	0.02 ± 0.00^{a}	0.05 ± 0.01^{a}
	1.0	0.09 ± 0.01^{b}	0.08 ± 0.01^{b}	0.08 ± 0.01^{b}	0.07 ± 0.01^{b}	0.10 ± 0.01^{a}
	1.5	0.05 ± 0.01^{a}	0.03 ± 0.01^{c}	0.05 ± 0.01^{a}	0.05 ± 0.01^{c}	0.15 ± 0.01^{c}
	2.0	0.03 ± 0.00^{a}	0.01 ± 0.00^{a}	0.02 ± 0.00^{c}	0.03 ± 0.01^{ac}	0.06 ± 0.03^{a}
NH_4NO_3	0.5	0.05 ± 0.01^{ad}	0.04 ± 0.01^{a}	0.06 ± 0.01^{a}	0.11 ± 0.01^{a}	0.04 ± 0.01^{a}
	1.0	0.12 ± 0.01^{b}	0.12 ± 0.01^{b}	0.12 ± 0.03^{b}	0.21 ± 0.01^{b}	0.10 ± 0.03^{b}
	1.5	0.07 ± 0.01^{ac}	0.07 ± 0.01^{c}	0.07 ± 0.01^{ac}	0.12 ± 0.01^{a}	0.07 ± 0.01^{ab}
	2.0	0.04 ± 0.01^{d}	0.02 ± 0.00^{a}	0.04 ± 0.01^{a}	0.07 ± 0.01^{c}	0.03 ± 0.01^{a}

Each value is a mean of two replicates; ± standard deviation among replicates; means with different letters within each column differ significantly ($p\leq 0.05$)

Table 4.8. Effect of Different Concentration of Nitrogen sources on the growth of Bacterial Isolates from sawdust

Nitrogen source ---------- Type	% Concentrat.	Isolates/Growth ---------- *Ps. aeruginosa* (I)	$(O.D)_{540nm}$ ---------- *Ps. aeruginosa* (J)	*Ps. aeruginosa* (K)	*Ps. aeruginosa* (L)	*Ps. aeruginosa* (O)
Tryptone	0.5	0.60 ± 0.01^{a}	0.54 ± 0.01^{a}	0.35 ± 0.04^{a}	0.44 ± 0.00^{a}	0.61 ± 0.02^{a}
	1.0	0.70 ± 0.01^{b}	0.69 ± 0.01^{b}	0.49 ± 0.04^{b}	0.54 ± 0.01^{b}	0.76 ± 0.04^{b}
	1.5	0.60 ± 0.01^{a}	0.47 ± 0.06^{a}	0.58 ± 0.01^{c}	0.50 ± 0.01^{c}	0.58 ± 0.06^{a}
	2.0	$0.49\pm.02^{c}$	0.38 ± 0.03^{c}	0.45 ± 0.02^{b}	0.40 ± 0.01^{d}	0.45 ± 0.04^{c}
Yeast extract	0.5	0.49 ± 0.03^{a}	0.51 ± 0.02^{a}	0.47 ± 0.01^{a}	0.51 ± 0.03^{a}	0.43 ± 0.01^{a}
	1.0	0.88 ± 0.01^{b}	0.91 ± 0.03^{b}	0.67 ± 0.01^{b}	0.59 ± 0.03^{b}	0.89 ± 0.08^{b}
	1.5	0.90 ± 0.01^{b}	0.90 ± 0.01^{b}	0.45 ± 0.04^{a}	0.42 ± 0.04^{c}	0.94 ± 0.04^{b}
	2.0	0.72 ± 0.01^{c}	0.71 ± 0.04^{c}	0.35 ± 0.04^{c}	0.30 ± 0.01^{d}	0.73 ± 0.13^{b}
$NaNO_3$	0.5	0.02 ± 0.00^{a}	0.02 ± 0.01^{a}	0.03 ± 0.00^{a}	0.05 ± 0.02^{a}	0.04 ± 0.01^{a}
	1.0	0.10 ± 0.01^{b}	0.07 ± 0.01^{b}	0.1 ± 0.01^{b}	0.09 ± 0.01^{b}	0.06 ± 0.01^{a}
	1.5	0.05 ± 0.01^{c}	0.06 ± 0.01^{b}	0.06 ± 0.00^{c}	0.09 ± 0.01^{b}	0.15 ± 0.01^{b}
	2.0	0.03 ± 0.01^{a}	0.02 ± 0.01^{a}	0.03 ± 0.01^{a}	0.05 ± 0.01^{a}	0.06 ± 0.01^{a}
KNO_3	0.5	0.06 ± 0.01^{a}	0.07 ± 0.02^{a}	0.04 ± 0.01^{a}	0.04 ± 0.02^{a}	0.05 ± 0.01^{a}
	1.0	0.11 ± 0.01^{b}	0.13 ± 0.01^{b}	0.12 ± 0.01^{b}	0.08 ± 0.01^{b}	0.10 ± 0.01^{b}
	1.5	0.13 ± 0.02^{b}	0.09 ± 0.01^{a}	0.08 ± 0.01^{b}	0.05 ± 0.01^{ab}	0.06 ± 0.01^{a}
	2.0	0.06 ± 0.01^{a}	0.07 ± 0.01^{a}	0.05 ± 0.03^{a}	0.03 ± 0.01^{a}	0.04 ± 0.01^{a}
NH_4NO_3	0.5	0.07 ± 0.01^{ac}	0.03 ± 0.01^{a}	0.06 ± 0.01^{ac}	0.06 ± 0.03^{a}	0.05 ± 0.01^{a}
	1.0	0.14 ± 0.01^{b}	0.12 ± 0.00^{b}	0.14 ± 0.01^{b}	0.13 ± 0.01^{b}	0.10 ± 0.01^{b}
	1.5	0.09 ± 0.01^{ad}	0.06 ± 0.01^{c}	0.08 ± 0.01^{a}	0.08 ± 0.01^{c}	0.18 ± 0.01^{c}
	2.0	0.05 ± 0.01^{c}	0.02 ± 0.00^{a}	0.03 ± 0.01^{c}	0.27 ± 0.33^{d}	0.10 ± 0.01^{b}

Each value is a mean of two replicates; ± standard deviation among replicates; means with different letters within each column differ significantly ($p \leq 0.05$)

Table 4.9. Effect of Different Concentration of Nitrogen sources on the growth of Bacterial Isolates from sawdust

Nitrogen source ---------- Type	% Concentrat	Isolates/Growth- ---------- *Ps. aeruginosa* (P)	$(O.D)_{540nm}$ ---------- *Ps. aeruginosa* (3)	*Ps. aeruginosa* (8)	*Thermoactinomyces vulgaris* (P3)	*Ps .aeruginosa* (S14)
Tryptone	0.5	0.47±0.01[a]	0.43±0.06[a]	0.61±0.02[a]	0.42±0.03[a]	0.47±0.06[a]
	1.0	0.56±0.05[b]	0.59±0.06[b]	0.80±0.04[b]	0.43±0.03[a]	0.60±0.01[b]
	1.5	0.47±0.01[a]	0.56±0.04[b]	0.55±0.02[a]	0.23±0.04[b]	0.44±0.04[a]
	2.0	0.32±0.01[c]	0.40±0.01[a]	0.46±0.02[c]	0.17±0.07[b]	0.30±0.02[c]
Yeast extract						
	0.5	0.45±0.01[a]	0.45±0.02[ac]	0.43±0.01[a]	1.12±0.08[a]	0.45±0.03[a]
	1.0	0.50 ±0.00[a]	0.55±0.07[bc]	0.91±0.04[b]	1.52±0.06[b]	0.58±0.03[b]
	1.5	0.45±0.02[a]	0.50±0.01[ac]	1.01±0.02[c]	1.40±0.03[c]	0.40±0.01[a]
	2.0	0.34±0.04[b]	0.40±0.01[ac]	0.71±0.03[d]	0.89±0.11[d]	0.29±0.02[c]
$NaNO_3$	0.5	0.03±0.01[ac]	0.03±0.00[a]	0.05±0.01[a]	0.02±0.00[a]	0.03±0.01[a]
	1.0	0.08±0.02[bc]	0.10±0.01[b]	0.07±0.00[b]	0.03±0.00[a]	0.06±0.00[b]
	1.5	0.05±0.01[abc]	0.06±0.01[c]	0.04±0.01[ac]	0.04±0.00[a]	0.03±0.00[ac]
	2.0	0.03±0.01[ac]	0.04±0.01[a]	0.02±0.00[c]	0.05±0.03[a]	0.02±0.01[a]
KNO_3	0.5	0.04±0.01[a]	0.05±0.01[ac]	0.04±0.03[ab]	0.04±0.01[a]	0.04±0.01[a]
	1.0	0.08±0.01[b]	0.12±0.01[b]	0.07±0.01[b]	0.09±0.01[b]	0.08±0.00[b]
	1.5	0.04±0.02[a]	0.07±0.01[c]	0.04±0.00[ab]	0.04±0.02[a]	0.05±0.02[a]
	2.0	0.02±0.00[a]	0.02±0.00[d]	0.03±0.01[ac]	0.02±0.00[a]	0.03±0.01[a]
NH_4NO_3	0.5	0.03±0.01[a]	0.04±0.01[a]	0.02±0.00[a]	0.04±0.01[a]	0.03±0.02[ac]
	1.0	0.09±0.01[b]	0.10±0.01[b]	0.07±0.00[b]	0.10±0.00[b]	0.08±0.01[b]
	1.5	0.06±0.02[c]	0.07±0.01[ab]	0.04±0.01[c]	0.07±0.01[c]	0.05±0.01[bc]
	2.0	0.03±0.01[a]	0.04±0.02[a]	0.02±0.01[a]	0.04±0.01[a]	0.03±0.01[ac]

Each value is a mean of two replicates; ± standard deviation among replicates; means with different letters within each column differ significantly ($p \leq 0.05$)

Table 4.10. Effect of Different Concentration of Nitrogen sources on the growth of Bacterial Isolates from sawdust

Nitrogen source		Isolates/Growth (O.D)540_{nm}				
Type	% Concentrat	*Actinomyces nueslundii* (3B)	*Anoxybacillus rupiensis* (5H)	*Actinomyces nueslundii* (6A1)	*Actinomyces nueslundii* (6A2)	*Actinomyces nueslundii* (6B)
Tryptone	0.5	0.68 ± 0.03^{ab}	0.40 ± 0.10^{a}	0.69 ± 0.08^{a}	0.56 ± 0.05^{ac}	0.70 ± 0.00^{a}
	1.0	1.04 ± 0.03^{b}	0.71 ± 0.01^{b}	0.99 ± 0.04^{b}	0.89 ± 0.08^{b}	0.87 ± 0.05^{b}
	1.5	0.66 ± 0.03^{a}	0.19 ± 0.06^{c}	0.81 ± 0.09^{a}	0.62 ± 0.01^{a}	1.03 ± 0.04^{c}
	2.0	0.84 ± 0.06^{c}	0.12 ± 0.02^{c}	0.64 ± 0.03^{a}	0.45 ± 0.04^{d}	0.21 ± 0.00^{d}
Yeast extract	0.5	0.75 ± 0.07^{a}	0.59 ± 0.03^{a}	0.51 ± 0.01^{a}	0.47 ± 0.01^{a}	0.46 ± 0.06^{a}
	1.0	1.08 ± 0.03^{b}	0.86 ± 0.08^{bc}	0.69 ± 0.04^{b}	0.60 ± 0.01^{b}	0.61 ± 0.04^{b}
	1.5	0.99 ± 0.03^{b}	0.78 ± 0.06^{bc}	1.13 ± 0.05^{c}	0.90 ± 0.01^{c}	1.11 ± 0.01^{c}
	2.0	0.50 ± 0.06^{c}	0.74 ± 0.06^{ac}	0.70 ± 0.03^{b}	0.62 ± 0.02^{b}	0.95 ± 0.06^{d}
$NaNO_3$	0.5	0.21 ± 0.01^{a}	0.04 ± 0.01^{a}	0.32 ± 0.05^{a}	0.33 ± 0.04^{a}	0.37 ± 0.07^{a}
	1.0	0.31 ± 0.01^{b}	0.03 ± 0.00^{a}	0.27 ± 0.05^{ab}	0.26 ± 0.07^{ab}	0.27 ± 0.04^{a}
	1.5	0.17 ± 0.03^{a}	0.02 ± 0.00^{b}	0.23 ± 0.04^{b}	0.21 ± 0.02^{b}	0.25 ± 0.00^{a}
	2.0	0.09 ± 0.00^{c}	0.01 ± 0.00^{c}	0.18 ± 0.08^{c}	0.17 ± 0.01^{b}	0.16 ± 0.07^{b}
KNO_3	0.5	0.13 ± 0.01^{a}	0.05 ± 0.01^{a}	0.08 ± 0.01^{a}	0.06 ± 0.01^{ab}	0.11 ± 0.02^{a}
	1.0	0.21 ± 0.01^{b}	0.12 ± 0.02^{b}	0.11 ± 0.01^{ba}	0.10 ± 0.03^{a}	0.18 ± 0.01^{b}
	1.5	0.12 ± 0.02^{ac}	0.06 ± 0.01^{a}	0.06 ± 0.02^{ac}	0.06 ± 0.04^{ab}	0.10 ± 0.02^{a}
	2.0	0.08 ± 0.02^{c}	0.03 ± 0.01^{a}	0.04 ± 0.01^{c}	0.04 ± 0.01^{b}	0.06 ± 0.02^{a}
NH_4NO_3	0.5	0.04 ± 0.01^{a}	0.06 ± 0.01^{a}	0.08 ± 0.03^{a}	0.10 ± 0.01^{a}	0.07 ± 0.01^{a}
	1.0	0.07 ± 0.01^{ba}	0.14 ± 0.06^{b}	0.12 ± 0.03^{a}	0.14 ± 0.01^{b}	0.13 ± 0.01^{b}
	1.5	0.06 ± 0.01^{a}	0.09 ± 0.02^{a}	0.08 ± 0.01^{a}	0.09 ± 0.01^{ac}	0.09 ± 0.02^{a}
	2.0	0.02 ± 0.00^{c}	0.05 ± 0.01^{a}	0.04 ± 0.01^{b}	0.06 ± 0.01^{ac}	0.05 ± 0.01^{a}

Each value is a mean of two replicates; ± standard deviation among replicates; means with different letters within each column differ significantly ($p \leq 0.05$

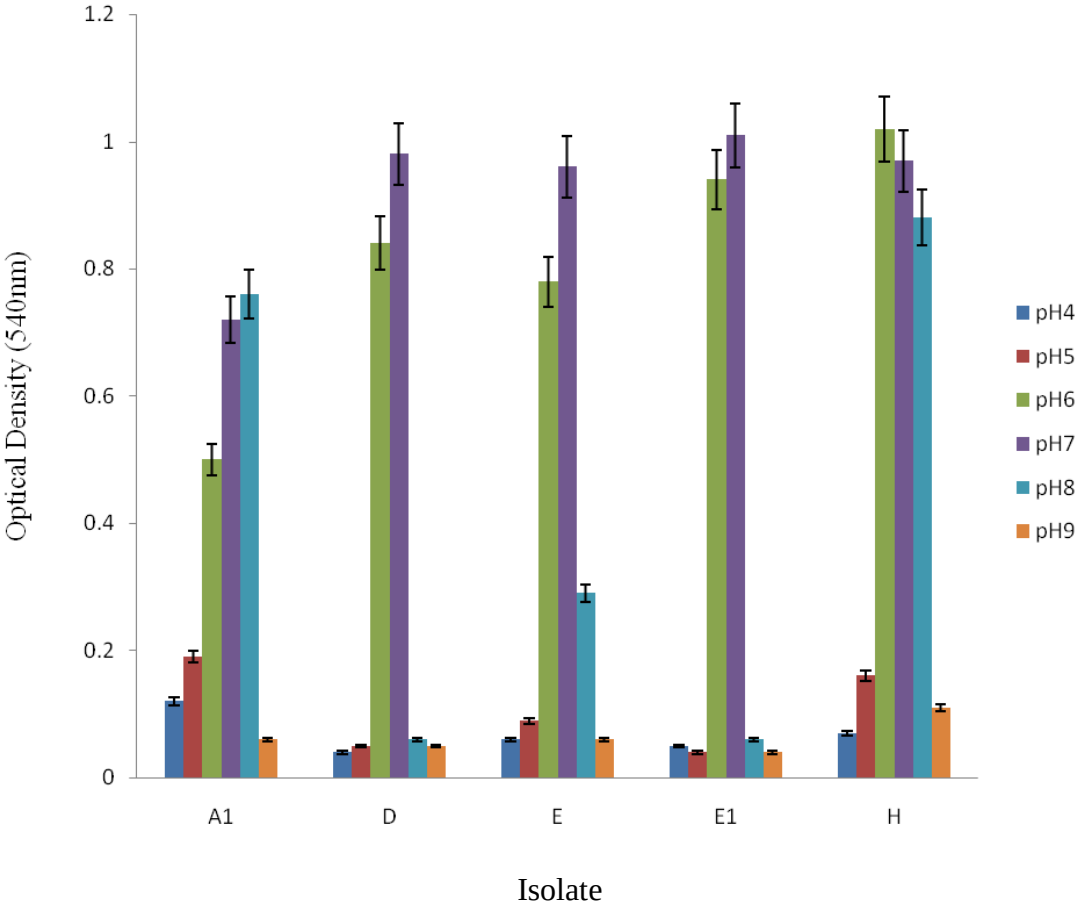


Figure 4.1. Effect of pH on the growth of the Isolates. Data are presented as mean of 2 replicates, standard deviation within the range 0-0.8 A1=*Roseomonas* sp., E1= *Anoxybacillus rupiensis*, D, E and H =*Pseudomonas aeruginosa*

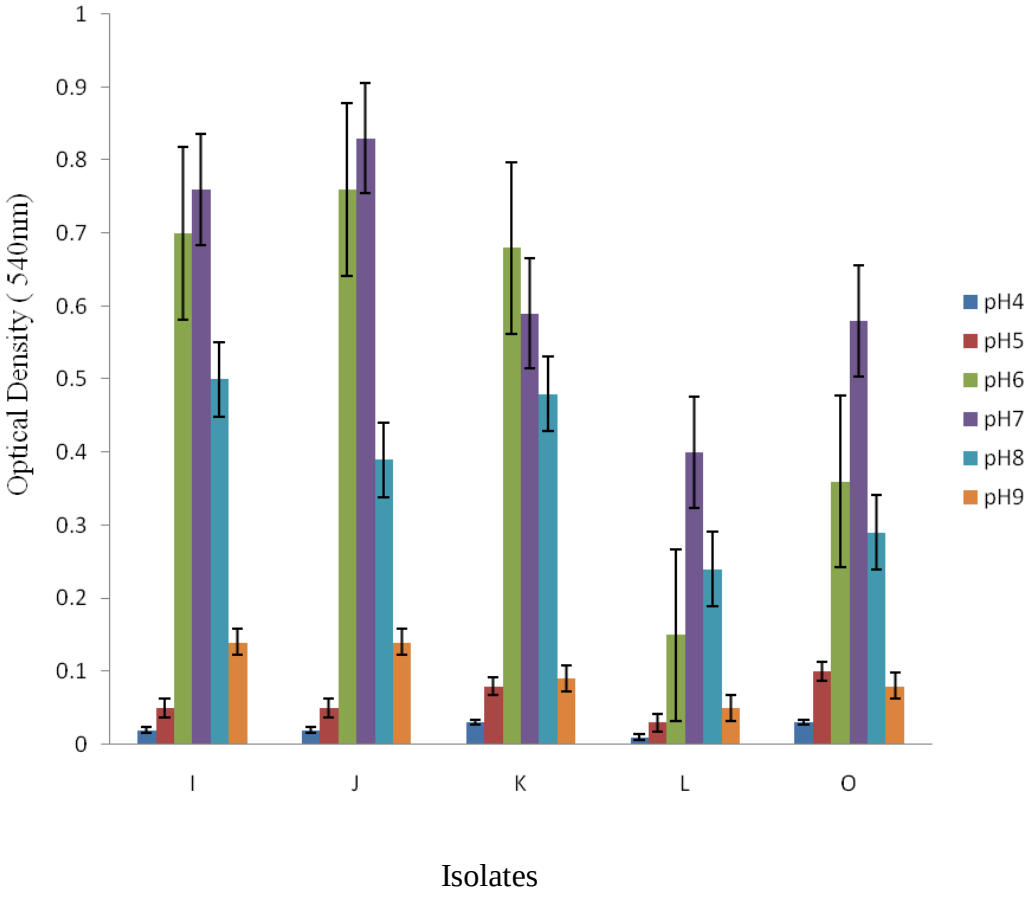


Figure 4.2. Effect of pH on the growth of the Isolates. Data are presented as mean of 2 replicates, standard deviation within the range 0-0.8 I-O=*Pseudomonas aeruginosa*

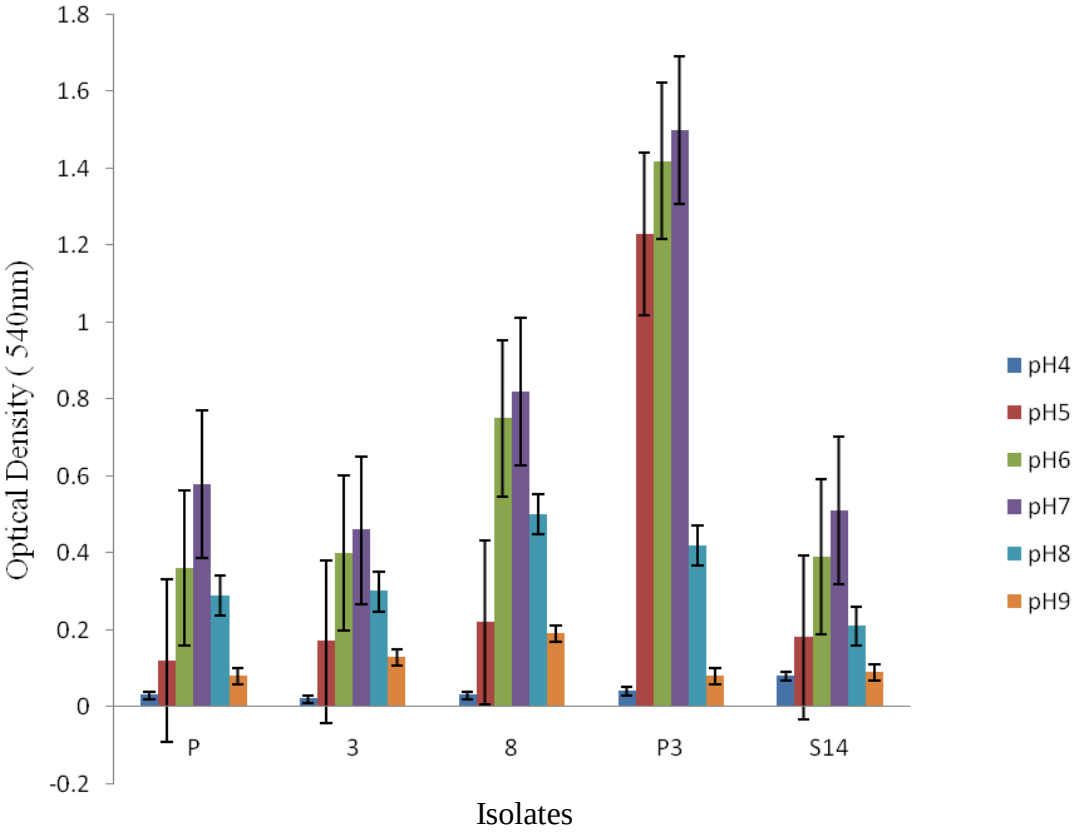


Figure 4.3. Effect of pH on the growth of the Isolates. Data are presented as mean of 2 replicates, standard deviation within the range 0-0.8 P–S14 = *Pseudomonas aeruginosa*, *P3* = *Thermoactinomyces vulgaris*.

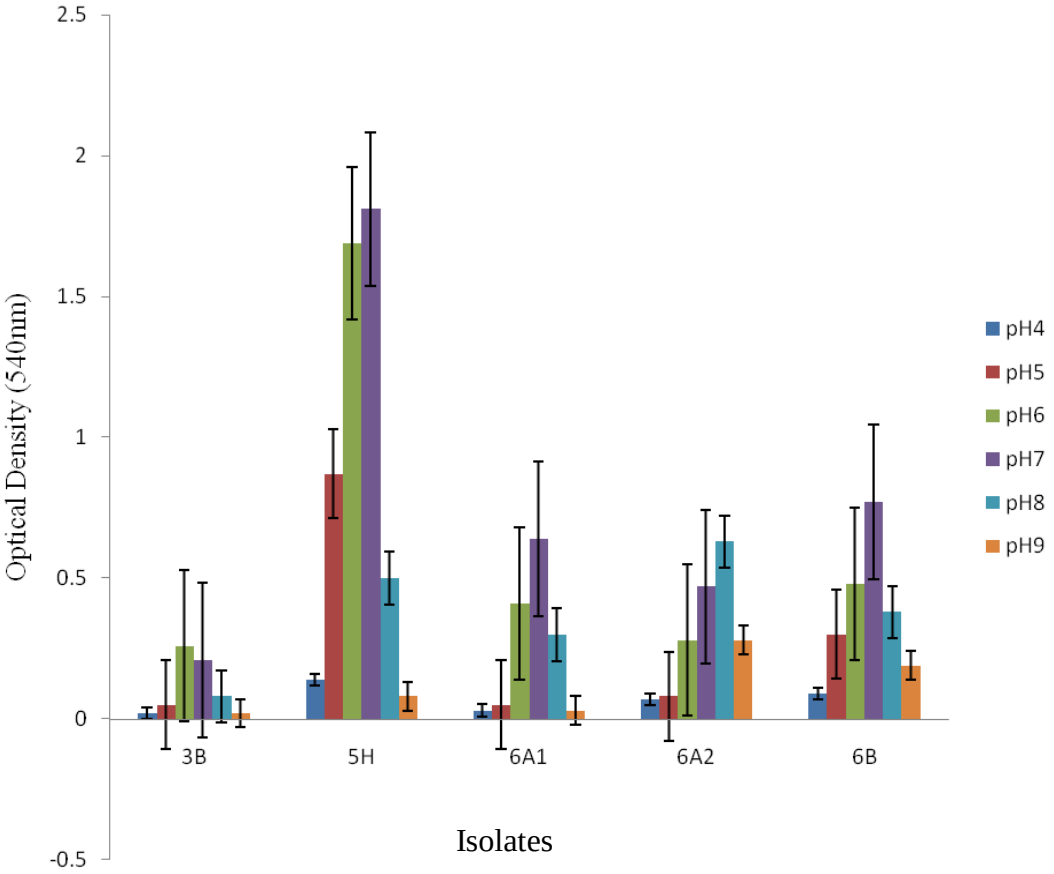


Figure 4.4. Effect of pH on the growth of the Isolates. Data are presented as mean of 2 replicates, standard deviation within the range 0-0.8 3B- 6B= *Actinomyces nueslundii*, 5H= *Anoxybacillus rupiensis*

4.6 **Effect of Temperature on the growth of the isolates**

Growth of the isolates in response to temperature changes were shown in Figures 4.5 to 4.8. The growth of the isolates, *Roseomonas* sp. (A1), *Pseudomonas aeruginosa* (D, E and H) and *Anoxybacillus rupiensis* (E1) showed that as temperature increased from 45^0C to 50^0C there were gradual increase in the growth of isolates D, E, E1 and H. The growth then decreased mildly beyond optimal temperature of 50^0C to 55^0C and sharply to 60^0C. However, the optimal temperature for A1 was 55^0C after which there was decrease in growth. The maximum growth of 0.86 $(O.D)_{540nm}$ was recorded at 50^0C for isolate E.

Figure 4.6 showed the growth profile of *Pseudomonas aeruginosa* (I, J, K, L and O). The growth increased gradually from 45^0C to 50^0C for all the isolates and decreased from 50^0C to 60^0C. The least growth of 0.10 $(O.D)_{540nm}$ was recorded for isolate I at 60^0C. Similar trend was observed in all the *Pseudomonas aeruginosa* (P, 3, 8, and S14) and *Thermoactinomyces vulgaris* (P3) where the increase in growth was also between the temperature of 45^0C and 50^0C. However, while the increment in the growth of P, 3 and S14 was gradual, that of 8 and P3 were sharp. The growth then decreased gradually for P, 3 and 8 whereas sharply decreased for P3 and S14. The highest growth value was noticed in P3 at temperature of 50^0C.

In Figure 4.8, three isolates, *Anoxybacillus rupiensis* (5H) and *Actinomyces nueslundii* (6A2 and 6B) had their maximum growth values at 55^0C while the highest growth was observed for 5H. 3B and 6A1 had their best growth at 50^0C. The increment in the growth was gradual for 6A1 and 6A2 from 45^0C to 50^0C. The highest growth 1.8 $(O.D)_{540nm}$ was observed for 5H at 55^0C. The decrease in the growth of 3B and 6A1 from 50^0C to 55^0C as well as from 55^0C to 60^0C for 5H was sharp.

4.7 **Effect of nitrogen sources on the cellulase production**

Effect of different nitrogen compounds on cellulase production by the isolates was investigated in the media having nitrogen sources at 1.0% (w/v) concentration (Figures 4.9-4.12). The growth of *Roseomonas* sp. (A1), *Pseudomonas aeruginosa* (D, E and H) and *Anoxybacillus rupiensis* (E1) was studied in relation to tryptone, Sodium nitrate ($NaNO_3$), tryptophan, yeast extract and Ammonium chloride (NH_4Cl) (Figure 4.9). The best utilized nitrogen containing compound for the five isolates was tryptone. This was followed by tryptophan and yeast extract when E1 was grown. The highest cellulase produced (3.0 U/ml), was by E1. This value was followed with 2.6

U/ml of cellulase activity by *Roseomonas* sp. (A1). $NaNO_3$ was the least utilized by all isolates except E which had its least cellulase produced in the medium containing tryptophan.

The best cellulase stimulating nitrogen compound for *Pseudomonas aeruginosa* (I, J, K, L and O) was tryptone (Figure 4.10). All the isolates metabolised the compounds to produce different amount of the enzyme. Isolate O produced the highest amount of cellulase and this immediately followed by K and J which had the least amount cellulase with tryptone as nitrogen source when compared with other isolates. However, the least cellulase produced in this figure was by K when tryptophan was incorporated into the medium.

Figure 4.11 showed that the highest amount of cellulase (2.3 U/ml) and (1.7 U/ml) were produced by *Pseudomonas aeruginosa* (8) and *Pseudomonas aeruginosa* (3) in the presence of tryptophan. Yeast extract gave the maximum cellulase production by *Pseudomonas aeruginosa* (S14) while the highest cellulase production was achieved by *Pseudomonas aeruginosa* (P) and *Thermoactinomyces vulgaris* (P3) in the medium containing tryptone. The least value of the enzyme (0.25 U/ml) was produced by P3 in the presence of NH_4Cl.

Actinomyces nueslundii (3B, 6A1 and 6B) produced cellulase amount when tryptone was included in their medium when compared to other nitrogen compounds (Figure 4.12). The same was true of *Anoxybacillus rupiensis* (5H) whereas tryptophan was the best cellulase stimulatory nitrogen compound for *Actinomyces nueslundii* (6A2) with the value of 1.9 U/ml. The highest cellulase amount was produced by 6B.

4.8 **Effect of incubation temperature on the synthesis of cellulase**

Cellulase production increased with temperature increased from 45^0C to 50^0C for isolates A1, D, E, E1 and H after which the synthesis decreased gradually to 60^oC (Figure 4.13). The highest value of cellulase (3.52 U/ml) was produced by *Anoxybacillus rupiensis* (E1) at optimal temperature of 50^0C while the least enzyme production was by *Pseudomonas aeruginosa* (D) at 60^0C. The same trend of increase in cellulase production from 45^0C to 50^0C was observed for isolates I, J, K, L and O (all *Pseudomonas aeruginosa*) (Figure 4.14). The highest cellulase activity (3.25 U/ml) was produced by isolate I and the least activity (0.56 U/ml) was produced by isolate J at 60^0C.

Cellulase production rose as the temperature was raised from 45^0C to 55^0C for *Pseudomonas aeruginosa* (8) and fell to 60^0C (Figure 4.15). In the case of other isolates *Pseudomonas aeruginosa* (P, 3 and S14) and *Thermoactinomyces vulgaris* (P3), there was increase in cellulase production as the temperature increased from 45^0C to 50^0C, optimum temperature , beyond which the synthesis fell down to 60^0C. The highest value (3.5 U/ml) was produced by 8 at optimal temperature of 55^0C.

The optimal temperature for cellulase production for all isolates was 50^0C (Figure 4.16). The highest cellulase activity (4.5 U/ml) was produced by *Actinomyces nueslundii* (6B) and the least value (0.8 U/ml) was also produced by the same organism at 60^0C. Cellulase level rose and then fell at the temperature above the optimum temperature of production.

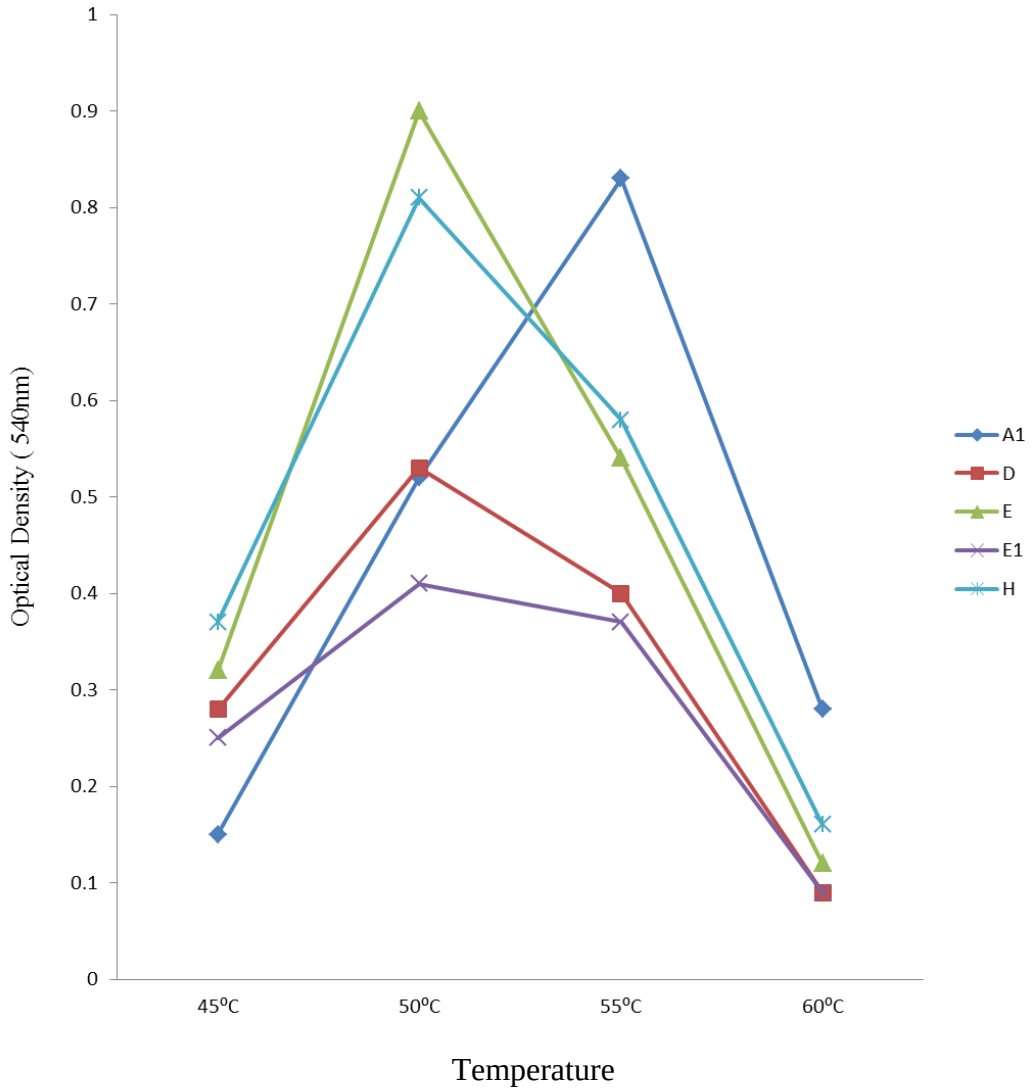


Figure 4.5. Effect of temperature on the growth of the isolates, Data are presented as a mean of two replicates, with standard deviation within the range 0-0.8, A1=*Roseomonas* sp., D, E and H=*Pseudomonas aeruginosa*, *E1= Anoxybacillus rupiensis.*

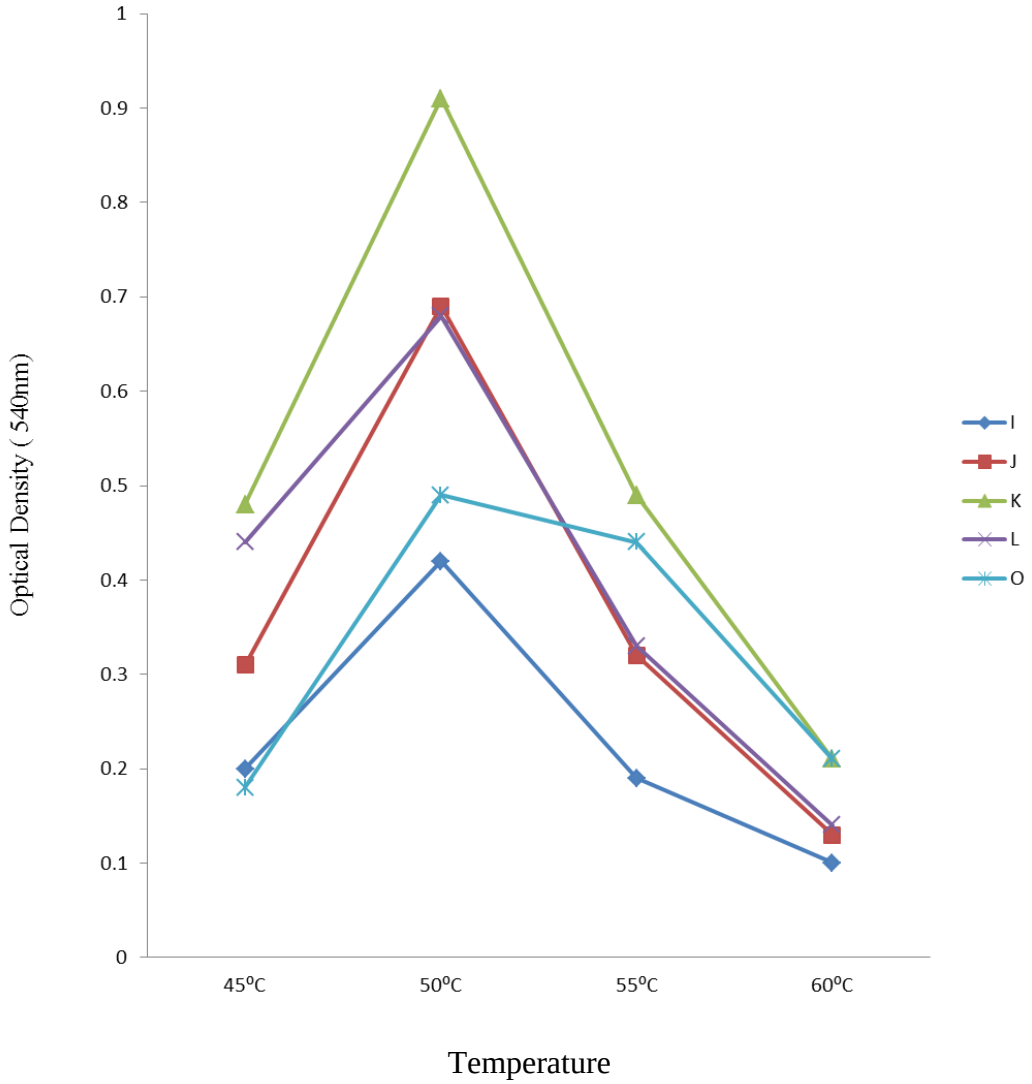


Figure 4.6. Effect of temperature on the growth of the isolates, Data are mpresented as a mean of two replicates, with standard deviation within the range 0-0.8. I-O= *Pseudomonas aeruginosa*

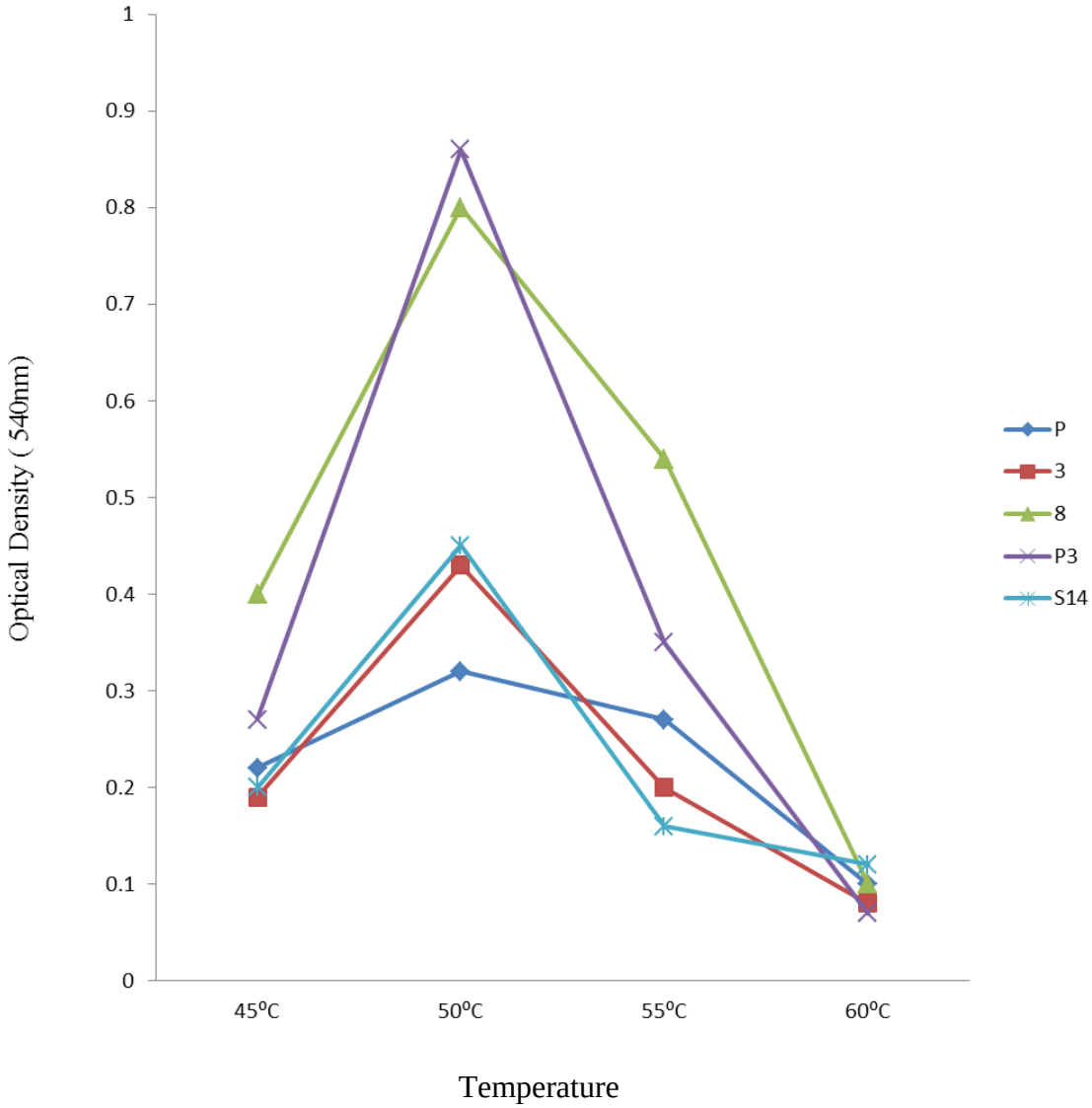


Figure 4. 7. Effect of temperature on the growth of the isolates, Data are presented as a mean of two replicates, with standard deviation within the range 0-0.8. P3=*Thermoactinomyces vugaris*, P-S14= *Ps. aeruginosa*

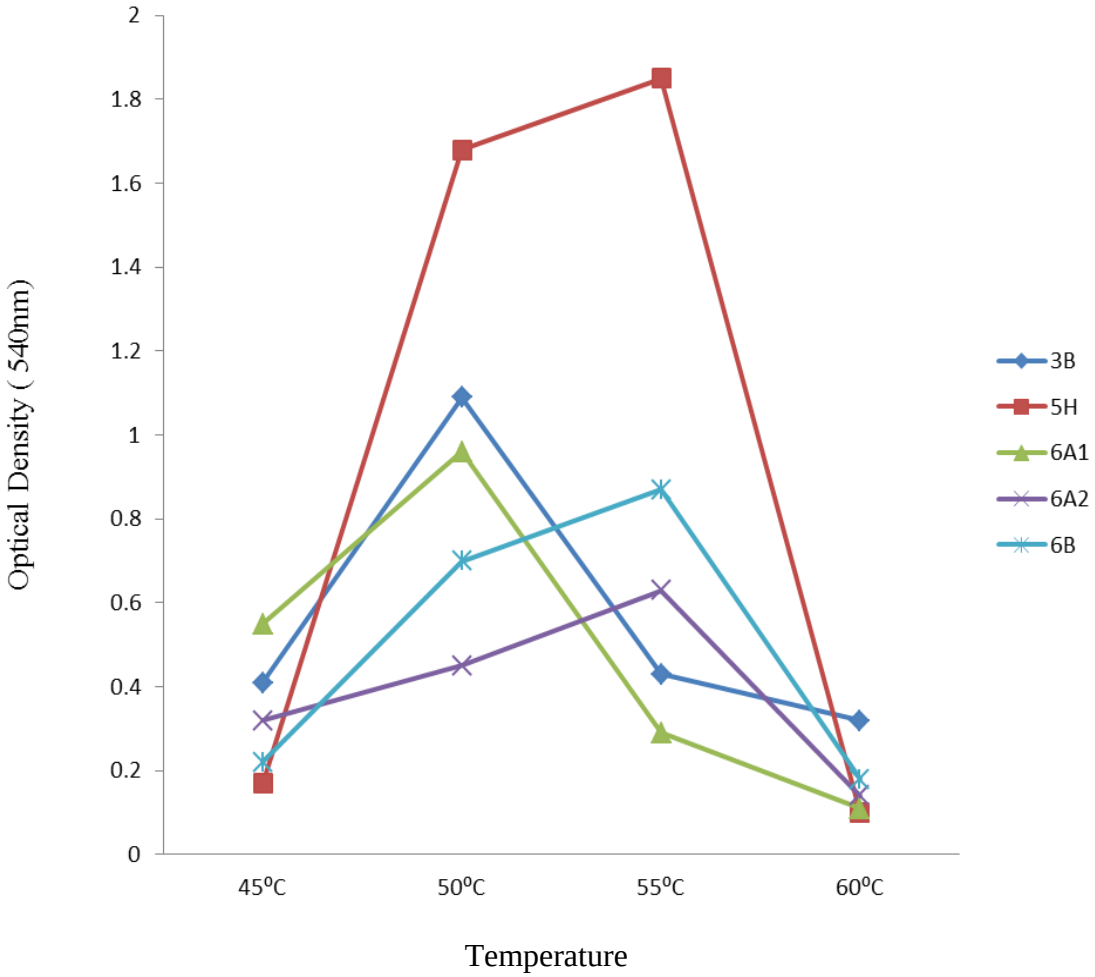


Figure 4.8. Effect of temperature on the growth of the isolates, Data are presented a as mean of two replicates, with standard deviation within the range 0-0.8.5H=*Anoxybacillus rupiensis*,3B-6B= *Actinomyces nueslundii*

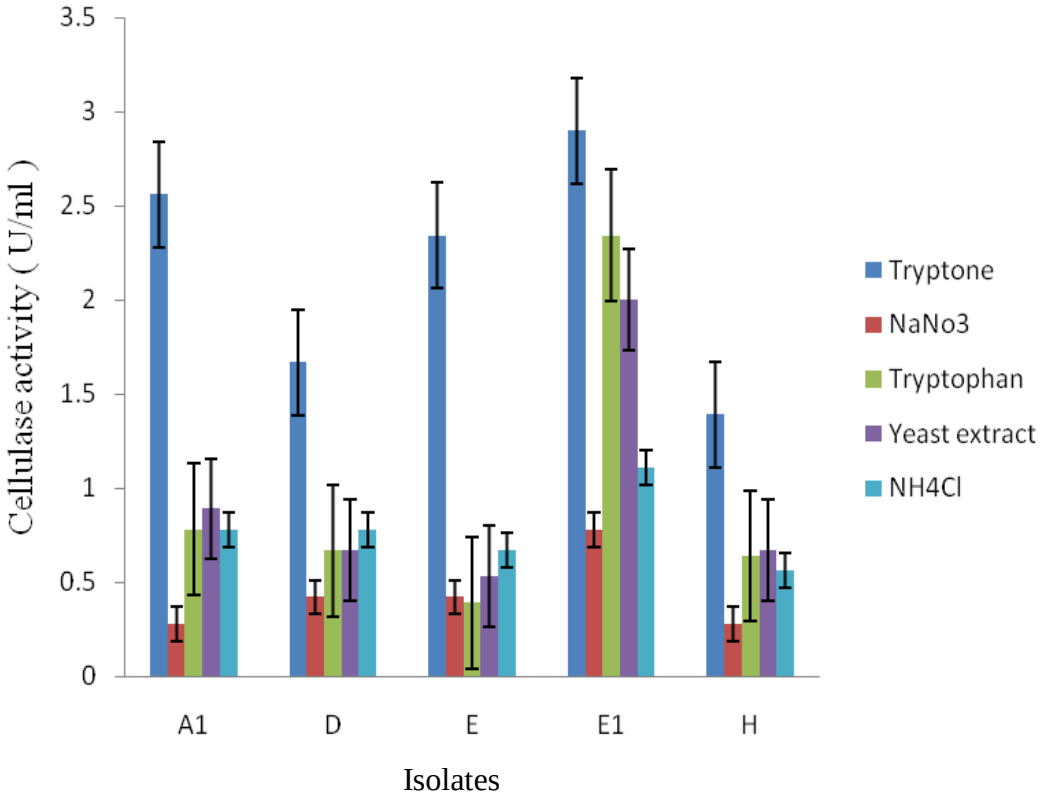


Figure 4.9. Effect of Nitrogen sources on cellulase synthesis by bacteria isolated rom sawdust. Data are presented as a mean of 2 replicates, with standard deviation within the range 0-0.3. A1= *Roseomonas* sp. D, E&H=*Pseudomonas aeruginosa* and E1=*Anoxybacillus rupiensis*.

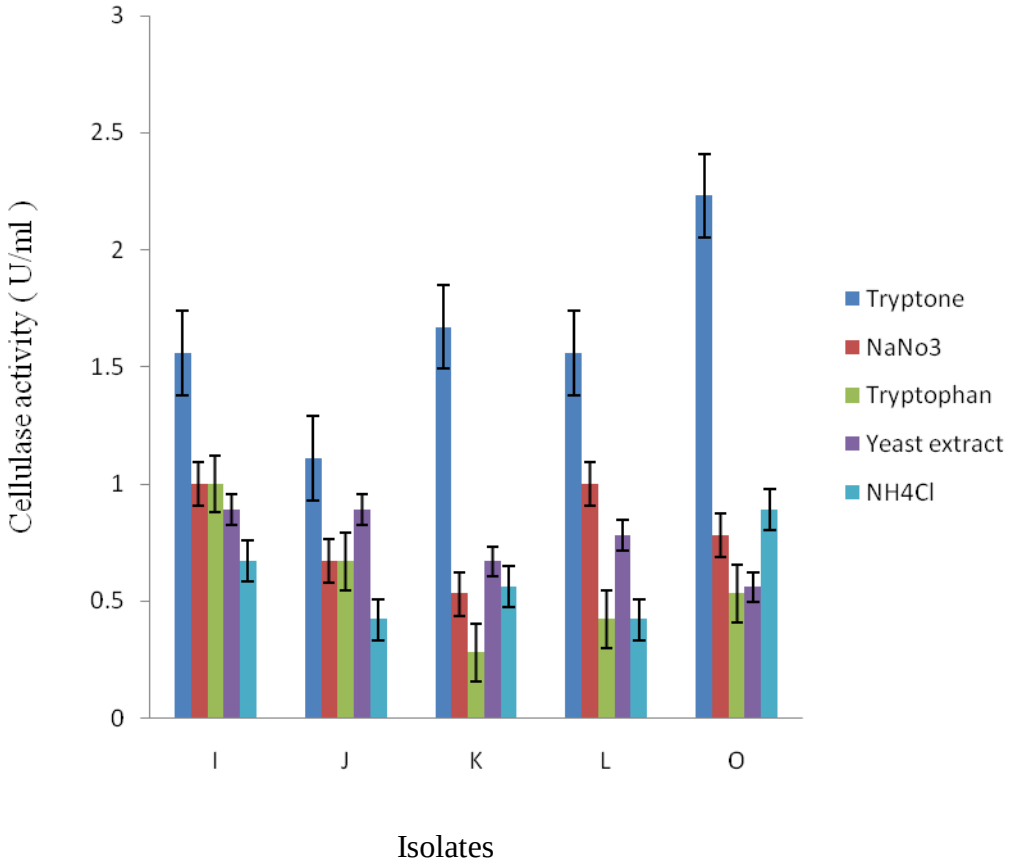


Figure 4.10. Effect of Nitrogen sources on cellulase synthesis by bacteria isolated from sawdust. Data are presented as a mean of 2 replicates, with standard deviation within the range 0-0.3. I-O=*Pseudomonas aaeruginosa*

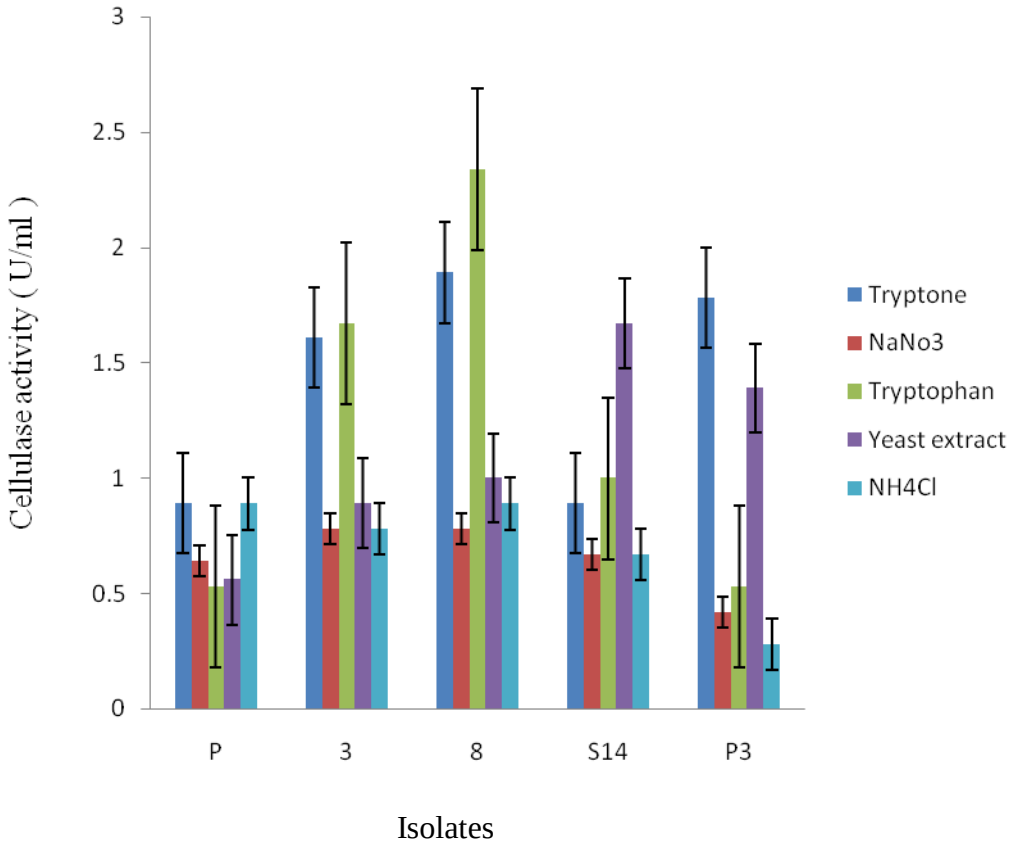


Figure 4.11. Effect of Nitrogen sources on cellulase synthesis by bacteria isolated from sawdust. Data are presented as a mean of 2 replicates, with standard deviation within the range 0-0.3. P-S14 = *Ps. aeruginosa*, *P3 = Thermoactinomyces vulgaris*

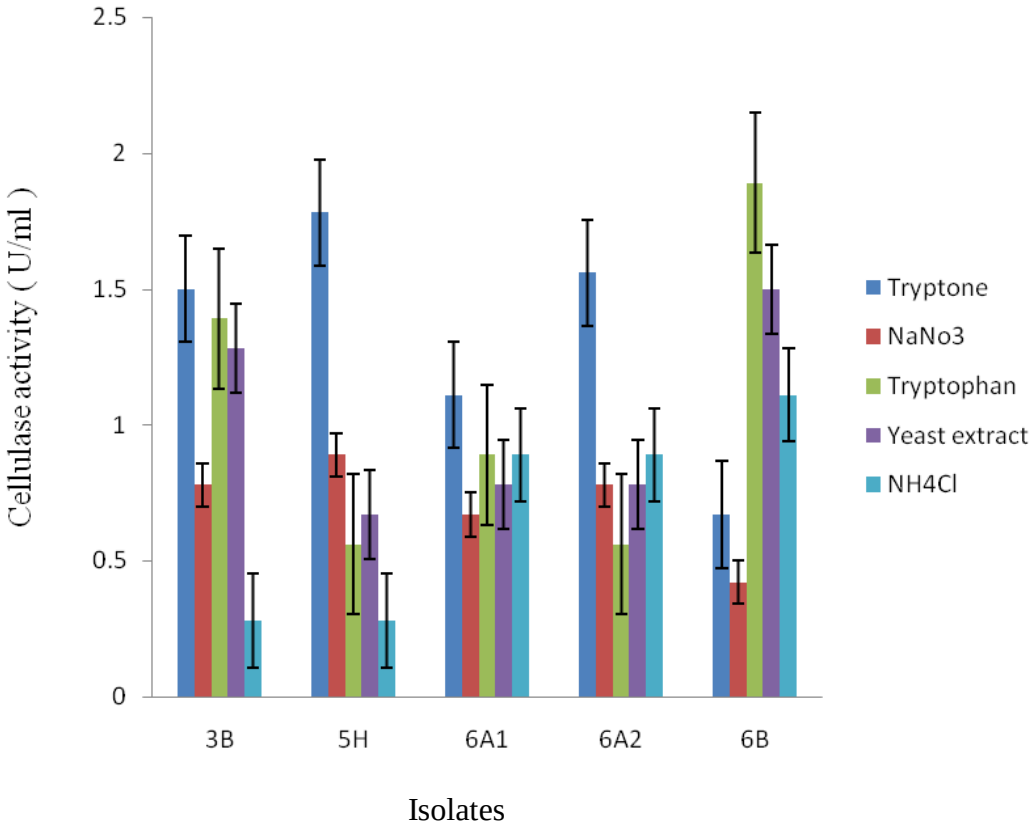


Figure 4.12. Effect of Nitrogen sources on cellulase synthesis by bacteria isolated isolated from sawdust. Data are presented as a mean of 2 replicates, with standard deviation within the range 0-0.3. 3B-6B= *Actinomyces nueslundii, 5H=Anoxybacillus rupiensis*

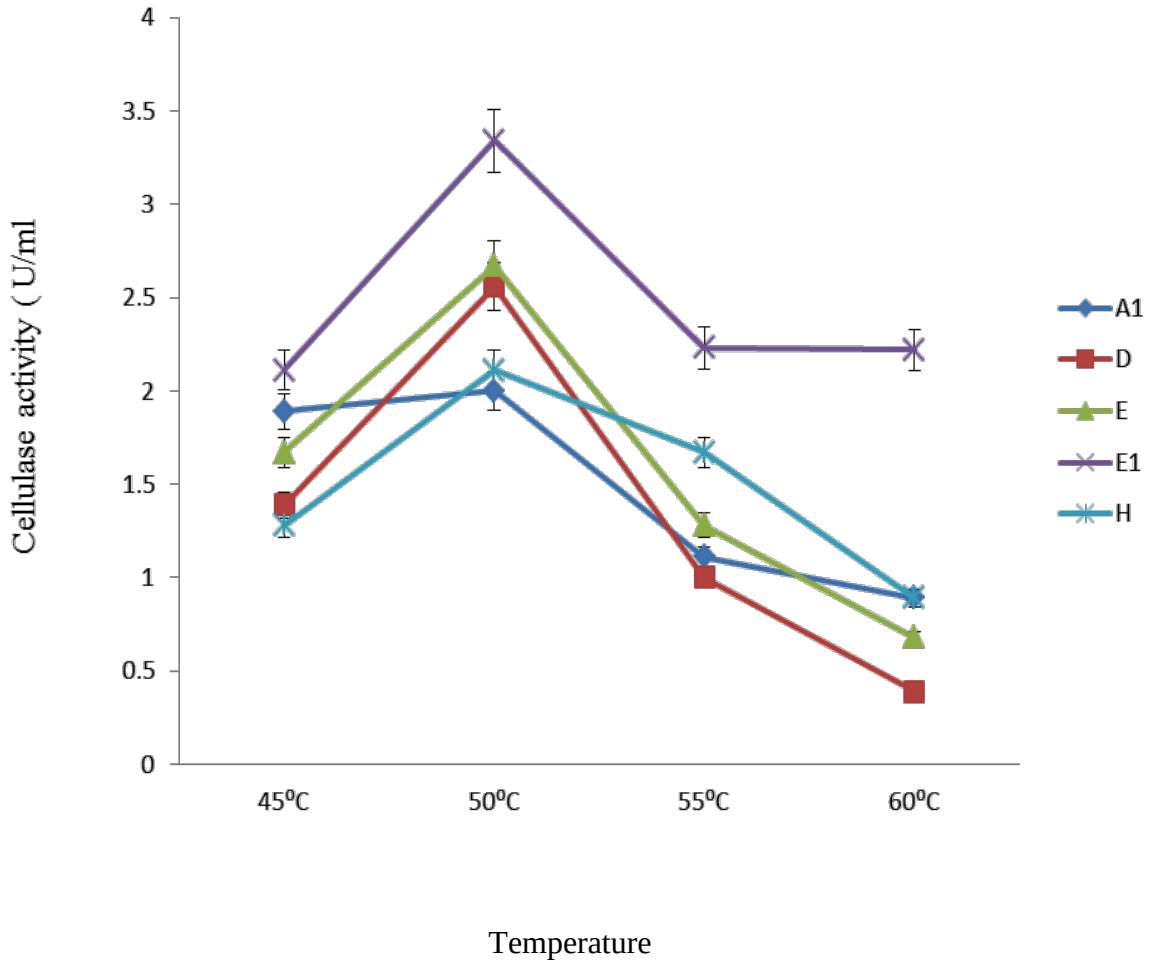


Figure 4.13. Effect of incubation temperature on the synthesis of cellulase by bacteria isolated from sawdust. Data are presented as a mean of 2 replicate with standard deviation within the range 0-0.3. A1= *Roseomonas* sp, D, E &H=*Pseudomonas aeruginosa*, E1=*Anoxybacillus rupiensis.*

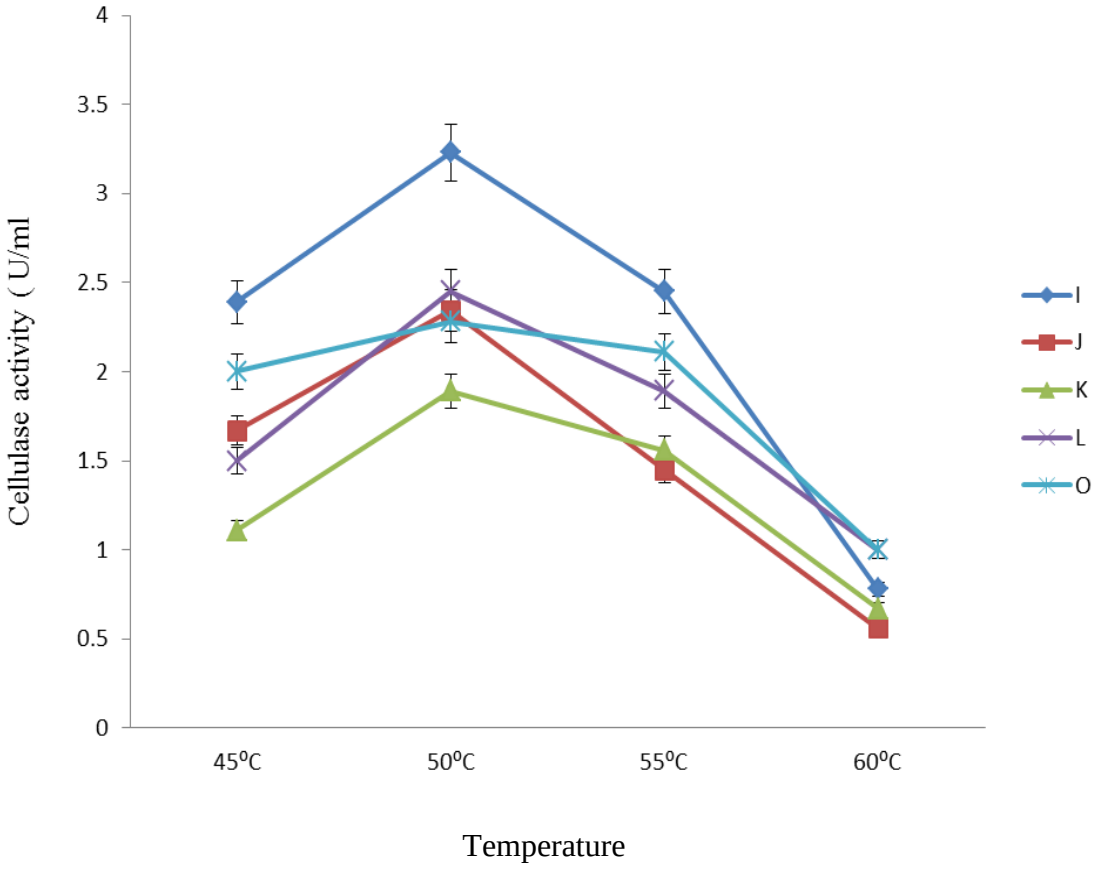


Figure 4.14. Effect of incubation temperature on the synthesis of cellulase by bacteria isolated from sawdust. Data are presented as a mean of 2 replicates, with standard deviation within the range0-0.3.I-O=*Ps. aeruginosa*

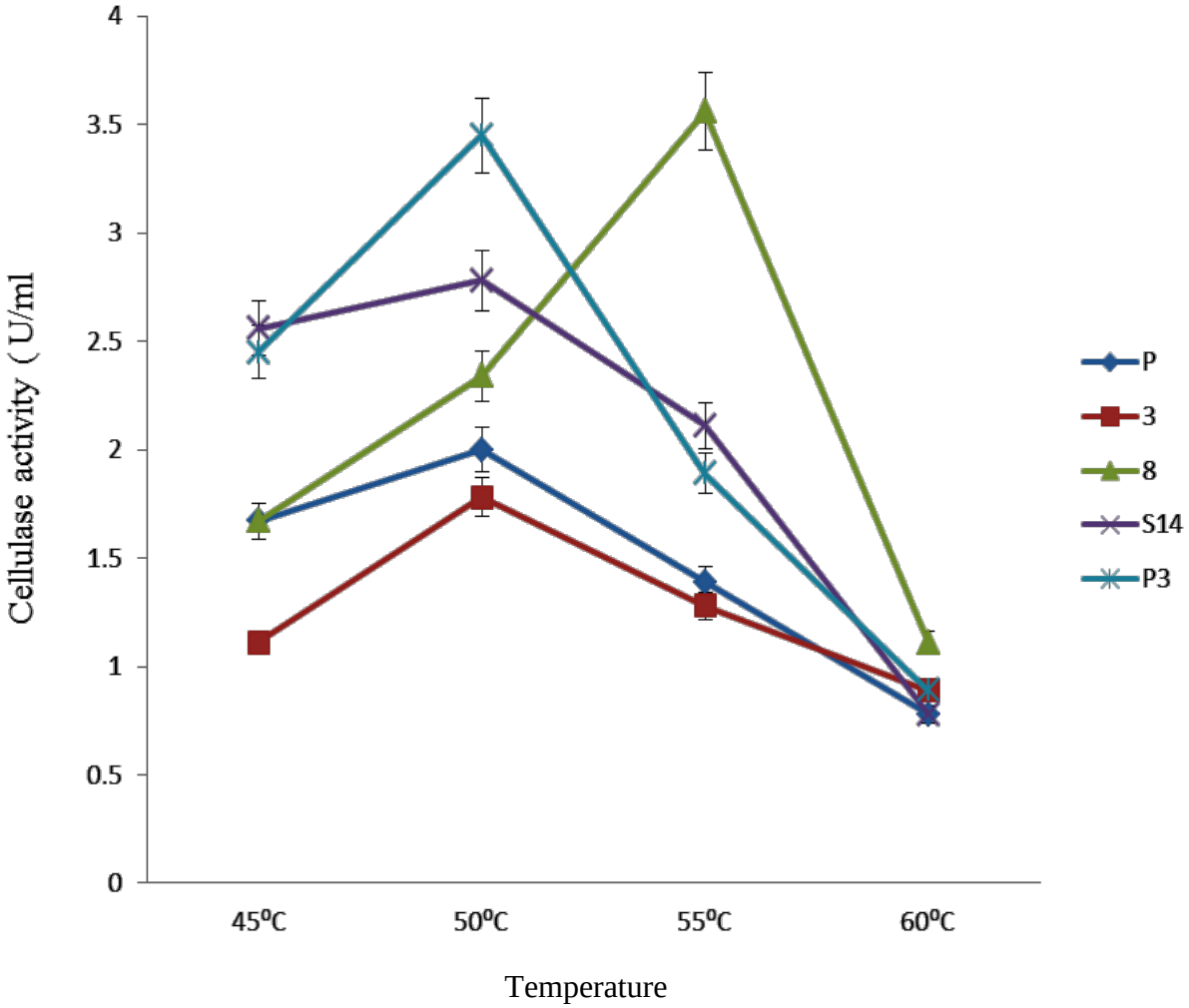


Figure 4.15. Effect of incubation temperature on the synthesis of cellulase by bacteria isolated from sawdust. Data are presented as a mean of 2 replicates with standard deviation within the range 0-0.3. P-S14=*P.s aeruginosa*, *P3= Thermoactinomyces vulgaris*

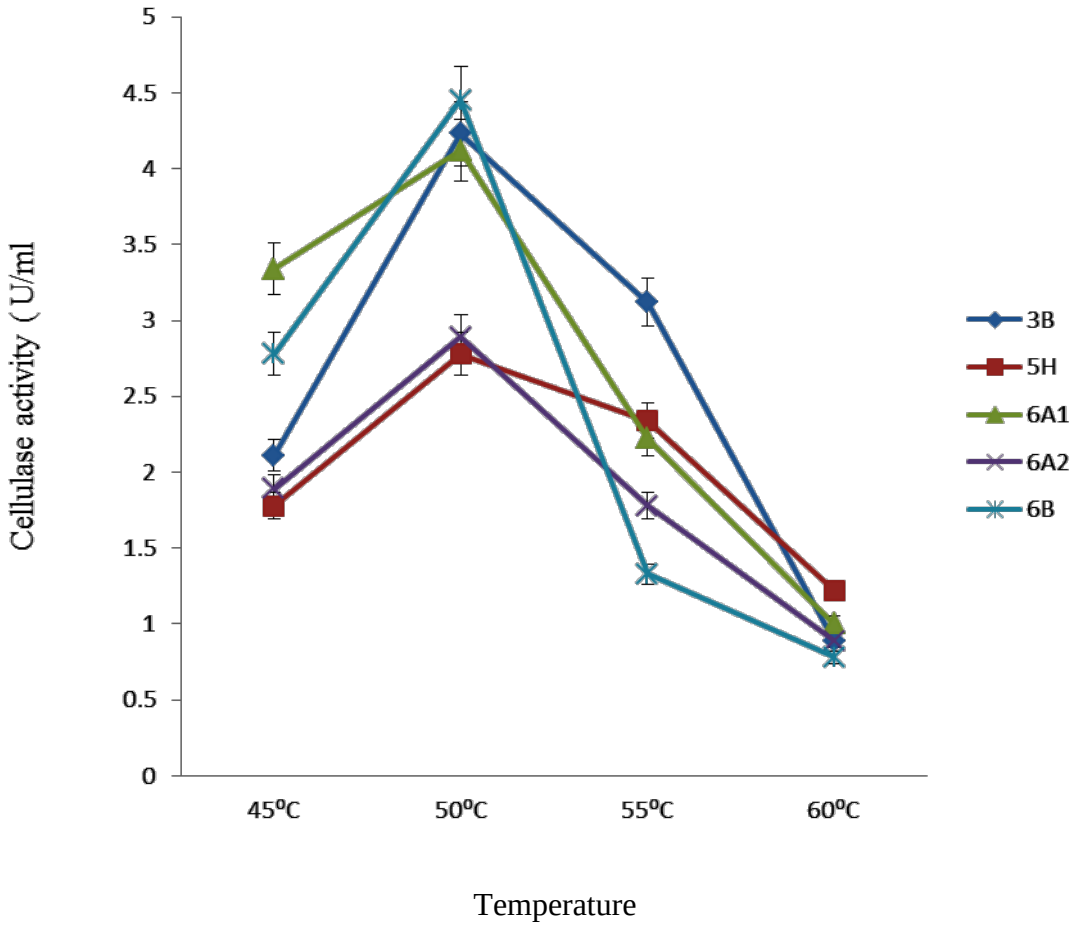


Figure 4.16. Effect of incubation temperature on the synthesis of cellulase by sbacteria isolated from sawdust. Data are presented as a mean of 2 replicates with standard deviation within the range 0-0.3. 3B-6B=*Actinomyces nueslundii, 5H= Anoxybacterium rupiensis*

4.9 **Effect of temperature on the activity of the purified cellulase**

For *Roseomonas* sp. cellulase activity increased from 45^0C and achieved the optimum at 60^0C. Thereafter there was decrease in activity. However, there was no noticeable difference in the activity of cellulase for *Pseudomonas aeruginosa* (D) at both 45^0C and 50^0C. The activity then increased to 55^0C and fell gradually to 70^0C. The cellulase activity of *Pseudomonas aeruginosa* (E and H) and *Anoxybacillus rupiensis* increased gradually from 45^0C to 55^0C and decreased thereafter to 70^0C. Cellulase from *Anoxybacillus rupiensis* gave the highest activity value (8.2 U/ml) at 55^0C while the least activity value (2.0 U/ml) was recorded at 70^0C incubation of isolate E.

Cellulase activity increased from 45^0C to 50^0C for isolates I and K while the increment was up 55^0C J, L and O (all *Pseudomonas aeruginosa*) (Figure 4.18). Highest enzyme activity (4.52 U/ml) was produced by L at optimal temperature of 55^0C. The decrease from the optimal temperature to 70^0C was gradual for the enzyme from J, K and O while from 65^0C to 70^0C was sharp for I and L.

Cellulase from all the isolates in Figure 4.19 had their activity increased from 45^0C to 55^0C except that from isolate S14 whose optimal temperature of activity was 50^0C. The increment activity was gradual for cellulase from *Ps. aeruginosa* (P, 3 and 8) between 45^0C and 55^0C while it was sharp between 50^0C and 55^oC enzyme from *Thermoactinomyces vulgaris* (P3). The highest activity value (6.4 U/ml) was obtained from enzyme produced by 8.

Cellulase activity of isolates 3B and 5H increased gradually from 45^0C to 50^0C and achieved a maximum at 55^0C, thereafter decreased to 70^0C (figure 4.20). The increase in the cellulase activity of 6A1 was gradual up 60^0C from 45C whereas the maximum activity was recorded for cellulase from isolate 6B at optimal temperature of 50^0C. The activity decreased gradually to 70^0C. The highest enzyme activity (7.8 U/ml) was obtained from cellulase produced by *Anoxybacillus rupiensis* (5H).

4.10 **Effect of pH on cellulase production**

Figure 4.21-4.24 depicts interaction of pH in relation to cellulase production by the isolates at different pH values. There was general increase in the production of cellulase as pH increased from 4.0 to 7.0 for most of the isolates.

The cellulases of D, E, E1 and H were optimally produced at pH 7.0 while that of A1 was produced optimally at pH 6.0 (Figure 4.21). The least value of 0.45 U/ml was produced by E at pH 9.0.

Isolates I, J, L and O had their maximum cellulase production at pH 7.0 while it was pH 6.0 for isolate K and the highest cellulase production was from isolate I (Figure 4.22). The amount of cellulase produced by *Ps. aeruginosa* (P and 3) increased gradually from pH 4.0 to 7.0 after which the production decreased sharply to pH 8.0 and mildly to pH 9.0 (Figure 4.23). Maximum production was at pH 7.0 for *Ps. aeruginosa* (P, 3 and S14) and *Thermoactinomyces vulgaris* (P3) whereas it was at pH 6.0 for *Ps. aeruginosa* (8).

The cellulase from *Anoxybacillus rupiensis* (5H) *Actinomyces nueslundii* (6A1, 6A2 and 6B) had their optimal production at pH7.0 while that of *Actinomyces nueslundii* (3B) was optimally produced at pH 6.0 (Figure 4.24). The highest cellulase activity (4.34U/ml) was produced by 6A1.

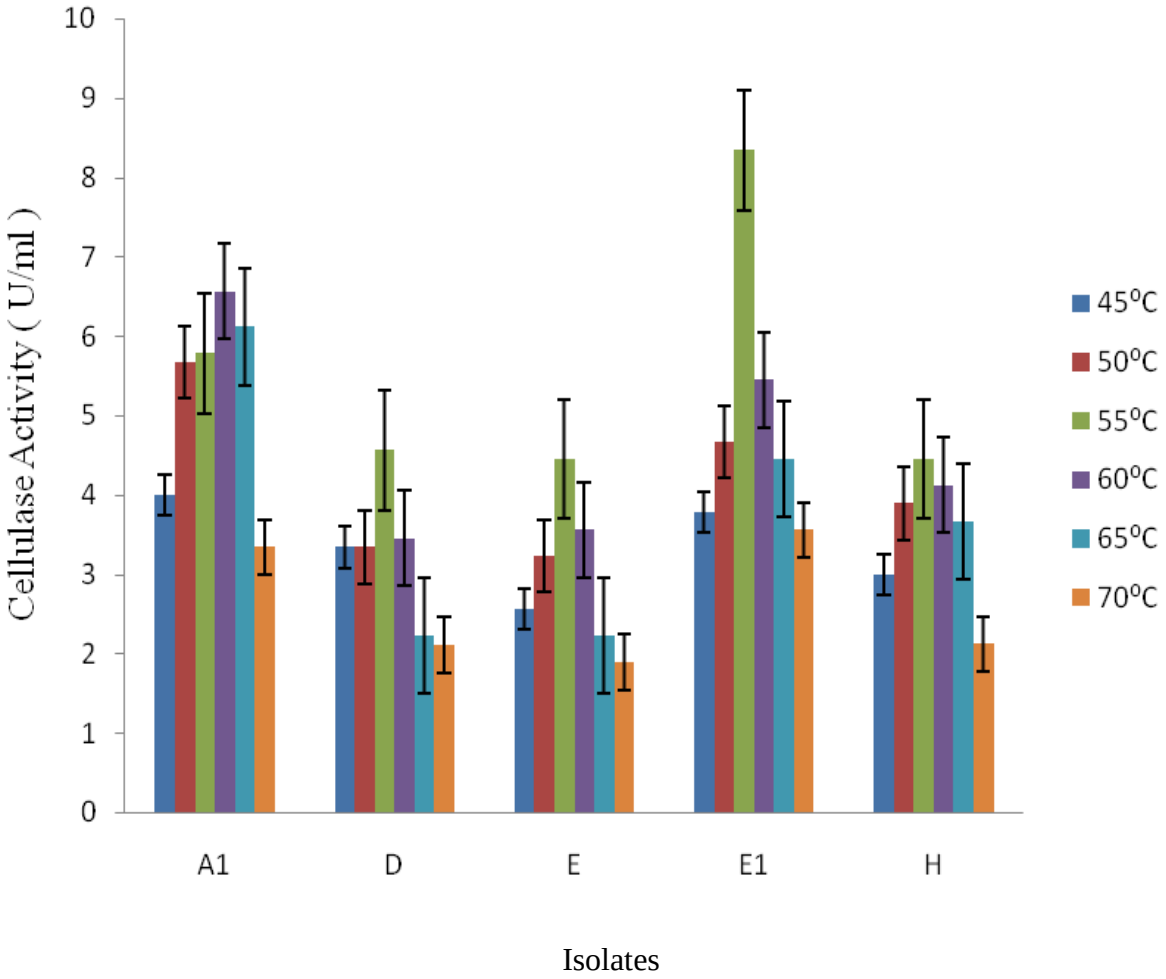


Figure 4.17. Effect of temperature on the activity of cellulase produced by bacteria isolated from sawdust. Data are presented as a mean of 2 replicates, with standard deviation within the range 0-0.3.A1= *Roseomonas* sp., D, E & H= *Pseudomonas aeruginosa*, E1= *Anoxybacillus rupiensis.*

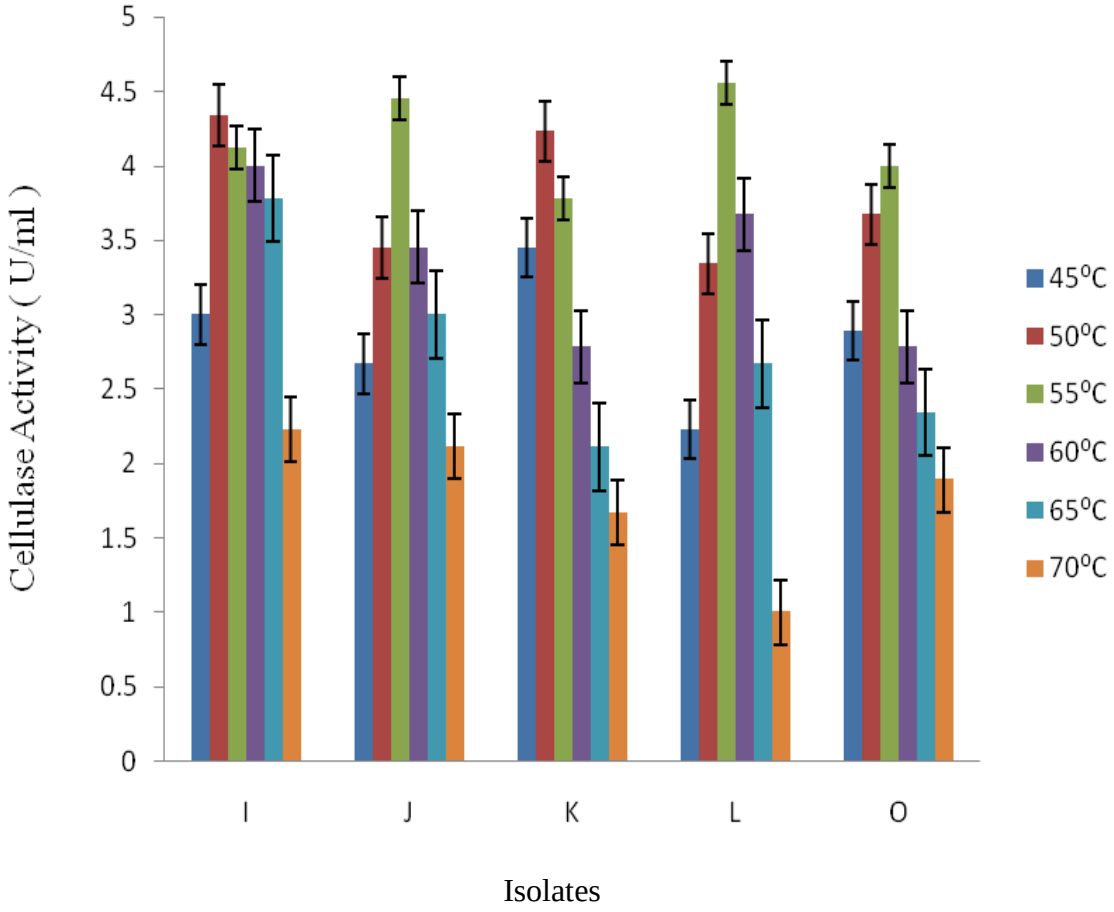


Figure 4.18. Effect of temperature on the activity of cellulase produced by bacteria isolated from sawdust. Data are presented as a mean of 2 replicates, with standard deviation within the range 0-0.3. *I-O=Pseudomonas aeruginosa*

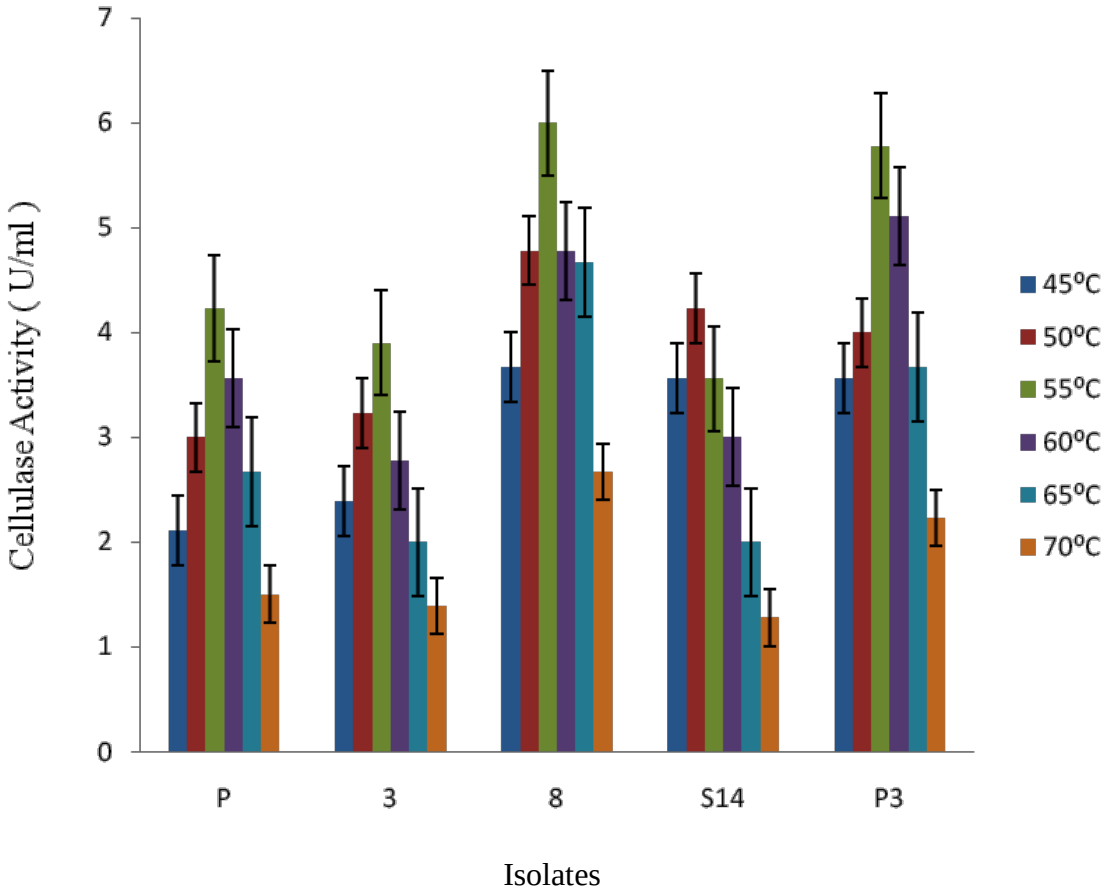


Figure 4.19. Effect of temperature on the activity of cellulase produced by bacteria isolated from sawdust. Data are presented as a mean of 2 replicates, with standard deviation within the range 0-0.3. P-S14=*Pseudomonas aeruginosa* ,*P3= Thermoactinomyces vulgaris*

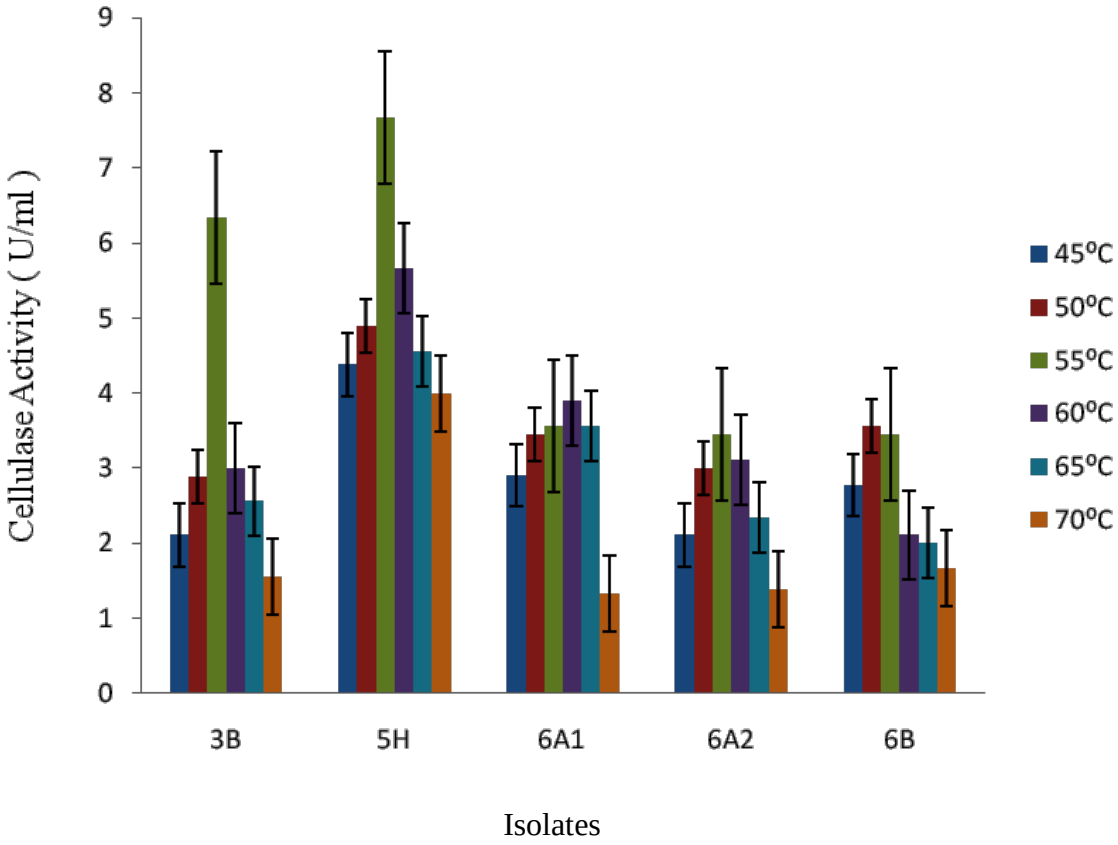


Figure 4.20. Effect of temperature on the activity of cellulase produced by bacteria isolated from sawdust. Data are presented as a mean of 2 replicates, with standard deviation within the range 0-0.3. 3B-6B= *Actinomyces nueslundii,5H= Anoxybacillus rupiensis*

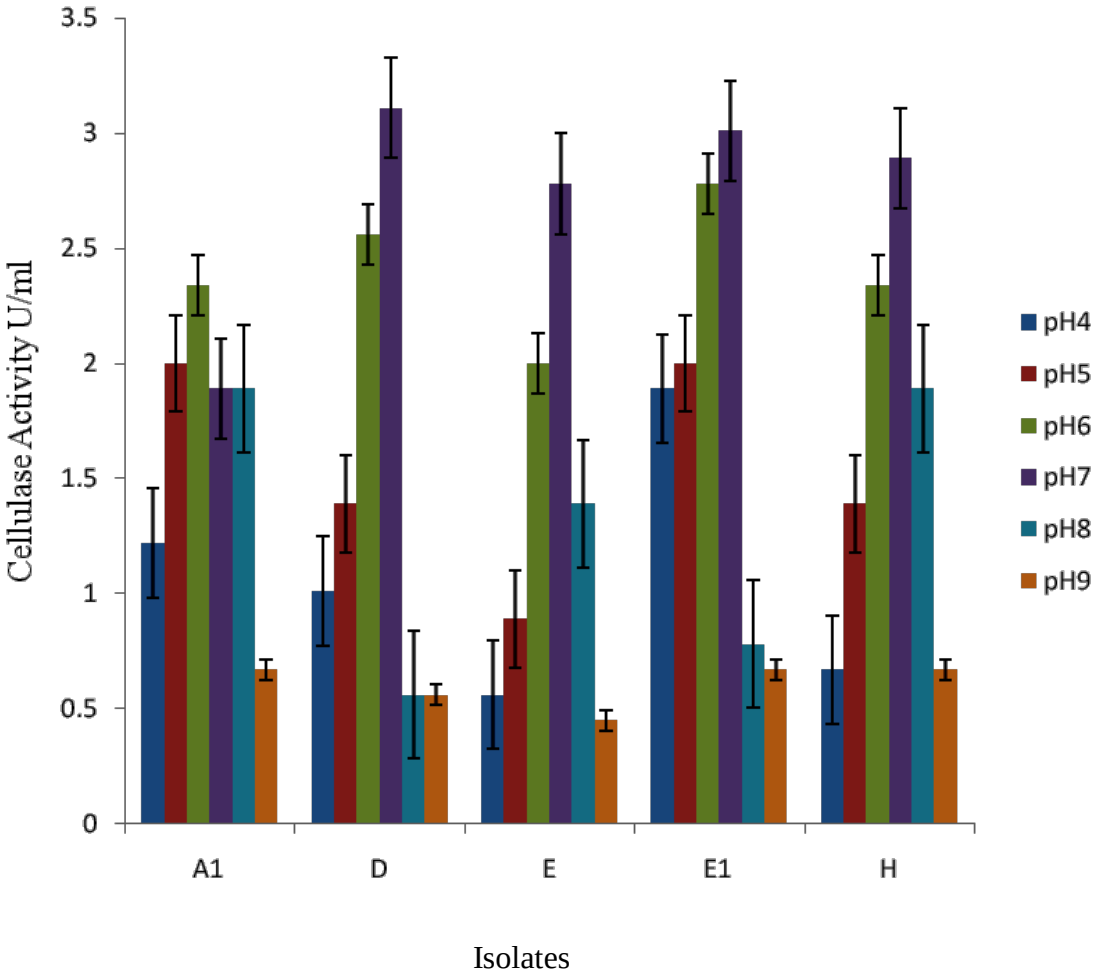


Figure 4.21. Effect of pH on the cellulase synthesis by bacteria isolated from sawdust. Data are presented as a mean of 2 replicates, with standard deviation within the range 0- 0.7. A1- *Roseomonas* sp. D, E& H= *Pseudomonas aeruginosa, E1= Anoxybacillus rupiensis.*

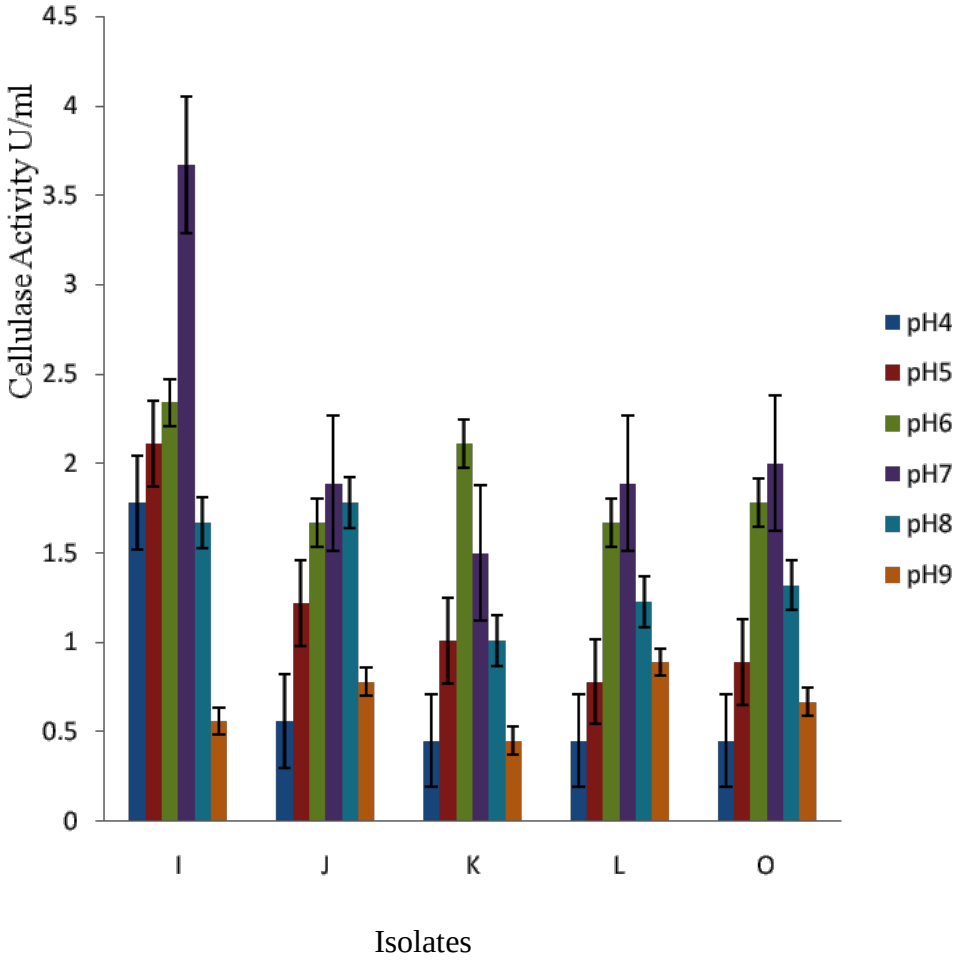


Figure 4.22. Effect of pH on the cellulase synthesis by bacteria isolated from sawdust. Data are presented as a mean of 2 replicates, with standard deviation within the range 0- 0.7.I-O= *Pseudomonas aeruginosa*

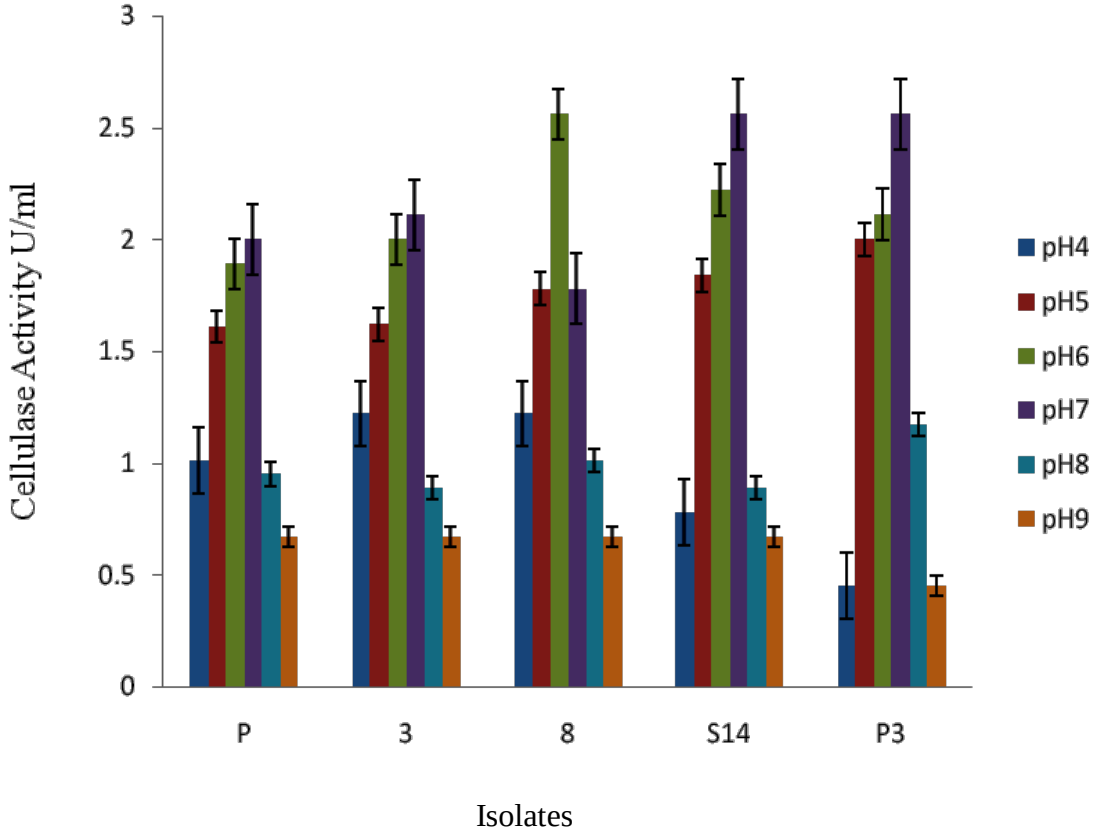


Figure 4.23. Effect of pH on the cellulase synthesis by bacteria isolated from sawdust. Data are presented as a mean of 2 replicates, with standard deviation within the range 0- 0.7.P-S14= *Ps. aeruginosa*, P3=*Thermoactinomyces vulgaris*

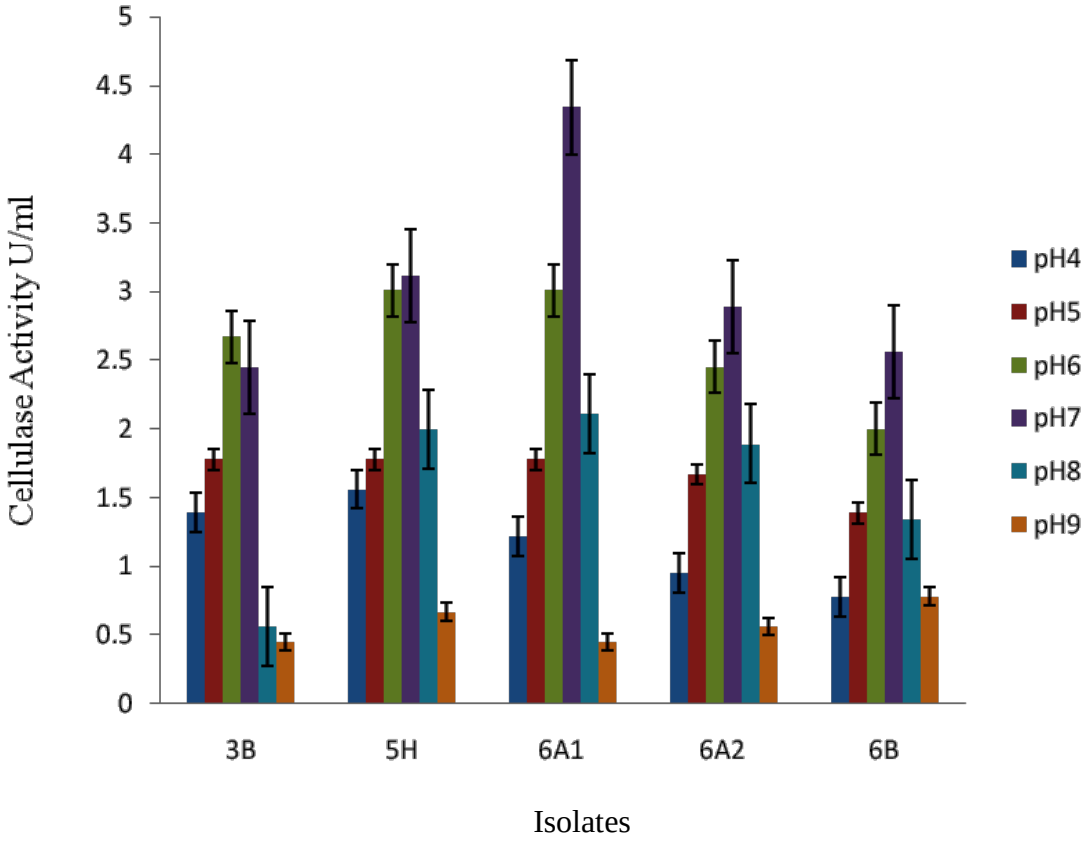


Figure 4.24. Effect of pH on the cellulase synthesis by bacteria isolated from sawdust. Data are presented as a mean of 2 replicates, with standard deviation within the range 0-0.7 .5H= *Anoxybacillus rupiensis*, 3B-6B= *Actinomyces nueslundii*

4.11 **Effect of pH on the activity of cellulase**

Activity of isolates A1, D, E, E1 and H cellulase at various pH values was determined using CMC (Figure 4.25). The cellulase of all the isolates had optimal activity at pH 7.0. The highest cellulase activity was for enzyme from E1 (4.7 U/ml). Enzyme activity increased as pH increased from 4.0 to 7.0 after which activity decreased for the cellulase from D, E and H. However, cellulase activity of enzyme from A1 and E1 increased to pH 9.0 after the initial decrease from pH 7.0 to 8.0.

Cellulase of *Ps. aeruginosa* (I, J, K, L and O) also had their optimal activity at pH 7.0 from where activity decreased to pH 9.0 (Figure 4.26). This optimal activity was followed by the activity at pH 8.0 for I and O while that of J, K and L was followed by activity at pH 6.0. The highest activity (3.8 U/ml) was obtained from cellulase produced by isolate I while the activity 0.5 U/ml was equally derived from the same organism at pH 4.0.

The cellulase of isolates P, 8, S14 and P3 had their optimal activity at pH7.0. There was no difference between the activity of cellulase from isolates 3 at pH 7.0 and 8.0. Apart from cellulase from isolates 8 and P3 whose activity increased at pH 9.0 having decreased between pH 7.0 and 8.0, cellulase from other isolates had their activity decreased down beyond the optimal activity. The highest activity was from isolate 8.

There was general gradual increase in the activity of cellulase from isolates 3B, 5H, 6a1, 6A2 and 6B from pH 4.0 to 6.0 (Figure 4.28). The optimal activity for cellulase from 3B, 6A1, 6A2 and 6B was at pH 7.0 while that of 5H was at pH 9.0. The increase in the activity between pH 6.0 and 7.0 was sharp for enzyme from 5H, 6A1, 6A2 and 6B. However, there was decrease in cellulase activity between pH 7.0 and 8.0 for the enzyme from 5H and 6B after which their enzyme activity increased at pH 9.0. The highest activity value was recorded for cellulase from 5H at pH 9.0.

4.12 **Screening of bacterial isolates for enzymatic activity**

The hydrolytic activities of the isolates were investigated by growing them in basal medium supplemented with CMC and then assaying for release of reducing sugar into the medium. All the twenty isolates tested exibited ability to produce reducing sugar. Isolates A1, E1, I, 8 and 5H produced significantly high amount of reducing sugar (0.78mg/ml, 0.84mg/ml, 0.72mg/ml, 0.86mg/ml and .092mg/ml respectively)

from CMC. The lowest value of 0.21mg/ml reducing sugar was observed with isolate P. (Table 4.11).

4.13 **Effect of different carbon sources on enzyme production**

High level of cellulase was recorded for *Roseomonas* sp. A1, *Ps. aeruginosa* D, I,8, *Anoxybacillus rupensis* E1and 5H, *Actinomyces nueslundii* 3B, 6A1, 6B and *Thermoactinomyces vulgaris* P3 in the presence of CMC 1%, lactose 1%, CMC1% for E1, I and 8, Galactose 2%, (3B) 1% (P3) 2% (6B) while it was CMC 1%and 1.5% for 5H and 6A1 respectively (Tables 4.12 and 4.13). The maximum level 10.01U/ml was observed for *Anoxybacillus rupiensis* E1. This was immediately followed by *Anoxybacillus rupiensis* 5H 9.22U/ml and *Roseomonas* sp. A1 9.00U/ml . Both glucose and sorbitol did not induce substantial amount of cellulase enzyme.

The highest Filter Paper Activity (FPA) 6.89U/ml was observed for A1 in 2% CMC as carbon source Table 4.14-4.15. This was followed by isolate 8 at 1% lactose as carbon source. Glucose was found to be poor FPA inducer.

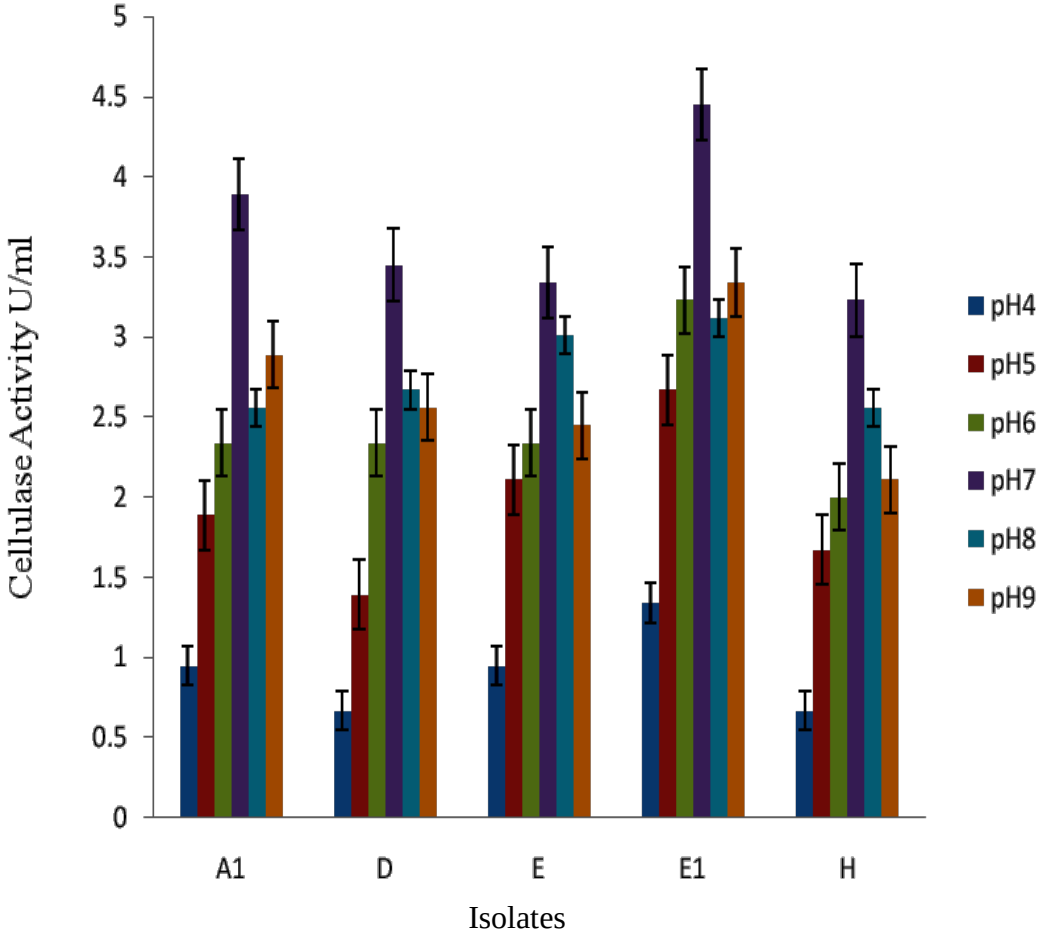


Figure 4.25. Effect of pH on the activity of cellulase produced by bacteria isolated from sawdust. Data are presented as a mean of 2 replicates, with standard deviation within the range 0-0.7. A1= *Roseomonas* sp, D, E & H= *Pseudomonas aeruginosa*, *E1= Anoxybacillus rupiensis.*

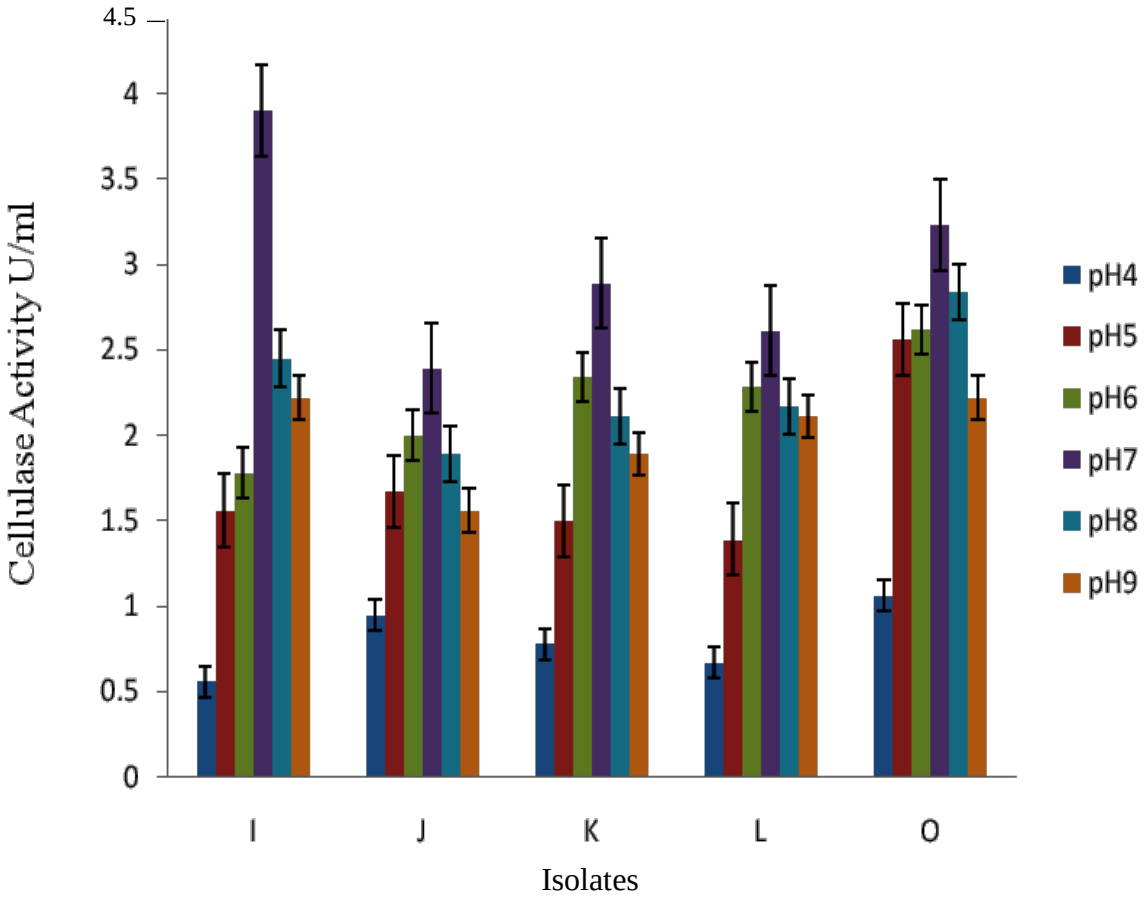


Figure 4.26. Effect of pH on the activity of cellulase produced by bacteria isolated from sawdust. Data are presented as a mean of 2 replicates, with standard deviation within the range 0-0.7 . I-O= *Pseudomonas aeruginosa*

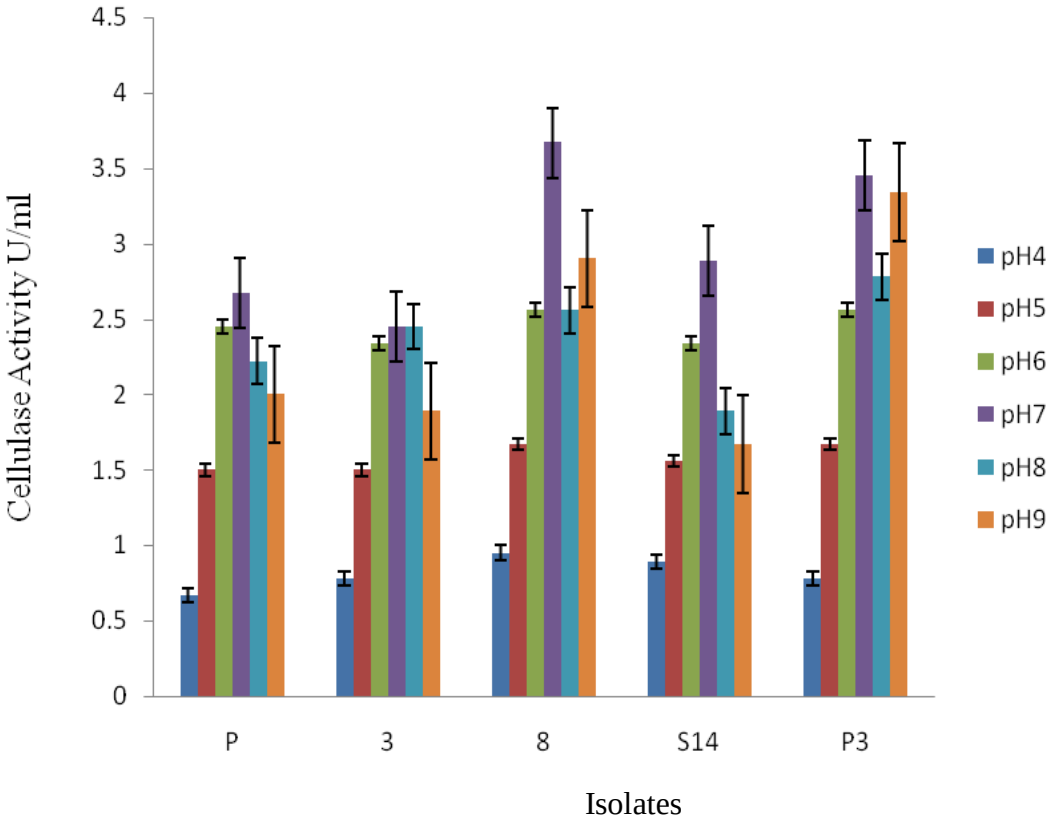


Figure 4.27. Effect of pH on the activity of cellulase produced by bacteria isolated from sawdust. Data are presented as a mean of 2 replicates, with standard deviation within the range 0-0.7. P-S14= *Pseudomonas aeruginosa*,P3= *Thermoactinomyces vulgaris*

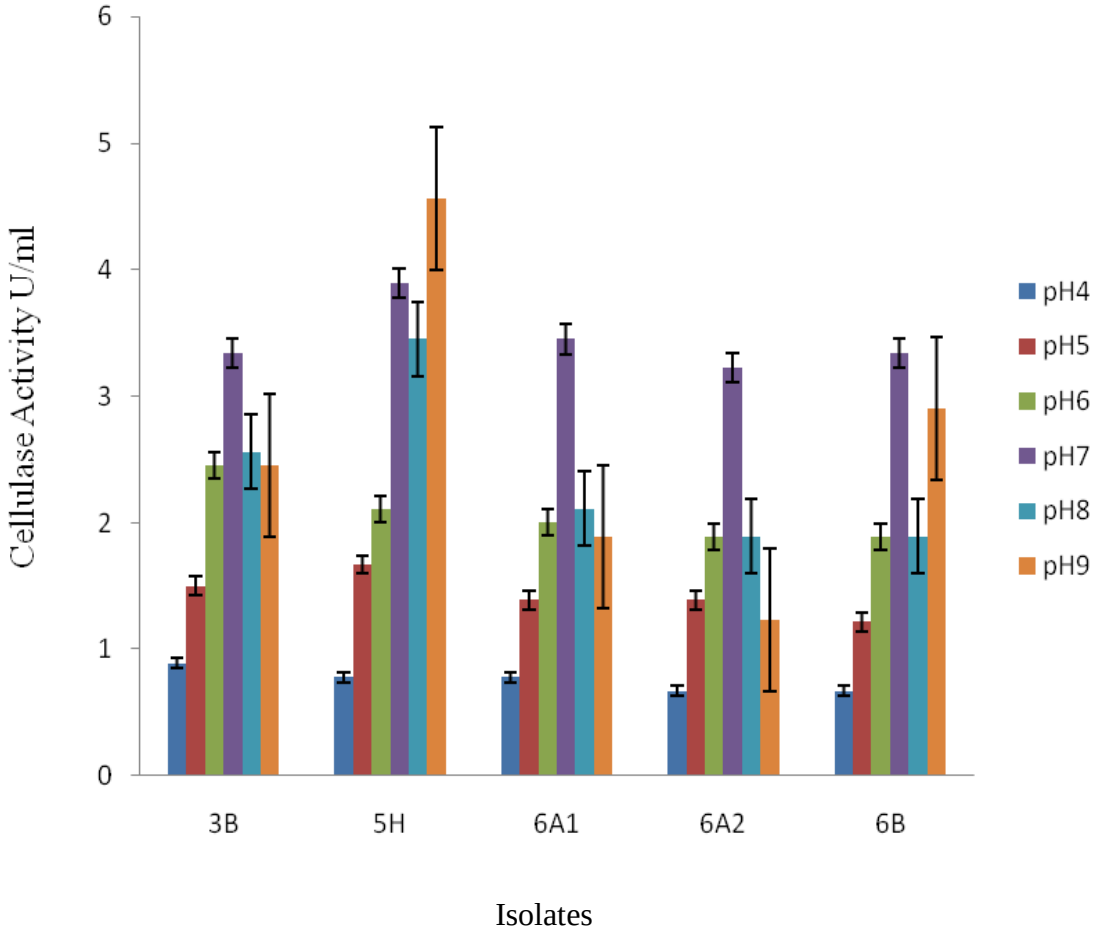


Figure 4.28. Effect of pH on the activity of cellulase produced by bacteria isolated from sawdust. Data are presented as a mean of 2 replicates, with standard deviation within the range 0-0.7 .3B-6B = *Actinomyces nueslundii*,5H= *Aoxybacillus rupiensis*

Table 4.11. Production of reducing sugar (mg/ml) by bacteria isolated from sawdust

Isolate code	Identity	Reducing sugar (mg/ml)
A1	*Roseomonas* sp.	$0.78\pm0.08^{a*}$
D	*Ps. aeruginosa*	0.42 ± 0.03^{bk}
E	*Ps. aeruginosa*	0.40 ± 0.06^{bk}
E1	*Anoxybacillus rupiensis*	0.84 ± 0.06^{al}
H	*Ps. aeruginosa*	0.34 ± 0.03^{bcgi}
I	*Ps. aeruginosa*	0.72 ± 0.06^{adhi}
J	*Ps. aeruginosa*	0.44 ± 0.11^{bk}
K	*Ps. aeruginosa*	0.37 ± 0.16^{b}
L	*Ps. aeruginosa*	0.34 ± 0.03^{begj}
O	*Ps. aeruginosa*	0.52 ± 0.06^{bfhik}
P	*Ps. aeruginosa*	0.21 ± 0.04^{cegj}
3	*Ps. aeruginosa*	0.62 ± 0.03^{dfhik}
8	*Ps. aeruginosa*	0.86 ± 0.03^{al}
P3	*Thermoactinomyces vulgaris*	0.62 ± 0.03^{fhik}
S14	*Ps. aeruginosa*	0.22 ± 0.06^{cegj}
3B	*Actinomyces nueslundii*	0.52 ± 0.06^{fhij}
5H	*Anoxybacillus rupiensis*	0.92 ± 0.06^{l}
6A1	*Actinomyces nueslundii*	0.62 ± 0.03^{dfhik}
6B	*Actinomyces nueslundii*	0.64 ± 0.00^{dfhik}
6A2	*Actinomyces nueslundii*	0.54 ± 0.08^{bfhik}

Figures with different superscripts depicts differences within mean values ($p< 0.05$) using Duncan`s Multiple Range Test.

Table 4.12: Effect of different concentrations of carbohydrates on the production of cellulase by bacterial isolates

Carbon source ---------- Type	% Concentratioon	Isolâtes/Enzyme activity(U/ml) ---------- *Roseomonas sp.* (A_1)	---------- *Ps. aeruginosa (*D)	*Anoxybacillus rupiensis.(* E1)	*Ps. aeruginosa (*I)	*Ps. aeruginosa (*8)
Galactose	0.5	0.67±0.02^{a}	3.67±0.04^{ab}	0.67±0.03^{a}	0.67±0.02^{a}	0.67±0.01^{a}
	1.0	1.78±0.01^{a}	5.89±0.01^{ab}	8.01±0.01^{b}	4.68±0.04^{b}	5.01±0.03^{b}
	1.5	7.12±0.03^{c}	6.34±0.06^{bc}	3.67±0.04^{d}	6.68±0.02^{b}	7.45±0.03^{b}
	2.0	7.79±0.03^{c}	4.78±0.02^{d}	5.01±0.02^{c}	8.01±0.02^{d}	8.01±0.03^{b}
	2.5	2.56±0.03^{b}	2.22±0.02^{d}	7.56±0.01^{b}	3.45±0.05^{c}	0.89±0.04^{a}
Lactose	0.5	1.33±0.02^{a}	0.5.89±0.0^{ab}	0.44±0.03^{a}	0.22±0.04^{a}	0.22±0.03^{a}
	1.0	5.89±0.06^{c}	6.78±0.02^{ab}	5.89±0.06^{b}	3.89±0.05^{b}	7.56±0.02^{b}
	1.5	2.66±0.01^{b}	4.45±0.03^{c}	3.45±0.01^{c}	4.67±0.06^{b}	3.22±0.03^{c}
	2.0	2.22±0.03^{b}	2.67±0.02^{d}	3.11±0.01^{c}	2.56±0.00^{c}	2.67±0.03^{c}
	2.5	2.22±0.04^{b}	2.67±0.03^{d}	3.11±0.09^{c}	1.78±0.02^{cd}	1.78±0.02^{d}
CMC	0.5	2.67±0.03^{a}	1.33±0.06^{a}	3.45±0.01d^{ac}	2.67±0.01^{a}	3.45±0.04^{a}
	1.0	9.00±0.03^{b}	2.89±0.03^{b}	10.01±0.01^{b}	8.34±0.03^{b}	8.56±0.01^{b}
	1.5	7.12±0.03^{c}	5.89±0.02^{c}	4.11±0.04^{ac}	4.67±0.11^{c}	6.34±0.01^{c}
	2.0	6.56±0.03^{c}	5.12±0.01^{c}	8.67±0.04^{bd}	8.45±0.06^{bd}	8.01±0.01^{b}
	2.5	3.22±0.02^{a}	2.89±0.04^{b}	5.78±0.04^{ce}	7.56±0.04^{bd}	8.01±0.01^{b}
Sorbitol	0.5	1.33±0.03^{a}	1.56±0.06^{a}	1.33±0.05^{ac}	1.56±0.03^{a}	1.56±0.03^{a}
	1.0	0.67±0.03^{a}	1.33±0.02^{a}	2.22±0.01^{bc}	1.33±0.04^{a}	0.89±0.05^{b}
	1.5	0.44±0.06^{a}	0.67±0.06^{b}	3.67±0.06^{abc}	0.44±0.02^{b}	0.89±0.04^{b}
	2.0	0.44±0.01^{a}	1.33±0.02^{a}	1.78±0.02^{a}	0.67±0.01^{c}	1.33±0.04^{c}
	2.5	2.00±0.01^{b}	1.78±0.06^{c}	1.78±0.06^{a}	0.44±0.03^{c}	0.09±0.01^{d}
Glucose	0.5	1.33±0.03^{a}	1.56±0.01^{a}	2.00±0.03^{a}	1.56±0.00^{a}	1.33±0.01^{a}
	1.0	1.33±0.01^{a}	0.89±0.01^{b}	1.33±0.01^{b}	2.00±0.03^{b}	1.33±0.01^{a}
	1.5	0.22±0.03^{b}	0.44±0.06^{c}	1.33±0.04^{b}	1.33±0.01^{a}	0.44±0.02^{b}
	2.0	0.22±0.01^{b}	0.44±0.02^{c}	0.44±0.03^{c}	0.44±0.06^{c}	0.22±0.00^{b}
	2.5	0.00±0.00^{c}	0.00±0.00^{d}	0.22±0.01^{c}	0.44±0.02^{c}	0.00±0.00c

Each value is a mean of two replicates; ± standard deviation among replicates; Means with different letters within each column differ significantly ($p \leq 0.05$) using Duncan`s Multiple Range Test.

Table 4.13. Effect of different concentrations of carbohydrates on the production of cellulase by bacterial isolates

Carbon source		Isolates/ Enzyme activity (U/ml)				
Type	% Concent	*Actinomyces nueslundii*. (3B)	*Thermoactinomyces vulgaris (*P3)	*Anoxybacillus rupiensis (*5H)	*Actinomyces nueslundii*. (6A1)	*Actinomyces nueslundii*. (6B**)**
Galactose	0.5	3.22±0.02^{a}	0.67±0.02^{a}	0.89±0.06^{a}	2.00±0.02^{a}	2.00±0.00^{a}
	1.0	4.67±0.04^{a}	8.67±0.04^{b}	7.12±0.02^{b}	2.67±0.01^{b}	4.45±0.03^{b}
	1.5	6.45±0.04^{b}	6.00±0.04^{c}	5.89±0.00^{c}	3.79±0.04^{c}	5.01±0.03^{b}
	2.0	6.78±0.01^{b}	3.45±0.00^{d}	2.00±0.03^{d}	5.67±0.02^{d}	7.78±0.02^{c}
	2.5	2.22±0.02^{c}	2.56±0.06^{d}	2.00±0.01^{d}	1.33±0.03^{a}	3.45±0.04^{d}
Lactose	0.5	0.89±0.02^{a}	0.67±0.04^{a}	0.22±0.03^{a}	0.44±0.04^{a}	4.45±0.03^{a}
	1.0	4.67±0.06^{b}	5.89±0.02^{b}	5.89±0.06^{b}	5.12±0.05^{b}	5.33±0.02^{b}
	1.5	3.45±0.01^{c}	3.89±0.03^{c}	4.67±0.01^{b}	3.67±0.06^{c}	4.89±003^{a}
	2.0	3.67±0.03^{bc}	3.67±0.02^{c}	2.22±0.01^{c}	2.89±0.0^{d}	3.44±0.03^{c}
	2.5	2.67±0.04^{d}	3.45±0.03^{d}	2.22±0.09^{c}	2.00±0.02^{d}	3.22±0.02^{c}
CMC	0.5	2.22±0.03^{a}	2.89±0.06^{a}	3.67±0.01^{a}	0.22±0.01^{a}	3.22±0.04^{a}
	1.0	2.89±0.03^{b}	3.22±0.03^{b}	9.22±0.01^{b}	2.89±0.03^{b}	4.67±0.01^{b}
	1.5	5.12±0.03^{cd}	5.67±0.02^{c}	7.45±0.04^{c}	8.89±0.11^{c}	5.34±0.01^{b}
	2.0	6.56±0.03^{c}	6.78±0.01^{d}	5.56±0.04^{d}	7.12±0.06^{cd}	6.56±0.01^{c}
	2.5	4.45±0.02^{d}	5.12±0.04^{ce}	3.23±0.04^{a}	4.45±0.04^{e}	6.56±0.01^{c}
Sorbitol	0.5	2.00±0.03^{a}	1.56±0.06^{a}	3.22±0.05^{a}	2.22±0.03^{a}	1.78±0.03^{a}
	1.0	0.66±0.03^{b}	0.89±0.02^{b}	2.89±0.01^{a}	0.89±0.04$^{\mathbf{b}}$	1.33±0.05^{a}
	1.5	0.66±0.06^{b}	0.44±0.06$^{\mathbf{b}}$	2.22±0.06^{b}	0.89±0.02^{b}	1.33±0.04^{a}
	2.0	0.89±0.01^{b}	0.44±0.02^{b}	2.00±0.02^{b}	0.89±0.01^{b}	0.44±0.04^{b}
	2.5	1.33±0.01^{c}	1.56±0.06^{c}	0.00±0.00^{c}	0.00±0.00^{c}	0.22±0.01^{b}
Glucose	0.5	1.33±0.03^{a}	1.89±0.01^{a}	1.56±0.03^{a}	2.00±0.00^{a}	2.00±0.01^{a}
	1.0	1.33±0.01^{a}	1.56±0.01^{b}	1.56±0.01^{a}	1.56±0.03^{b}	1.33±0.01^{b}
	1.5	0.44±0.03^{b}	1.33±0.06^{c}	1.33±0.04^{a}	1.33±0.01^{b}	1.33±0.02^{b}
	2.0	0.00±0.00^{c}	0.44±0.02^{d}	0.44±0.03^{b}	0.44±0.06^{c}	1.56±0.00^{b}
	2.5	0.00±0.00^{c}	0.44±0.00^{d}	0.00±0.00^{c}	0.22±0.02^{c}	0.44±0.00^{c}

Each value is a mean of two replicates; ± standard deviation among replicates;
Means with different letters within each column differ significantly ($p \leq 0.05$) using Duncan`s Multiple Range Test.

Table 4.14. Effect of different concentrations of carbohydrates on the filter paper activity(PFA) by bacterial isolates

Carbon source ---------- Type	% Concent.	Isolates/FPA ---------- (U/ml) ---------- *Roseomonas* sp. (A_1)	*Ps. aeruginosa* (D)	*Anoxybacillus rupiensis.*(E1)	*Ps. aeruginosa* (I)	*Ps. aeruginosa* (8)
Galactose	0.5	1.1±0.02^{a}	1.0±0.04^{ab}	1.67±0.03^{ab}	0.4±0.02^{a}	0.8±0.01^{a}
	1.0	2.01±0.01^{a}	2.34±0.01^{ab}	2.07±0.01^{ab}	1.7±0.04^{b}	2.01±0.03^{b}
	1.5	4.52±0.03^{b}	3.54±0.06^{bc}	3.52±0.04^{ab}	5.9±0.02^{b}	4.45±0.03^{ab}
	2.0	4.11±0.03^{c}	4.32±0.02^{d}	4.01±0.02^{ab}	2.3±0.02^{a}	1.1±0.03^{b}
	2.5	3.56±0.03^{d}	1.52±0.02^{d}	4.56±0.01^{c}	1..2±0.05^{c}	0.2±0.04^{c}
Lactose	0.5	2.13±0.02^{ab}	1.3±0.04^{ab}	1.45±0.03^{a}	2.34±0.04^{a}	3.45±0.03^{a}
	1.0	4.03±0.06^{ab}	2.01±0.02^{ab}	4.11±0.06^{b}	3.11±0.05^{b}	6.56±0.02^{b}
	1.5	5.13±0.01^{b}	3.89±0.03^{c}	2.67±0.01^{b}	5.01±0.06^{c}	3.11±0.03^{a}
	2.0	2.03±0.03^{c}	3.67±0.02^{a}	2.13±0.01^{a}	2.67±0.00^{a}	3.45±0.03^{c}
	2.5	2.13±0.04^{d}	2.45±0.03^{d}	0.89±0.09^{c}	1.1±0.02^{a}	1.3±0.02^{d}
CMC	0.5	1.67±0.03^{a}	1.3±0.06^{a}	1.1±0.01d^{ac}	1.25±0.01^{ad}	1.25±0.04^{a}
	1.0	3.11±0.03^{b}	2.01±0.03^{b}	1.8±0.01^{b}	1.5±0.03^{b}	3.11±0.01^{b}
	1.5	5.13±0.03^{a}	3.11±0.02^{c}	1.8±0.04^{ac}	1.71±0.11^{bc}	4.34±0.01^{b}
	2.0	6.89±0.03^{c}	2.95±0.01^{b}	2.01±0.04^{ad}	3.5±0.06^{abd}	4.81±0.01^{a}
	2.5	2.13±0.02^{c}	3.11±0.04^{d}	1.3±0.04^{e}	2.13±0.04^{acd}	2.45±0.01^{c}
Sorbitol	0.5	1.3±0.03^{a}	1.68±0.06^{a}	1.68±0.05^{ac}	1.33±0.03^{a}	1.89±0.03^{a}
	1.0	0.67±0.03^{a}	2.3±0.02^{b}	2.23±0.01^{bc}	1.14±0.04^{b}	2.89±0.05^{b}
	1.5	1.56±0.06^{a}	0.67±0.06^{b}	2.79±0.06^{abc}	1.18±0.02^{a}	1.78±0.04^{a}
	2.0	2.0±0.01^{b}	2.46±0.02^{a}	2.28±0.02^{a}	1.67±0.01^{dc}	1.78±0.04^{c}
	2.5	1.3±0.01^{c}	2.46±0.06^{c}	1.78±0.06^{d}	2.46±0.03^{d}	1.78±0.01^{d}
Glucose	0.5	1.0±0.03^{a}	1.3±0.01^{a}	0.45±0.03^{a}	1.0±0.00^{a}	0.34±0.01^{a}
	1.0	0.89±0.01^{b}	0.89±0.01^{b}	0.89±0.01^{a}	1.0±0.03^{b}	0.00±0.01^{b}
	1.5	1.4±0.03^{c}	0.34±0.06^{c}	1.34±0.04^{b}	0.45±0.01^{a}	0.00±0.02^{c}
	2.0	0.67±0.01^{d}	0.13±0.02^{d}	0.67±0.03^{a}	0.45±0.06^{c}	0..34±0.00^{a}
	2.5	0.00±0.00^{d}	0.00±0.0^{e}	0.00±0.00^{c}	0.24±0.02^{d}	0.00±0.0^{e}

Each value is a mean of two replicates; ± standard deviation among replicates; Means with different letters within each column differ significantly ($p \leq 0.05$) using Duncan`s Multiple Range Test.

Table 4.15: Effect of different concentrations of carbohydrates on the filter paper activity (FPA) by bacterial isolates.

Carbon source ---------- Type	% Concent.	Isolates/ FPA ---------- (U/ml) ---------- *Actinomyces nueslundii.*(3B)	*Thermoact. vulgaris* (P3)	*Anoxybacillus rupiensis* (5H)	*Actinomyces nueslundii*. (6A1)	*Actinomyces nueslundii*. (6B)
Galactose	0.5	1.32±0.02^{a}	0.57±0.02^{ab}	0.78±0.06^{ab}	0.57±0.02^{a}	1.14±0.00^{a}
	1.0	0.93±0.04^{a}	1.32±0.04^{ab}	0.89±0.02^{ab}	0.57±0.01^{b}	1.17±0.03^{b}
	1.5	1.32±0.04^{b}	1.50±0.04^{bc}	1.32±0.00^{ab}	1.50±0.04^{b}	1.68±0.03^{ab}
	2.0	2.07±0.01^{c}	0.00±0.00^{d}	1.68±0.03^{ab}	2.07±0.02^{a}	0.78±0.02^{b}
	2.5	1.32±0.02^{d}	0.57±0.06^{d}	0.78±0.01^{c}	0.18±0.03^{c}	1.50±0.04^{c}
Lactose	0.5	0.93±0.02^{ab}	0.57±0.04^{ab}	0.18±0.03^{a}	0.57±0.04^{a}	1.50±0.03^{a}
	1.0	1.14±0.06^{ab}	1.50±0.02^{ab}	1.32±0.06^{b}	1.14±0.05^{b}	1.14±0.02^{b}
	1.5	1.32±0.01^{b}	.93±0.03^{c}	0.93±0.01^{b}	1.14±0.06^{c}	0.57±003^{a}
	2.0	1.32±0.03^{c}	0.93±0.02^{a}	0.78±0.01^{a}	0.78±0.0^{a}	0.57±0.03^{c}
	2.5	0.93±0.04^{d}	0.93±0.03^{d}	0.78±0.09^{c}	0.57±0.02^{a}	0.57±0.02^{d}
CMC	0.5	1.3±0.03^{a}	2.93±0.06^{a}	2.14±0.01d^{ac}	1.93±0.01^{ad}	1.78±0.04^{a}
	1.0	1.58±0.03^{b}	2.68±0.03^{b}	1.18±0.01^{b}	1.93±0.03^{b}	2.68±0.01^{b}
	1.5	1.93±0.03^{a}	1.93±0.02^{c}	1.78±0.04^{ac}	1.93±0.11^{bc}	1.93±0.01^{b}
	2.0	1.93±0.03^{c}	2.14±0.01^{b}	1.93±0.04^{ad}	1.93±0.06^{abd}	1.93±0.01^{a}
	2.5	2.68±0.02^{c}	3.64±0.04^{d}	4.72±0.04^{e}	4.54±0.04^{acd}	1.30±0.01^{c}
Sorbitol	0.5	2.07±0.03^{a}	2.14±0.06^{a}	1.86±0.05^{ac}	1.68±0.03^{a}	1.45±0.03^{a}
	1.0	1.57±0.03^{a}	3.28±0.02^{b}	3.46±0.01^{bc}	1.68±0.04^{b}	2.28±0.05^{b}
	1.5	3.07±0.06^{a}	2.68±0.06^{b}	3.28±0.06^{abc}	2.32±0.02^{a}	2.28±0.04^{a}
	2.0	2.28±0.01^{b}	2.68±0.02^{a}	3.07±0.02^{a}	2.50±0.01^{dc}	1.86±0.04^{c}
	2.5	1.93±0.01^{c}	3.64±0.06^{c}	1.68±0.00^{a}	3.07±0.00	0.93±0.01^{d}
Glucose	0.5	0.50±0.03^{a}	0.32±0.01^{a}	0.72±0.03^{a}	1.50±0.00^{a}	0.90±0.0
	1.0	0.93±0.01^{b}	1.82±0.01^{b}	1.46±0.01^{b}	1.18±0.03^{b}	3.54±0.01^{b}
	1.5	0.98±0.03^{b}	0.98±0.06^{c}	2.18±0.04^{c}	1.64±0.01^{a}	3.36±0.02^{c}
	2.0	3.80±0.0^{c}	2.00±0.02^{d}	1.50±0.03^{a}	1.28±0.06^{c}	1.36±0.00^{a}
	2.5	2.00±0.00^{c}	0.78±0.0^{e}	0.98±0.00	1.10±0.02^{d}	0.60±0.0^{d}

Each value is a mean of two replicates; ± standard deviation among replicates; Means with different letters within each column differ significantly ($p \leq 0.05$) using Duncan`s Multiple Range Test.

4.14 **Time course of enzyme production**

The results presented in figure 4.29 show the time course of cellulase production by isolates. Maximum enzyme production occurred at 72h for isolates A1, 8, 5H and 6A1. Isolate E1 has its best cellulase production at 48h, this value is compared with that of 8. The enzyme production decreased to 72h for E1 and 96h for A1, 8, 5h and 6A1. However the production picked up again and continued to 144h for A1, 5H and 6A1.

4.15 **Effect of mineral salts on enzyme production**

The effect of supplementation of mineral salts in the basal medium for enzyme production of cellulase and filter paper activity by bacterial isolates was studied (Table 4.16, 4.17). *Anoxybacillus rupiensis* 5H showed an increase in cellulase synthesis in the medium containing $CaCl_2$, $FeSO_4$ and KCl. NaCl was also a good factor in medium supplemented with it for *Anoxybacillus rupiensis* E1 and *Ps. aeruginosa* 8. Medium containing KCl similarly improved the enzyme production by *Thermoactinomyces vulgaris* and *Actinomyces nueslundii*. The presence of $CuCl_2$, $ZnCl_2$ and $AlCl_3$ did not elicit good production for all the bacterial isolates as the production of the cellulase decreased with their presence.

4.16 **Effect of lignocellulosic substrates on the enzyme production**

Ability of different lignocellulosic substrates to elicit the production of lignocellulosic enzymes by the isolates is shown in Table 4.18. For isolates *Roseomonas* sp.(A1), *Anoxybacillus rupiensis*(E1), *Pseudomonas aeruginosa* (8) and *Actinomyces nueslundii* (6A1) sugarcane bagasse produced the highest cellulase enzyme (6.66U/ml, 6.14U/ml,4.1 and 4.68U/ml in 1%, 1%,2% and 1% respectively)whereas the highest cellulase production by isolate *Anoxybacillus rupiensis* (5H) was in 2% corncob. However, there is no significant difference in amount of cellulase produce by 5H between 1% and 2% corncob.

The highest filter paper activity of 4.86U/ml (A1), 6.98U/ml (E1), 5.85U/ml (8), 6.14U/ml (5H) and 4.86/Uml (6A1) was detected from culture supernatants obtained from corncob for E1and sugarcane baggase for others. Sawdust was very poor in the production of both cellulase and filter paper activity in all cases.

4.17 **Cellulase production under optimized conditions**

Figure 4.30 shows the results of production of cellulase under the optimized medium and growth conditions stated earlier (i.e optimum temperature, pH, time of incubation etc). The result showed an increase in cellulase production by *Roseomonas* sp. A1, *Anoxybacillus rupiensis* E1, *Pseudomonas aeruginosa* 8, *Anoxybacillus rupiensis* 5H and *Actinomyces nueslundii* 6A1under optimized conditions. Based on the activity of enzyme produced by the bacterial isolates under optimized conditions, *Roseomonas* sp. A1, *Anoxybacillus rupiensis* E1 and *Anoxybacillus rupiensis* 5H were selected for purification and cellulase activity studies. These isolates were grown under the optimized conditions and the supernatant obtained after centrifugation was used for further studies.

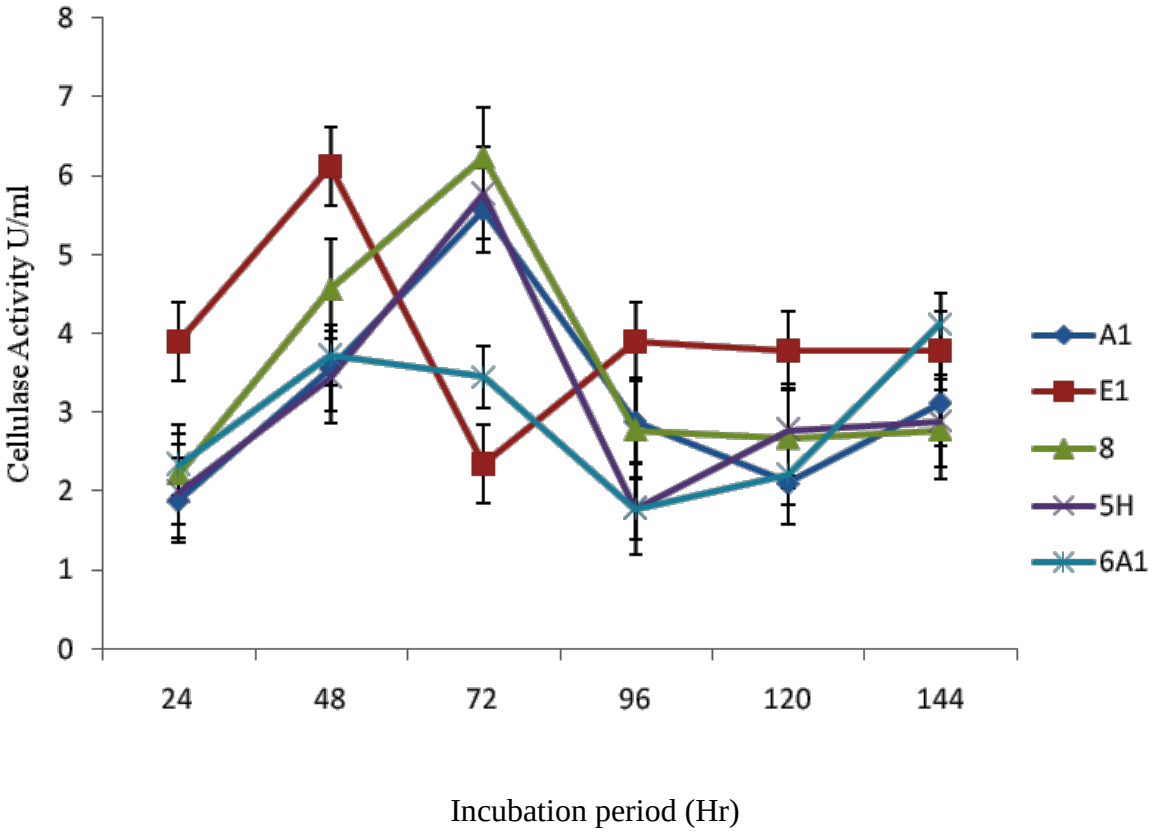


Figure. 4.29. Effect of hours of incubation on the cellulase production by the bacterial isolates. Data are presented as mean of 2 replicates, having standard deviation within the range 0-0.5 A1=*Roseomonas* sp. E1= *Anoxybacillus rupiensis*, 8= *Pseudomonas aeruginosa*, 5H= *Anoxybacillus rupiensis* & 6A1= *Actinomyces nueslundii*

Table 4.16. Effect of different mineral salts on cellulase synthesis by bacterial isolates

Mineral salts	Bacterial isolates/ cellulase activity (U/ml)				
	Roseomonas sp.A1	*Anoxybacillus rupiensis* E1	*Pseudomonas aeruginosa* 8	*Anoxybacillus rupiensis* 5H	*Actinomyces nueslundii* 6A1
Control	4.45±0.03[a]	6.67±0.06[a]	5.56±0.04[a]	5.56±0.05[a]	7.23±0.22[a]b
$CaCl_2$	4.23 ±0.14[a]	5.12 ±0.03[b]	4.78±0.01[a]	6.67±0.15[b]	6.67±0.03[a]
$FeSO_4$	4.56±0.06[a]	6.67±0.06[a]	6.12 ±0.15[b]	6.12±0.19[a]	7.23±0.03[ab]
$MgSO_4$	4.45±0.09[a]	7.78±0.01[ac]	5.56±0.06[a]	4.78±0.06[b]	6.67±0.00[a]
NaCl	5.00±0.03[a]	7.23±0.03[ac]	6.67 ±0.06[b]	5.56±0.13[a]	7.23±0.05[ab]
KCl	6.12±0.04[b]	6.67±0.14[a]	5.56±0.01[a]	6.67±0.21[b]	7.78±0.15[ab]
$CuCl_2$	1.11±0.07[c]	1.78±0.03[d]	1.67±0.13[c]	0.56±0.05[c]	1.11±0.07[c]
$ZnCl_2$	1.11±0.03[c]	1.67±0.00[d]	0.78±0.04[d]	0.50±0.08[c]	1.22±0.00[c]
$AlCl_3$	2.34±0.09[d]	3.34±0.04[e]	2.00±0.11[c]	1.78±0.00[d]	2.22±0.12[d]

Each value is a mean of two replicates± stands for standard deviation among replicates; Means with different letters within each column differ significantly ($p \leq 0.05$). using Duncan`s Multiple Range Test.

Table 4.17. Effect of different mineral salts on Filter paper activity (FPA) by bacterial isolates

Mineral salts	Bacterial isolates/ FPA (U/ml)				
	Roseomonas sp.A1	*Anoxybacillus rupiensis sp.* E1	*Pseudomonas aeruginosa* 8	*Anoxybacillus rupiensis* 5H	*Actinomyces nueslundii* 6A1
Control	3.22 ±0.05[a]	2.50±0.05[a]	2.84± 0.13[a]	2.95±0.02[a]	2.45±0.05[a]
$CaCl_2$	3.78±0.15[a]	2.74±0.04[a]	3.11±0.06[a]	3.22±0.16[a]	2.72±0.01[a]
$FeSO_4$	3.78±0.08[a]	2.61±0.07[a]	3.00±0.02[a]	3.34±0.08[a]	2.61±0.14[a]
$MgSO_4$	3.84±0.10[a]	2.74±0.01[a]	3.06±0.05[a]	3.45±0.23[a]	2.67±0.03$_a$
NaCl	4.00±0.00[a]	2.78±0.03[a]	3.61±0.21[a]	3.84±0.00[a]	3.34±0.15[b]
KCl	4.06±0.17[a]	2.67±0.13[a]	3.11±0.03[a]	3.89±0.04[a]	2.89±0.25[a]
$CuCl_2$	0.67±0.07[c]	0.72± 0.12[c]	0.61±0.09[c]	0.61±0.00[c]	0.78±0.09[d]
$ZnCl_2$	0.56±0.14[c]	0.95±0.01[c]	0.50±0.01[c]	0.67±0.00[c]	0.67±0.12[d]
$AlCl_3$	1.39±0.03[b]	1.28±0.05[b]	1.06± 0.06[b]	1.50±0.11[b]	1.67±0.00[c]

Each value is a mean of two replicates± stands for standard deviation among replicates; Means with different letters within each column differ significantly ($p \leq 0.05$).

using Duncan`s Multiple Range Test.

Table 4.18. Effect of different lignocellulosic substrates on cellulase and filter paper activity (FPA) production by bacterial isolates

Isolates	Concentration % lignocelluloses % Conc.	Substrate/ Enzyme activity U/ml: Cellulase SD	Cellulase CC	Cellulase SB	FPA SD	FPA CC	FPA SB
A1	1	4.1±0.01^{a}	4.68±0.06^{a}	6.66±0.33^{a}	2.9± 0.07^{a}	4.41± 0.71^{a}	3.56± 1.27^{bc}
	2	3.51± 0.04^{a}	2.93± 0.34^{bc}	4.68± 0.56^{bc}	1.6± 0.00^{a}	3.92± 1.84^{b}	4.86± 0.00^{a}
	3	2.93± 0.00^{b}	3.51± 0.03^{b}	3.24± 0.01^{b}	1.6± 0.37^{ab}	1.94± 0.14^{c}	3.56± 0.14^{bc}
E1	1	3.51± 0.07^{a}	5.49± 0.1.13^{ab}	6.14± 0.24^{a}	1.92± 0.28^{a}	6.98± 0.71^{a}	3.92± 1.56^{b}
	2	3.34± 0.13^{a}	5.94± 1.70^{b}	5.49± 1.15^{ab}	1.31± 0.00^{b}	6.79± 1.56^{ab}	5.2± 1.13^{a}
	3	1.76± 0.05^{b}	6.3± 0.07^{b}	4.34± 0.08^{b}	1.31± 0.28^{ab}	4.23± 1.72^{a}	5.2± 1.70^{a}
8	1	2.4± 1.13^{a}	2.7± 1.56^{a}	3.1± 0.00^{a}	0.63± 0.09^{a}	4.23± 0.00^{a}	5.85± 0.00^{a}
	2	2.0± 0.34^{a}	3.5± 0.17^{b}	4.1± 0.08^{b}	0.63± 0.14^{b}	5.49± 1.70^{b}	3.24± 0.00^{b}
	3	1.7± 0.07^{b}	2.1± 0.00^{a}	2.78± 0.15^{ac}	0.63± 0.57^{c}	2.61± 0.17^{ac}	2.93± 0.14^{b}
5H	1	4.1± 1.41^{a}	7.17± 1.82^{a}	6.02± 0.03^{a}	3.6± 0.00^{ab}	2.61± 0.00^{a}	4.41± 1.17^{a}
	2	5.3± 0.15^{b}	7.76± 0.57^{ab}	5.65± 0.05^{a}	4.20± 0.17^{b}	2.61± 0.71^{a}	6.14± 0.00^{b}
	3	2.6± 0.00^{c}	4.5± 0.71^{c}	5.54± 0.17^{b}	2.9± 1.13^{ac}	3.56± 0.00^{b}	3.78± 0.14^{c}
6A1	1	2.34± 0.14^{a}	2.72± 1.17^{a}	4.68± 0.57	1.31± 0.14^{a}	0.63± 0.00^{a}	3.56± 0.28^{a}
	2	2.88± 0.42^{b}	2.9± 0.08^{a}	4.3± 0.28^{b}	0.63± 0.00^{b}	3.6± 0.28^{c}	4.86± 0.00^{b}
	3	2.25± 0.00^{a}	1.4± 0.00^{b}	2.6± 0.14^{c}	0.99± 0.00^{c}	2.93±1.06^{b}	2.61± 0.08^{c}

SD—Sawdust: CC---Corn cob: SB—Sugar cane bagasse; Each value is a mean of two replicates; ± stands for standard deviation among replicates; Means with different letters within each column differ significantly ($p \leq 0.05$) using Duncan`s Multiple Range Test.

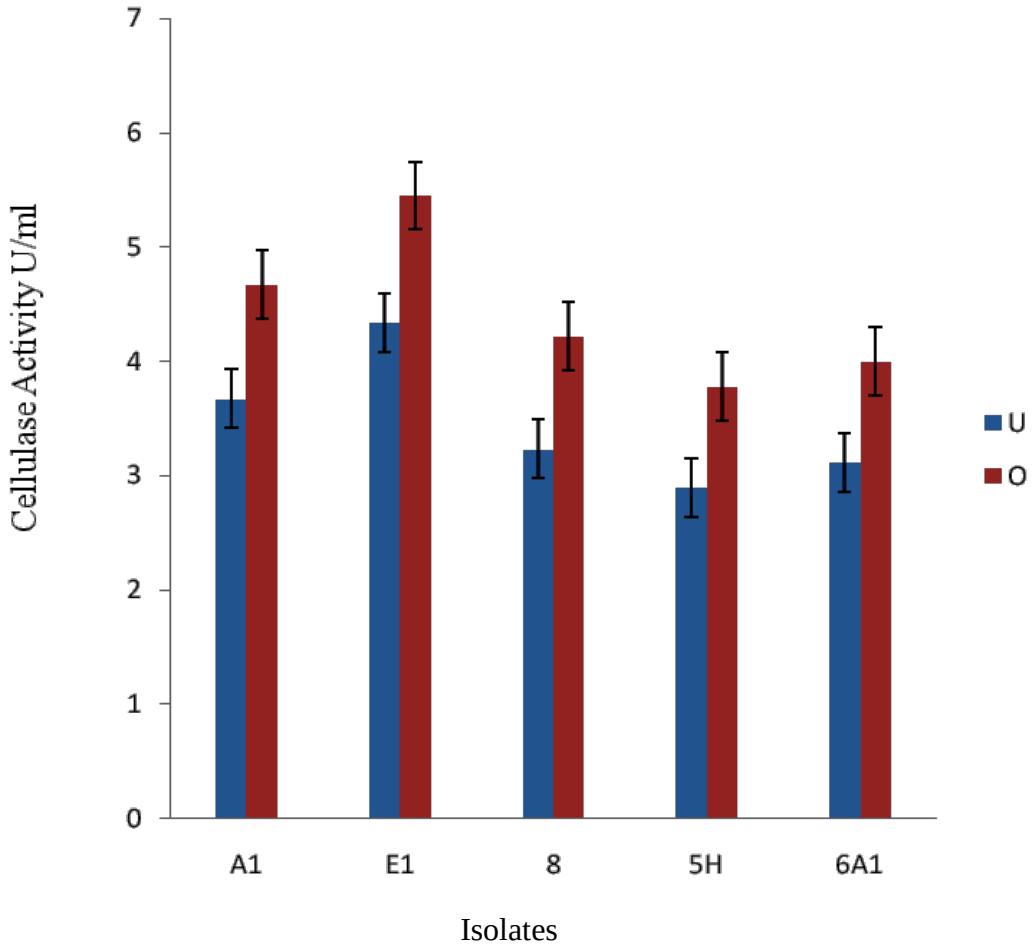


Figure 4.30. Cellulase production under unoptimized and optimized conditions A1= *Roseomonas* sp., E1= *Anoxybacillus rupiensis* sp., 8= *Pseudomonas aeruginosa*, 5H= *Anoxybacillus rupiensis*, 6A1= *Actinomyces nueslundii* U=Unoptimized, O=Optimized

4.18 **Enzyme purification**

The supernatant obtained after the growth of isolates in CMC under optimized conditions by *Roseomonas* sp. A1, *Anoxybacillus rupiensis* E1 and *Anoxybacillus rupiensis* 5H were purified using ammonium sulphate precipitation (20-80% saturation), dialysis and G-75 gel filtration chromatography. When the crude supernatant of *Roseomonas* sp. was fractionated on the Sephadex column, cellulase activity was detected in fractions 9 to 21 with peak at 13to 15. For *Anoxybacillus rupiensis* E1, enzyme activity was observed in fractions 3 to 16

with its peaks in fractions 5 to 6 and 9 to 11 while for *Anoxybacillus rupiensis* 5H, cellulase activity was in fractions 6 to 17 and with the peak in fraction 8 to 9. Tables 4.19, 4.20 and 4.21 give the summary of the purification procedures of cellulase from culture supernatants of *Roseomonas* sp. A1, *Anoxybacillus rupiensis* E1 and *Anoxybacillus rupiensis* 5H respectively.

4.19 **Molecular weight determination**

Cellulase of the isolates was purified by chromatography fractionation and separated into bands by sodium dodecyl sulphate polyacrylamide (SDS-PAGE) gel electrophoresis to show the molecular mass of enzyme for A1, E1 and 5H respectively (Figure 4.34).

Table 4.19: Various steps in purification of cellulase from *Roseomonas* sp. (A1)

Purification Step	Total volume (ml)	Protein content mg/ml	Total Protein (mg/ml)	Total activity (U)	Specific activity (U/mg protein)	Yield (%)	Purification fold
Crude Extract	200	1.33	266	1112	4.2	100	-
Ammonium sulphate precipitation	90	0.93	84	668	8.0	60	1.90
Dialysed partially purified enzyme	25	0.48	12	166	13.5	15	3.21
Sephadex G-75	30	0.2	6	120	20	10.7	4.76

Table 4.20: Various steps in purification of cellulase from *Anoxybacillus rupiensis*. (E1)

Purification Step	Total volume (ml)	Protein content (mg/ml)	Total Protein (mg/ml)	Total activity (U)	Specific activity (U/mg protein)	Yield (%)	Purification fold
Crude Extract	200	1.44	288	1224	4.25	100	-
Ammonium sulphate precipitation	120	0.87	104	740	7.1	60.45	1.67
Dialysed partially purified enzyme	25	0.64	16	200	12.5	16.34	2.94
Sephadex G-75	30	0.2	6	160	26.7	13.1	6.3

Table 4.21: Various steps in purification of cellulase from Anoxybacillus rupiensis (5H)

Purification Step	Total volume (ml)	Protein content (mg/ml)	Total Protein (mg/ml)	Total activity (U)	Specific activity (U/mg protein)	Yield (%)	Purification fold
Crude Extract	200	1.16	232	1156	4.98	100	-
Ammonium sulphate precipitation	150	0.91	136	820	6.03	70	1.21
Dialysed partially purified enzyme	25	0.48	12	138	11.5	11.94	2.30
Sephadex G-75	30	0.13	4	88	22.0	7.6	4.41

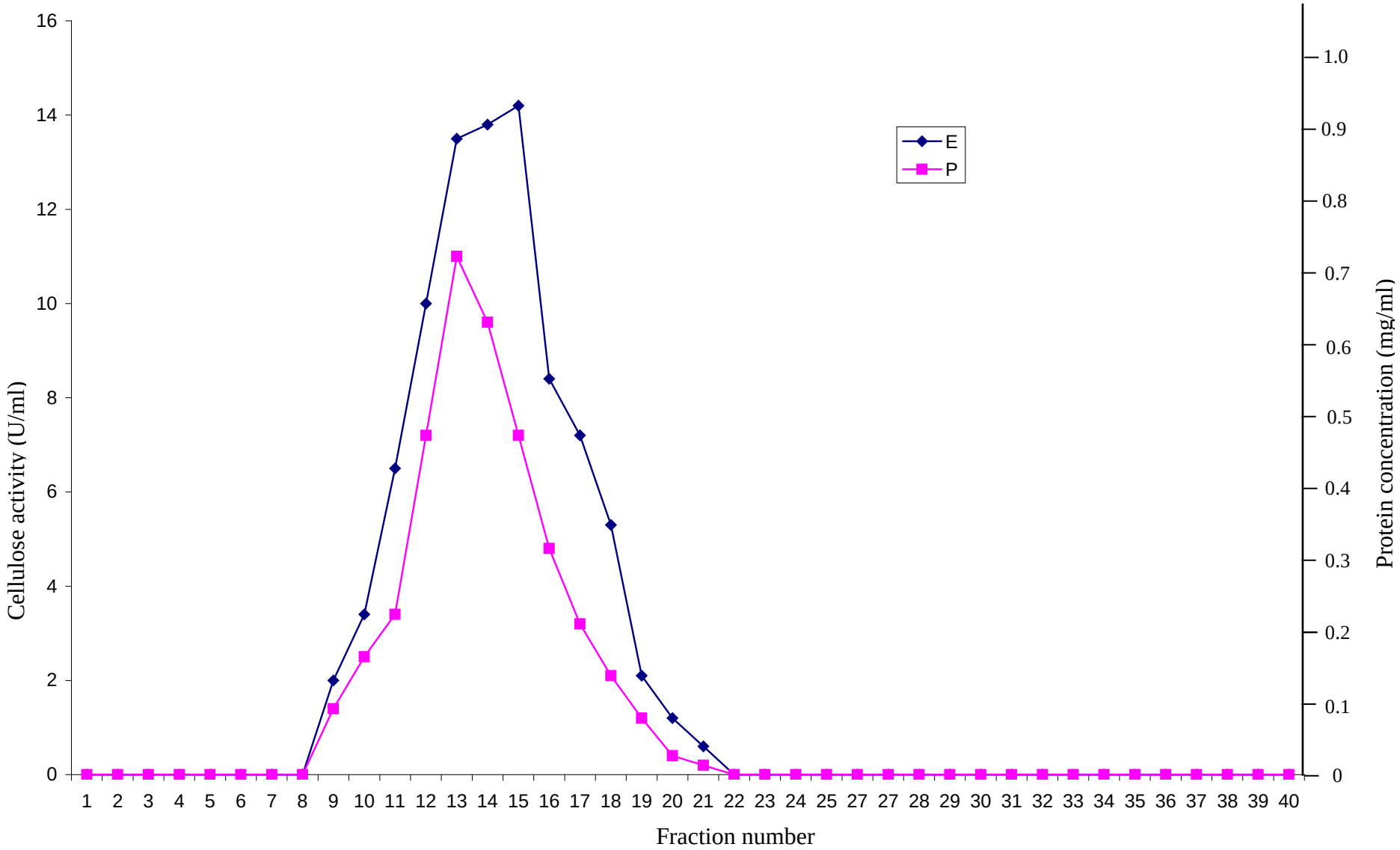


Figure 4.31. Sephadex G-75 chromatography of *Roseomonas* sp. (A1) = Cellulase (E) Enzyme activity (p) Protein concentration. protein (mg/ml)

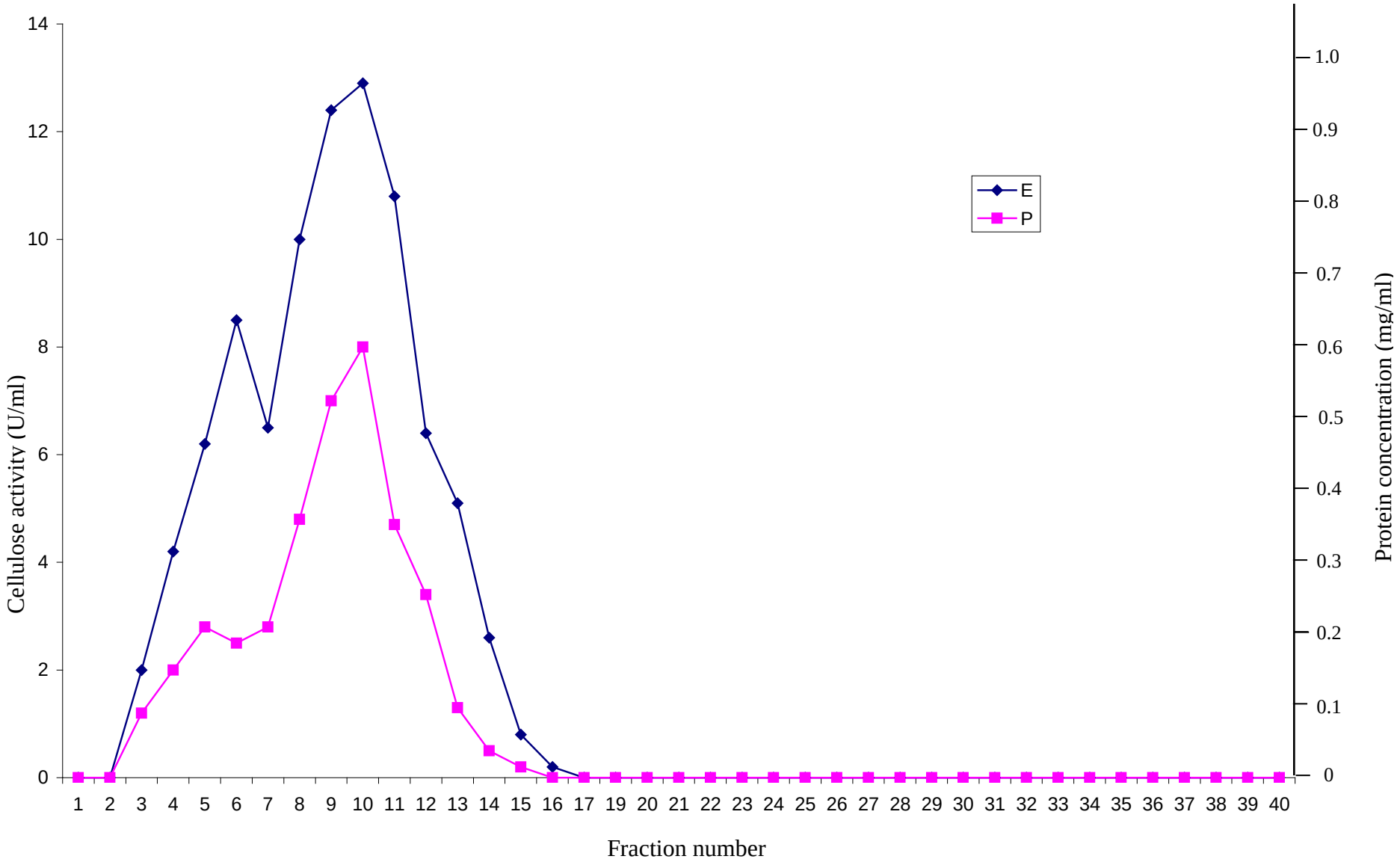


Figure 4.32. Sephadex G-75 chromatography of *Anoxybacillus rupiensis* (E1) = Cellulase (E) Enzyme activity (p) Protein concentration. Protein (mg/ml)

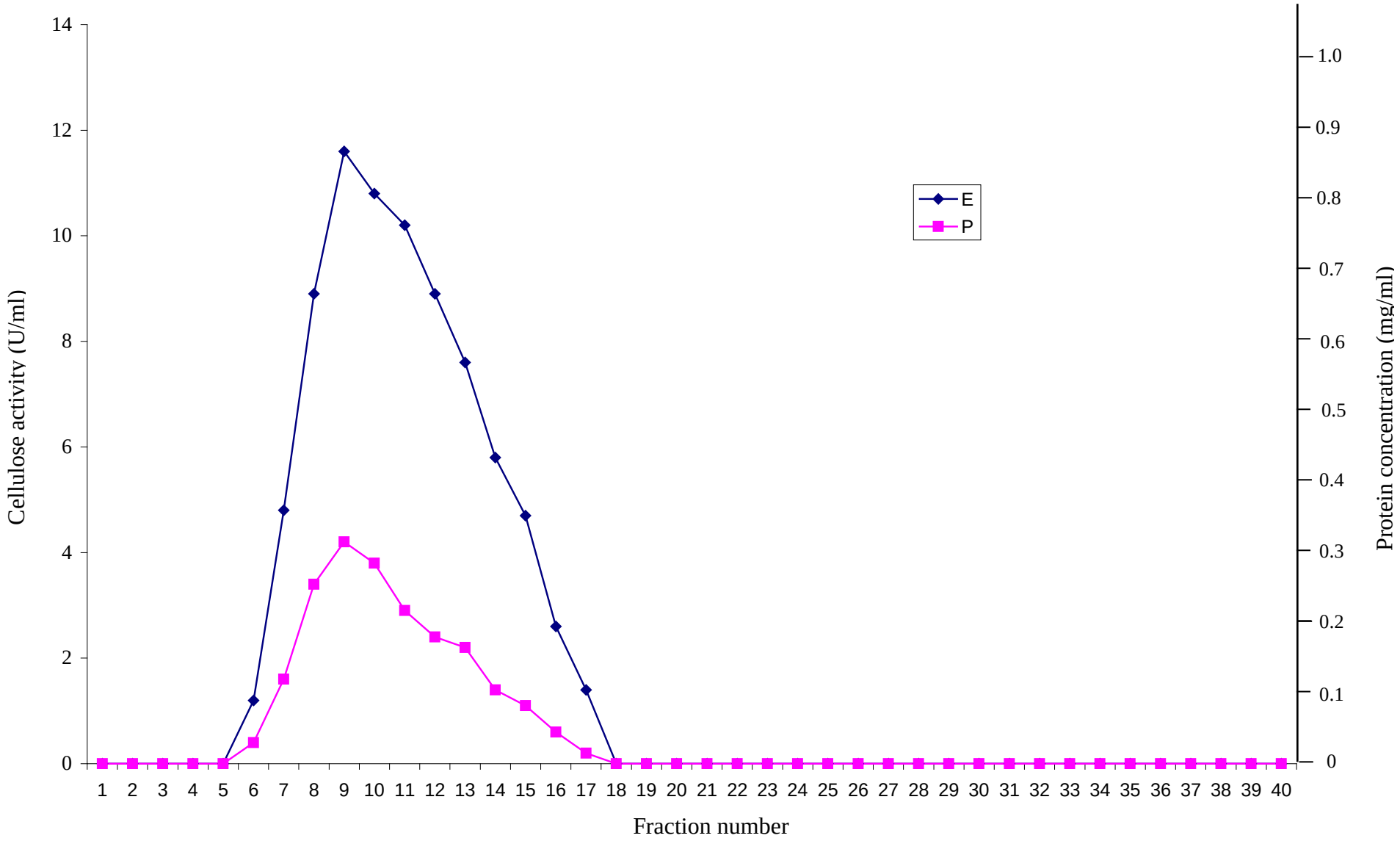


Figure 4.33. Sephadex G-75 chromatography of *Anoxybacillus rupiensis* (5H) = Cellulase (E) Enzyme activity (p) Protein concentration. Protein (mg/ml).

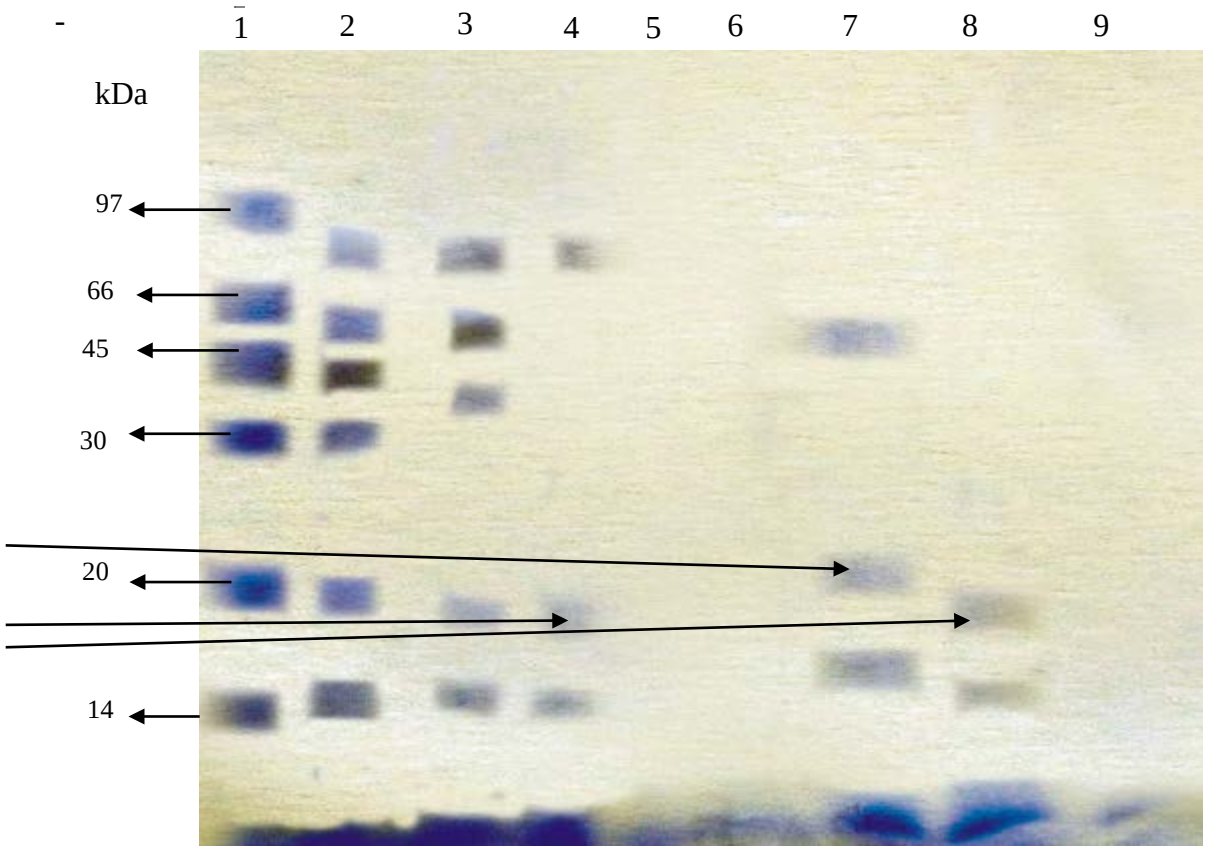


Figure 4.34: Electrophoretogram of cellulase from *Roseomonas* sp.A1, *Anoxybacillus rupiensis* E1. and *Anoxybacillus rupiensis*5H as obtained from SDS-PAGE. Lane 1= Molecular makers,2 and 3 are crude extracts from the isolates, lanes 5, 6 and 9 are blanks, lane 4, 7and 8 are 17.4kDa, 22.6kDa and 16kDa, sephadexG-75 fraction of the A1, E1 and 5H respectively.

4.20 **Characterization of Enzymes**

4.2.1 **Effect of temperature on cellulase activity of purified enzyme from isolates**

The effect of temperature on enzyme activity was assayed by measuring the activity using standard methods at specific temperatures ranging from 45^0C to 80^0C. The optimal activity (5.4U/ml) occurred at 70^0C for cellulase from *Roseomonas sp* A1. while it is 65^0C for both *Anoxybacillus rupiensis* E1 (8.3U/ml) and *Anoxybacillus rupiensis* 5H (7.6U/ml) (Figure 4.35). At 80^0C the activity of A1, E1 and 5H are 60%, 67% and 61% respectively.

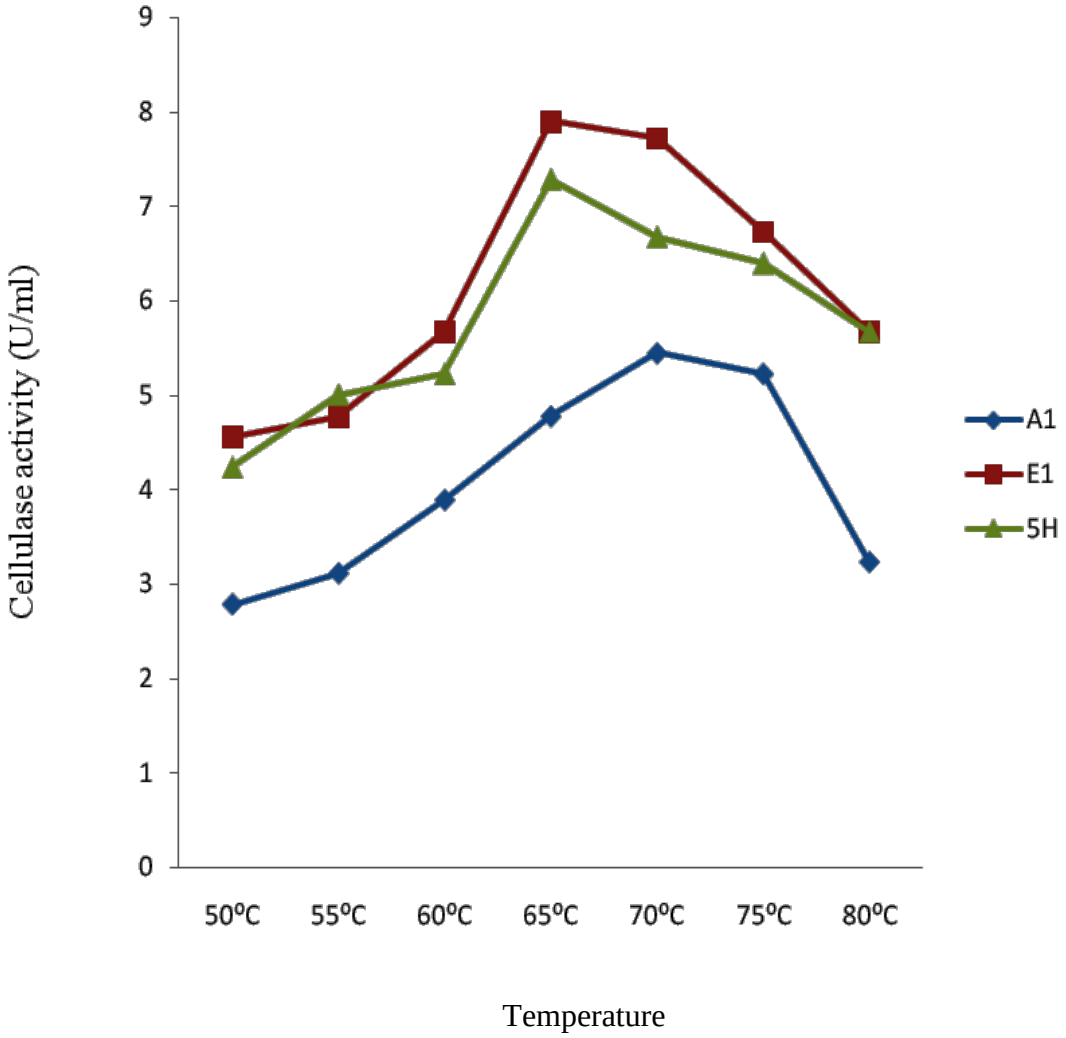


Figure-4.35. Effect of temperature on the activity of cellulase produced by bacteria isolated from sawdust. Data are presented as a mean of 2 replicates, with standard deviation within the range 0-0.3. A1=*Roseoonas* sp.E1 = *Anoxybacillus rupiensis*, 5H= *Anoxybacillus rupiensis*

4.2.2 **Effect of temperature on cellulase stability**

Cellulase of *Roseomonas* sp. A1 and *Anoxybacillus rupiensis* E1 achieved pick at 60^0C for 30min (Figure 4.36). While A1 enzyme declined mildly, that of E1 declined sharply. Cellulase of 5H achieved pick at 65^0C and then declined sharply to 70^0C and mildly to 75^0C.

4.2.3 **Thermal stability of enzyme**

Thermal stability experiments for cellulases were performed by incubating the enzyme at different temperatures and determining the relative activity at various time intervals. Figure 4.37 shows the thermal stability kinetics of *Roseomonas* sp. A1 cellulase. The cellulase was stable for 40min when held at temperature of 60^0C while stability decreased with increase in time at temperature of 65^0C.

Thermal stability of *Anoxybacillus rupiensis* E1 cellulase is shown in Figure 4.38. The enzyme was fully stable at 50^0C for 40mins after which the stability decreased up to 80% at 2hrs. However, there was decrease in stability after 10min at 55^0C, 60^0C and 65^0C. The half lives of the enzyme were 30min at 65^0C and 70min at 60^0C.

Thermoal stability of *Anoxybacillus rupiensis* 5H cellulase is shown in Figure 4.39. The cellulase was stable for 40min at up to temperature of 55^0C for isolate 5H while stability decreased with increase in time beyond 20min and 10min at temperature of 60^0C and 65^0C respectively. The half lives of the enzyme were 47min at 65^0C and 63min at 60^0C.

The activity of 5H enzyme was stable at 50^0C and 55^0C for 40min. The stability of the enzyme was 80 and 70% at 40min for 60^0C and 65^0C. At the end of 1hr the activity of the enzyme has decreased to 82%, 75% and 50% for 50^0C, 55^0C and 60^0C respectively. The enzyme retained 55% and 35% activity by the end of 120min for 50^0C and 55^0C.

4.2.4 **Effect of pH on cellulase activity of purified enzyme**

The activity of *Roseomonas* sp. A1, *Anoxybacillus rupiensis* E1 and *Anoxybacillus rupiensis* 5H at various pHs were determined. Cellulase from A1 had its optimum activity at pH 7.0 (Fig. 4.40) While there are two optimal pHs for E1 and 5H at both 7.0 and 9.0 after which their activities decreased for the three isolates. Overall the enzyme was active over a wide range of pH.

4.2.5 **Effect of pH on the cellulase stability of purified enzyme**

The stability of the enzyme was determined by incubating it at 50^{o}C for 2hrs at different pH values and the residual activity measured by the standard assay method. Figure 4.41 shows the pH stability curves for cellulase from *Roseomonas* sp. A1, *Anoxybacillus rupiensis* E1 and *Anoxybacillus rupiensis* 5H. A1 was stable at pH 7 to 8 while the stability for E1 and 5H was between pHs 7 to 9.

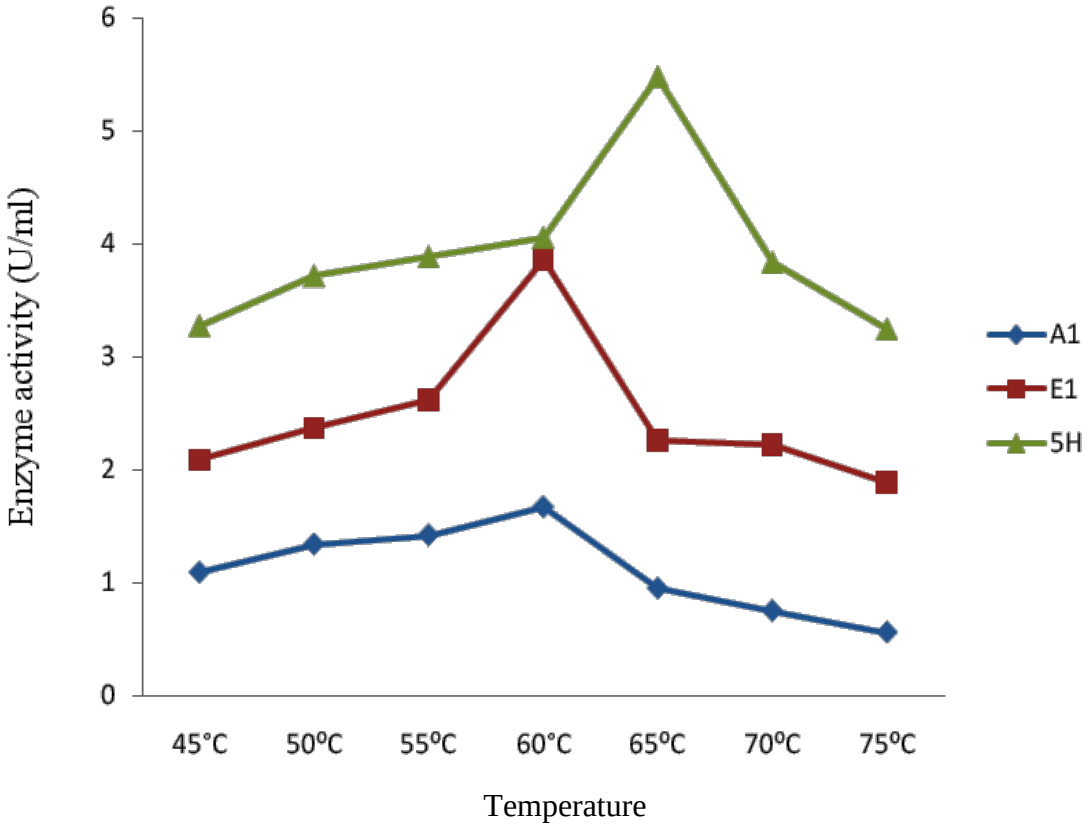


Figure 4.36. Effect of temperature on the stability of cellulase enzyme by the isolates. *Roseomoas* sp. (A1), *Anoxybacillus rupiensis* (E1) and *Anoxybacillus rupiensis* 5H

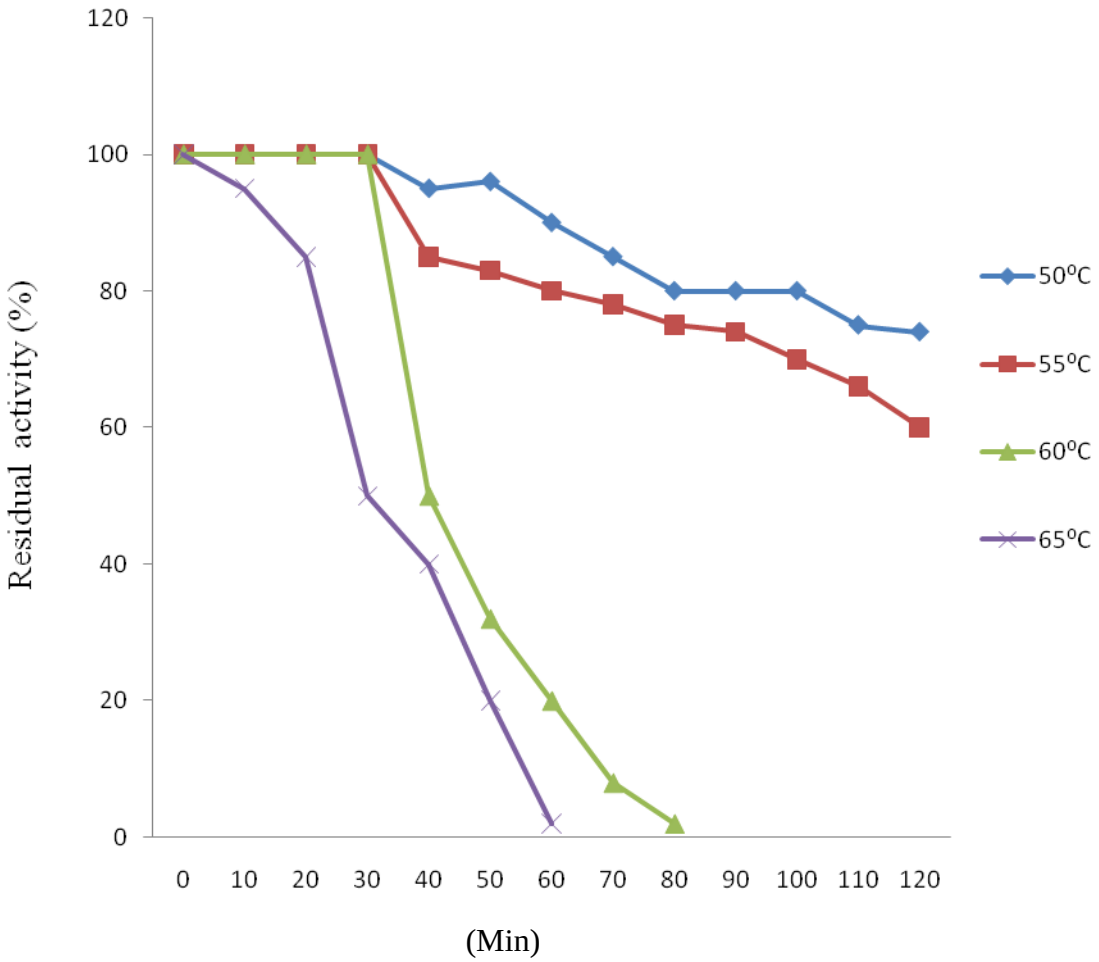


Figure 4.37. Thermostability of cellulase from *Roseomonas* sp A1 at different temperatures. Data are presented as a mean of 2 replicates. Standard deviation were less than 10% in all cases.

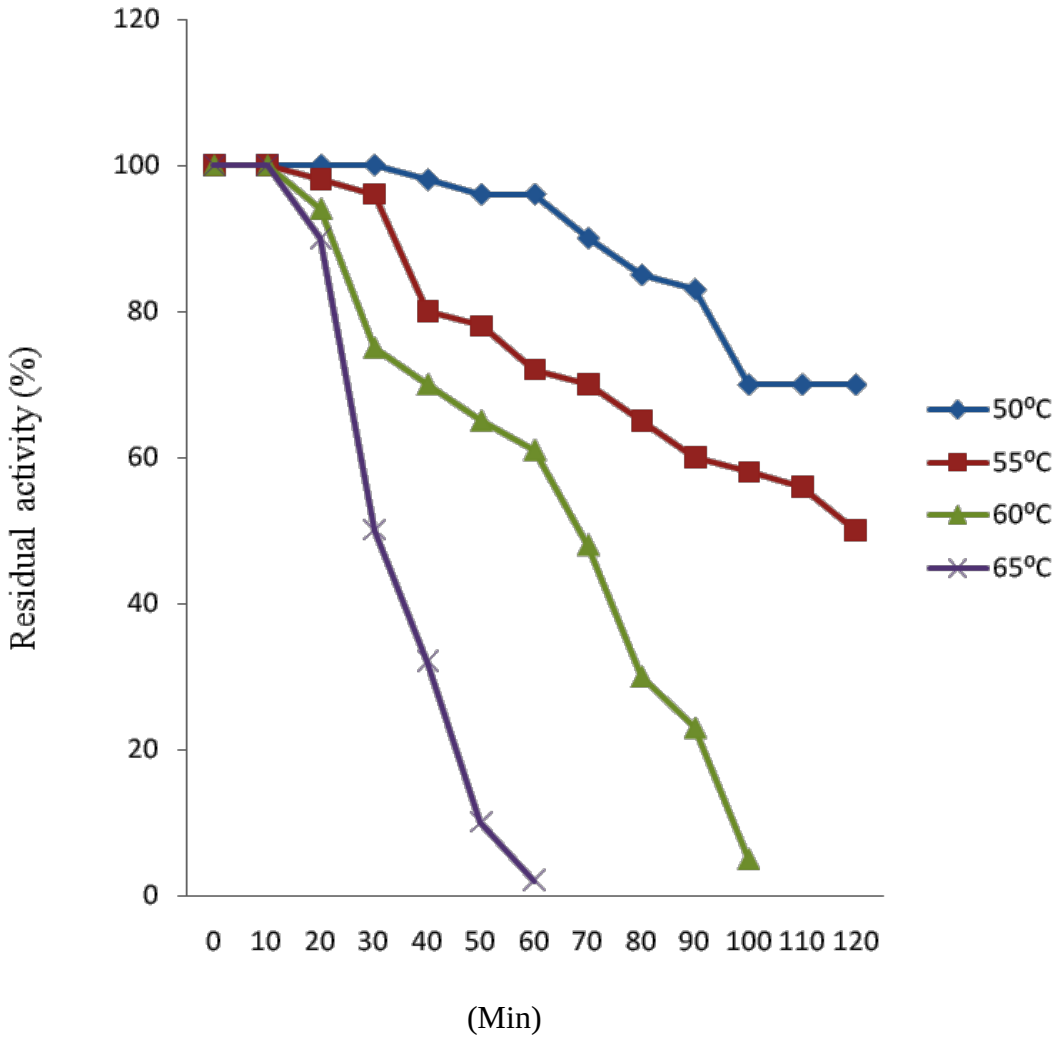


Figure 4.38.Thermostability of cellulase from *Anoxybacillus rupiensis.* E1 at different temperatures. Data are presented as a mean of 2 replicates. Standard deviation were less than 10% in all cases.

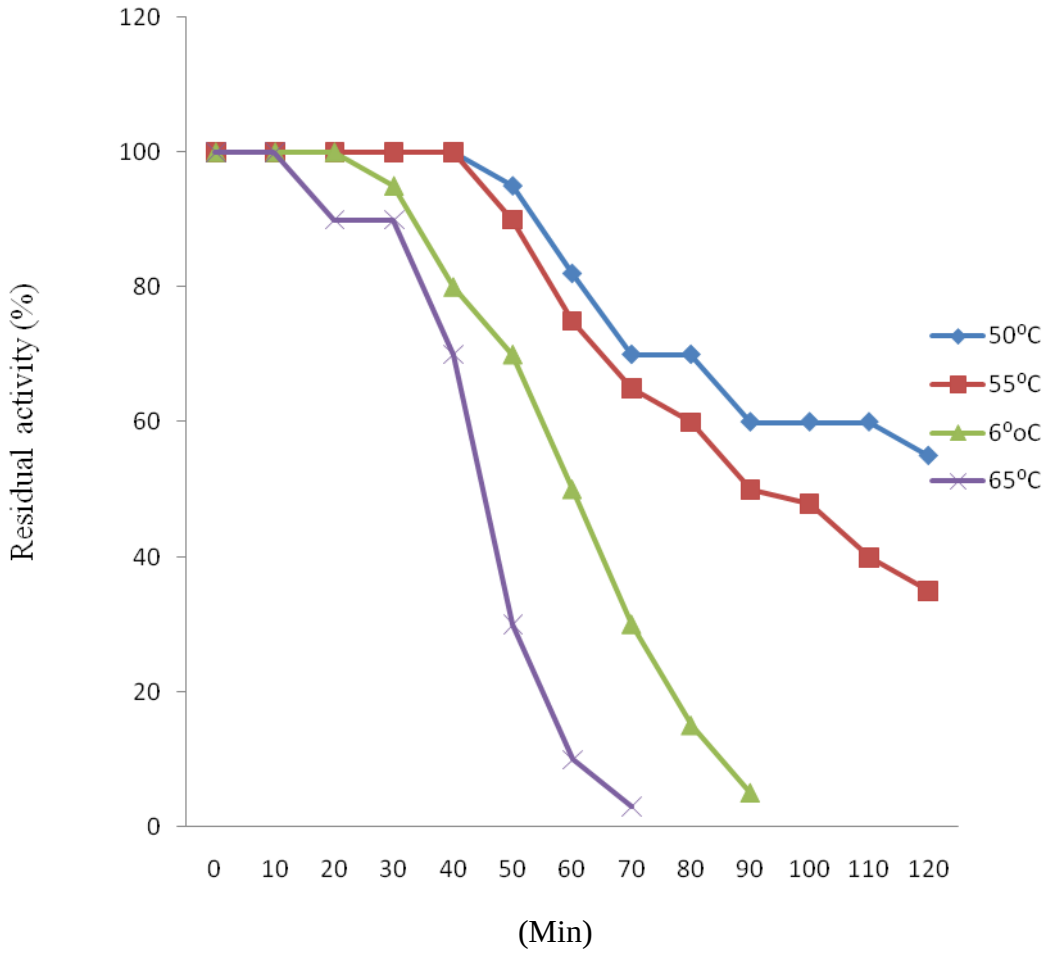


Figure 4.39. Thermostability of cellulase from *Anoxybacillus rupiensis* 5H at different temperatures. Data are presented as a mean of 2 replicates. Standard deviation were less than 10% in all cases.

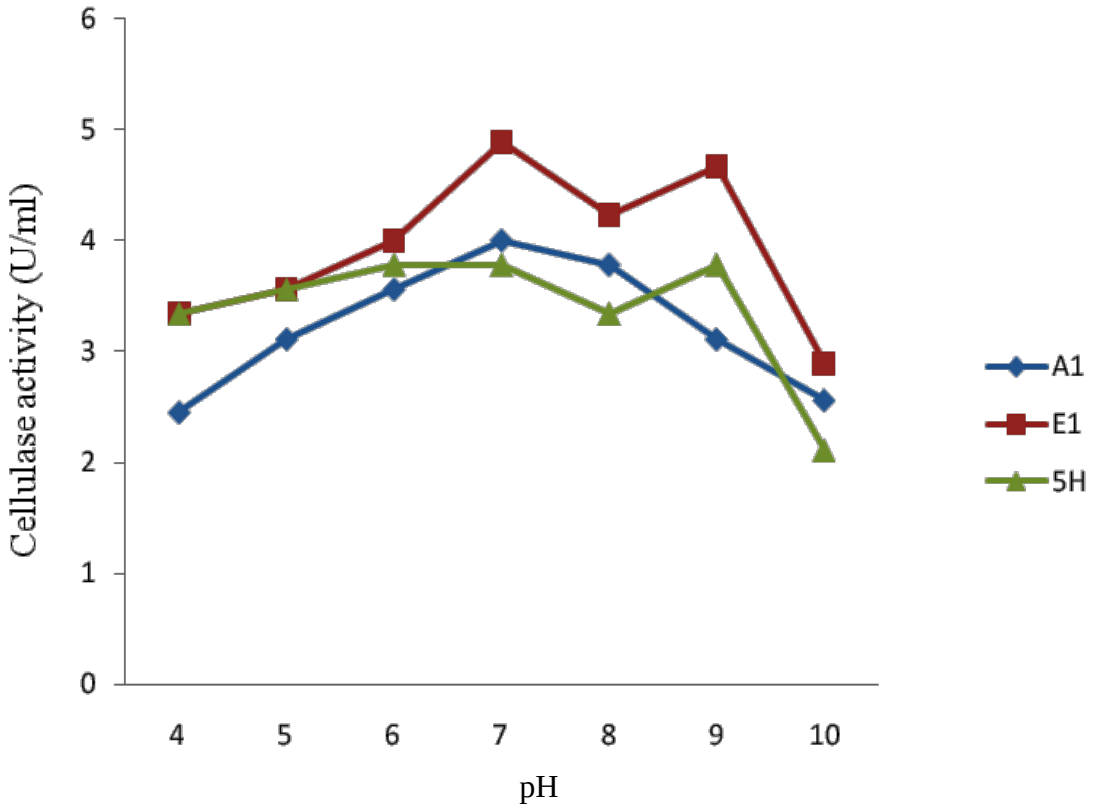


Figure 4.40. Effect of pH on the activity of purified cellulase produced by bacteria isolated from sawdust. Data are presented as a mean of 2 replicates, with standard deviation within the range 0-0.3. A1=*Roseomonas* sp, E1= *Anoxybacillus rupiensis*, 5H = *Anoxybacillus rupiensis*

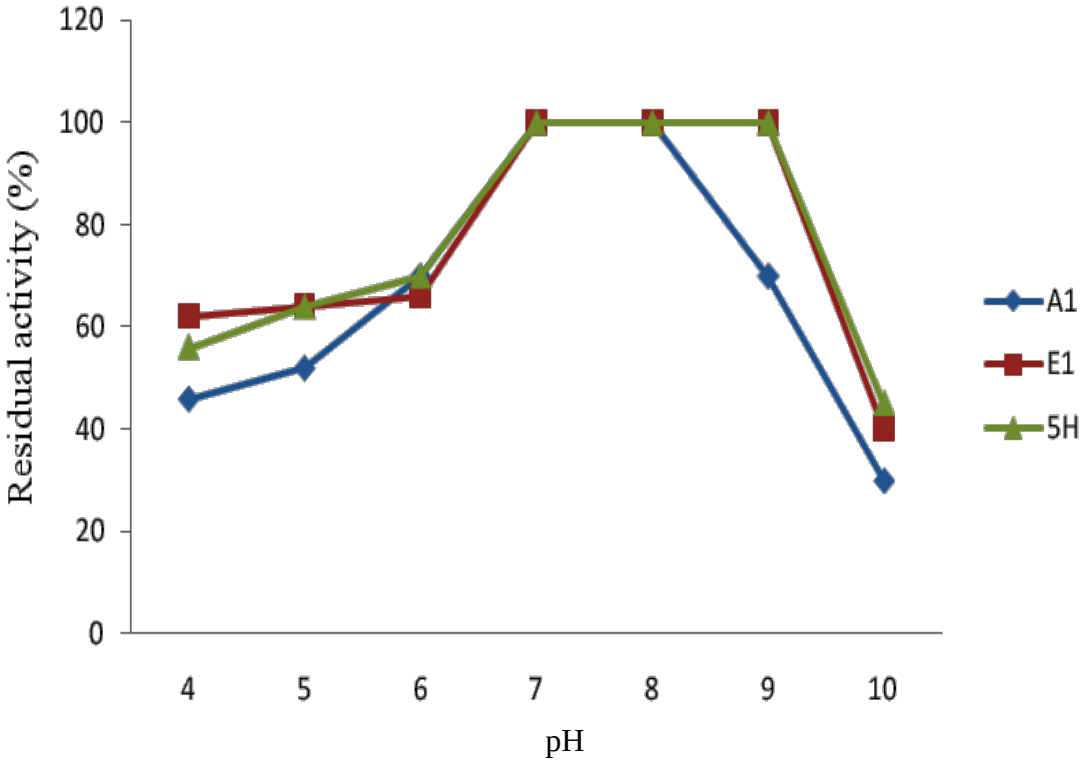


Figure 4. 41. Effect of pH on stability of purified cellulase from *Roseomonas* sp. A1, *Anoxybacillus rupiensis*.E1 and *Anoxybacillus rupiensis* 5H. Data are presented as a mean of 2 replicates. Standard deviation were less than 10% in all cases. The enzyme activities were expressed relative to the maximal values (4.0U/ml for A1, 4.89U/ml for E1 and 3.78U/ml for 5H).

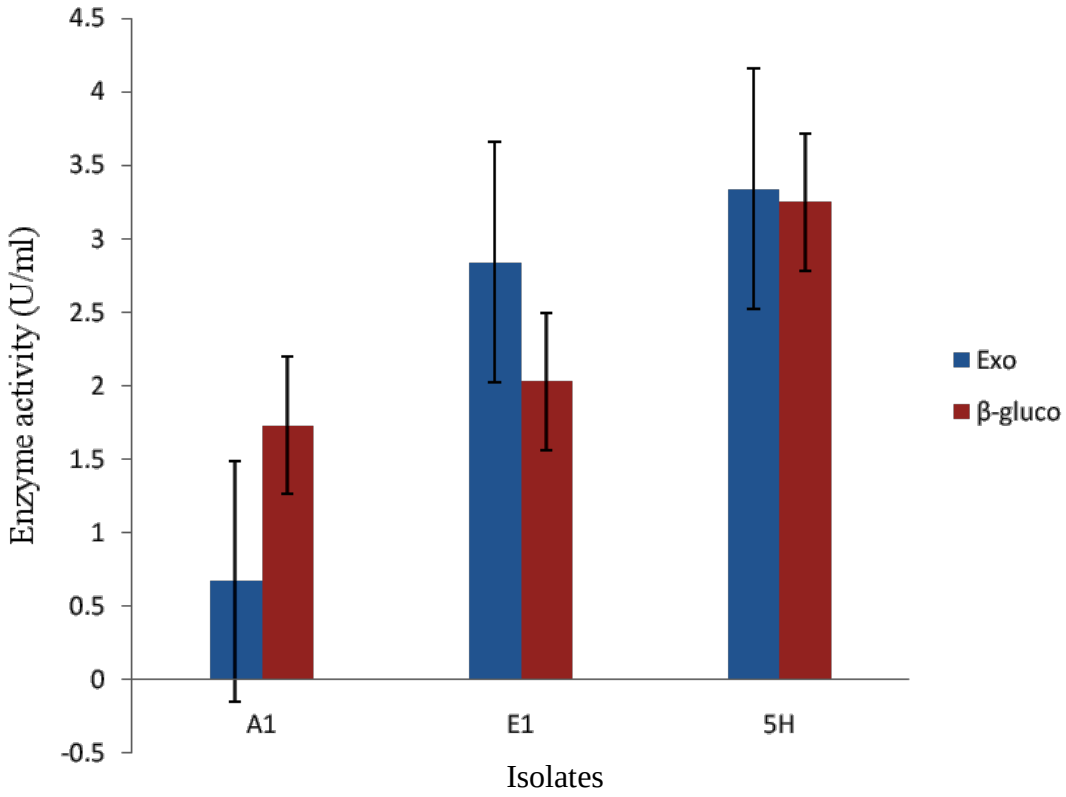


Figure 4.42. Production of Exoglucanase and beta-glucosidase enzymes by *Roseomonas* sp. (A1), *Anoxybacillus rupiensis.* (E1) and *Anoxybacillus rupiensis* 5H. *Exo= Exoglucanase, β-gluco= β-glucosidase*

4.2.6 **Time course for purified cellulase activity**

Studies on the effect of time on cellulase activity by *Roseomonas* sp. A1,*Anoxybacillus rupiensis* E1 and *Anoxybacillus rupiensis* 5H is shown in figure 4.43 Incubation for 30min was required for maximum activity by A1 and 5H while it was 40min for E1 after which there was drop in the activity as time increased.

4.2.7 **Effect of substrate concentration on the enzyme activity**

There was a gradual increase in cellulase activity as the concentration of CMC increased , it reached a maximum at 2.0% for *Roseomonas* sp. (A1) and *Anoxybacillus rupiensis* (5H) and 2.5% for *Anoxybacillus rupiensis* (E1). Further increase in substrate concentration was accompanied by a reduction in enzyme activity (Figure 4.44).

4.2.8 **Effect of metal ions, SDS, Tween 80 and EDTA on cellulase activity**

For *Roseomonas* sp. A1 cellulase activity increased in the presence of Na^{+} from 1mM to 10mM. Activity decreased as Ca^{2+}, Zn^{2+} and EDTA concentrations increased. However, lower concentration of Ca^{+} and Fe^{2+} lead to increment in the enzyme activity. Cu, Mg, Hg, Zn ions, Urea, SDS, EDTA and Tween 80 are all inhibitory at all concentrations. 10mM concentration of Tween 80 yielded increase in the activity of the enzyme (Table 4.22).

Increment in the activity of cellulase from *Anoxybacillus rupiensis* E1 was observed at 1mM for Na^{+} , Ca^{2+} and Fe^{2+} while Hg^{2+}, Cu^{2+}, Zn^{2+} Urea and SDS reduced activity of the enzyme Table 4.23. More so, Tween 80 at10mM concentration there was increment in the activity of the enzyme. All the concentrations of Mg^{2+} enhanced the activity of cellulase.

In the case *Anoxybacillus rupiensis* 5H, Na^{+} and Tween 80 were able to improve the activity of the enzyme at 1mM and 10mM while 0.1mM reduced its activity. All the concentrations of Ca^{2+} and Mg^{2+} were able to increase the activity of the enzyme. Hg^{2+} Zn^{2+} and EDTA were inhibitory to the activity at all concentrations. However, SDS at minimal concentration was able to enhance the activity of the enzyme (Table 4.24).

4.2.9 **Substrate specificity**

The activity of cellulase from isolates was tested on some cellulose and hemicelluloses related materials (Table 4.25). The results show that the cellulase from all the isolates were specific for cellulosic materials as they did not hydrolyze oat-spelt xylan and starch, hence there was no production of xylanase and amylase.

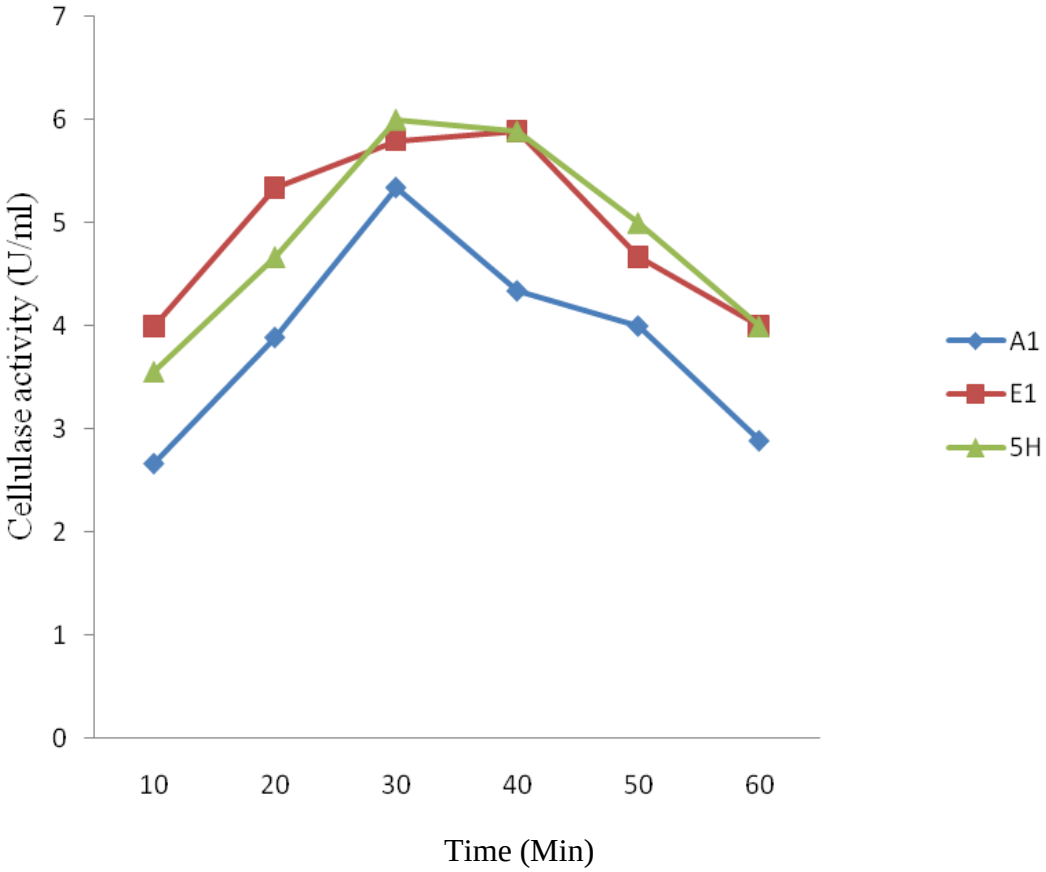


Figure 4.43. Effect of reaction time on the activity of purified cellulase from *Roseomonas* sp. A1, *Anoxybacillus rupiensis* E1 , *Anoxybacillus rupiensis* 5H. Data are presented as a mean of 2 replicates. Standard deviations were less than 10% in all cases.

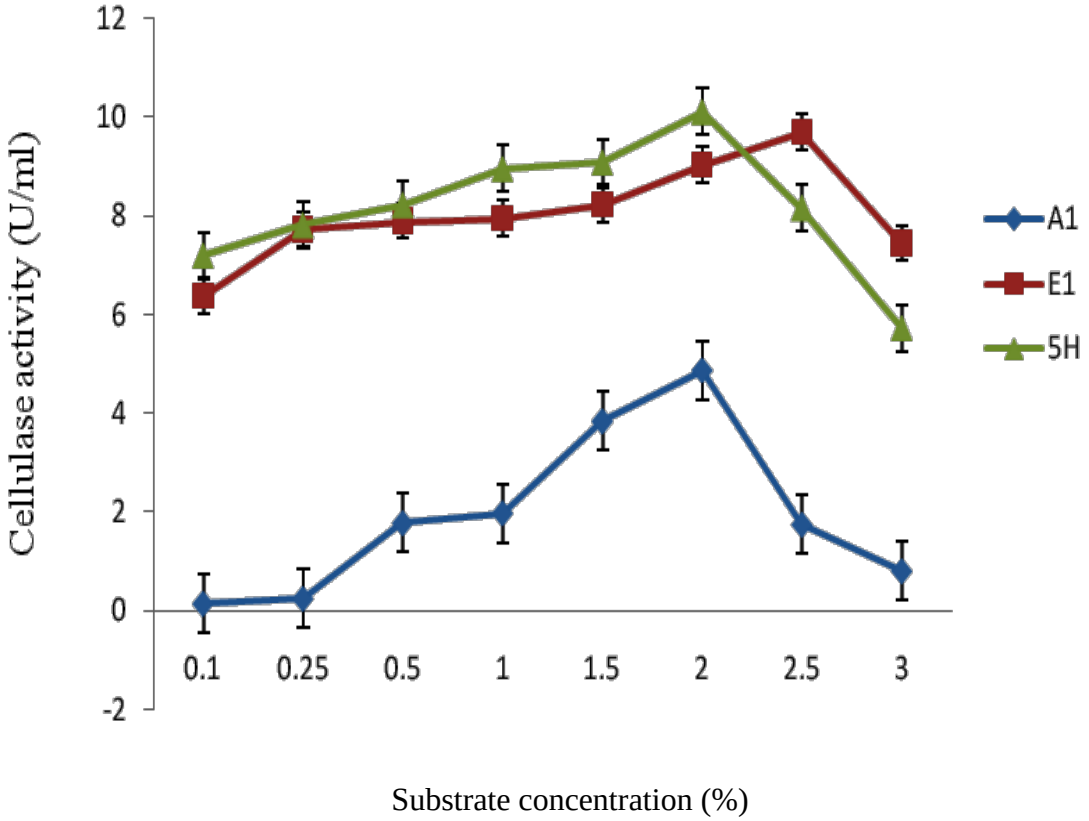


Figure 4.44. Effect of different concentrations of substrate on the activity of purified cellulase from A1= *Roseomonas* sp. E1 = *Anoxybacillus rupiensis*, 5H= *Anoxybacillus rupiensis* Data are presented as a means of 2 replicates.

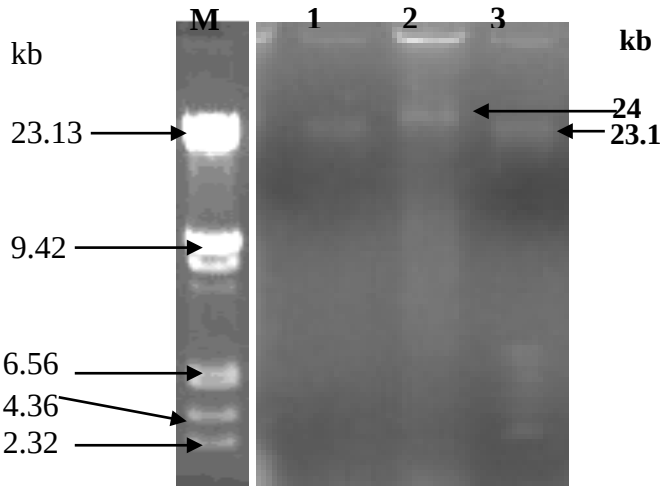


Figure 4.45. Agarose gel electrophoresis of plasmid DNAs recovered from the bacterial isolates. Lane M = Hind III digested DNA markers; Lane 1 = Isolate A1; Lane 2 = Isolate 5H; Lane 3 = isolate E1

Table 4.22. Effect of metal ions, SDS, Tween 80 and EDTA on activity of purified cellulase from *Roseomonas* sp. A1

Metal ions	Concentration of metal ions (mM)/ Relative cellulase activity (%)		
	0.1mM	1mM	10mM
Control	100±0.00[a]	100 ±0.00[a]	100±0.00[a]
Na	89± 4.24[b]	108±0.00[a]	114±4.24[a]
Mn	23±0.17[d]	33±1.41[e]	10±0.00[a]
Ca	106±1.83[a]	101±0.00[a]	89±2.83[b]
Cu	92±0.00[b]	78±0.71[c]	29±0.00[e]
Mg	75 ±2.83[c]	82±0.00[c]	93±1.41[b]
Hg	18±1.41[d]	36±0.00[e]	34±2.83[e]
Fe	102±2.83[a]	110±1.14[a]	92±0.00[b]
Zn	98±1.83[a]	88±1.83[c]	72±1.14[cd]
Urea	54±2.83[c]	67±0.00[d]	82±1.14[c]
SDS	62±1.41[c]	68±2.83[c]	79±0.00[c]
EDTA	92±4.24[ab]	87±0.00[c]	85±1.83[bc]
Tween80	84±0.00[b]	94±1.41[b]	101±1.41[a]

EDTA-Ethylene diamine tetraacetic acid. Each value is a mean of two replicates; ± stands for standard deviation among replicates; Means with different letters within each column differ significantly (≤ 0.05) using Duncan`s Multiple Range Test. 100% activity correspond to 24.6 U/ml

Table 4.23. Effect of metal ions, SDS, Tween 80 and EDTA on activity of purified cellulase from Anoxybacillus *rupiensis sp*. E1

Metal ions	Concentration of metal ions (mM)/Relative cellulase activity (%)		
	0.1mM	1mM	10mM
Control	100±0.00[a]	100 ±0.00[a]	100±0.00[a]
Na	94±0.00[ab]	101±0.00[a]	94±0.71[a]
Mn	62± 2.83[c]	66±4.24[c]	98±0.00[a]
Ca	91±0.71[a]	108±0.00[a]	186±1.41[b]
Cu	68±4.24[c]	48± 2.83[c]	82±2.83[c]
Mg	110±1.41[a]	112±2.83[b]	116±0.00[a]
Hg	47±2.83[c]	48±0.00[c]	45±0.00[d]
Fe	98±0.00[a]	107±2.83[a]	84±0.71[c]
Zn	87±0.00[b]	89±0.71[b]	72±2.83[d]
Urea	54±1.41[c]	67±0.00[b]	87±0.71[c]
SDS	84±0.00[b]	89±1.14[b]	92±0.00[a]
EDTA	90±0.71[ab]	81±4.24[b]	76±2.83[c]
Tween80	84±0.71[b]	92±2.83[a]	102±1.41[a]

EDTA-Ethylene diamine tetraacetic acid. Each value is a mean of two replicates; ± stands for standard deviation among replicates; means with different letters within each column differ significantly (≤ 0.05) using Duncan`s Multiple Range Test. 100% activity corresponds to 30.1 U/ml.

Table 4.24: Effect of metal ions, SDS, Tween 80 and EDTA on activity of purified cellulase from *Anoxybacillus rupiensis*. 5H

Metal ions	Concentration of metal ions (mM)/Relative cellulase activity (%)		
	0.1mM	1mM	10mM
Control	100±0.00[a]	100 ±0.00[a]	100±0.00[a]
Na	96±1.41[a]	122±0.71[a]	118±1.41[a]
Mn	65±0.00[c]	87±4.24[b]	126±0.00[a]
Ca	102±4.24[a]	118±0.00[a]	171±2.83[b]
Cu	81±0.71[b]	66±1.41[b]	102±2.83[a]
Mg	108±2.83[a]	103±2.83[a]	130±4.24[ab]
Hg	37±1.41[c]	76± 4.24[b]	58±0.00[d]
Fe	101±0.71[a]	106±1.14[a]	87±0.00[c]
Zn	85±2.83[b]	72±2.83[a]	69±1.14[d]
Urea	55±1.41[c]	70±1.41[b]	78±0.71[cd]
SDS	102±0.00[a]	86±1.41[b]	72±0.00[d]
EDTA	98±4.24[a]	96±0.00[a]	86±4.24[c]
Tween80	88±1.41[b]	102±0.71[a]	103±1.41[a]

EDTA-Ethylene diamine tetraacetic acid. Each value is a mean of two replicates; ± standard deviation among replicates; means with different letters within each column differ significantly (≤ 0.05) using Duncan`s Multiple Range Test. 100% activity corresponds to 32.2 U/ml.

Table 4. 25. Activity of partially purified cellulase from *Roseomonas* sp. (A1), *Anoxybacillus rupiensis* (E1) and *Anoxybacillus rupiensis* (5H) against various substrates.

Substrates	*Roseomonas*.sp. A1 Cellulase (U/ml)	*Anoxybacillus rupiensis* E1 Cellulase (U/ml)	*Anoxybacillus rupiensis* 5H Cellulase (U/ml)
CMC	3.89	7.78	5.45
Oat-spelt Xylan	0.00	0.00	0.00
Salicin	3.34	5.45	3.78
Starch	0.00	0.00	0.00

CMC: Carboxylmethylcellulose

4.2.10 **Kinetic Studies**

Affinity of 72h *Roseomonas* sp. A1 cellulase for CMC as substrate was examined by using Lineweaver-Burk plot (Figure 4.46). The enzyme reaction followed Michaelis-Menten kinetics with K_m and V_{max} values of 12.5mg/ml and 1.54U/mg respectively when the concentration of CMC was varied from 0.2-1.0mg/ml.

The same trend was observed with the CMC as substrate for *Anoxybacillus rupiensis* E148h old and 5H 72h old. While K_m and V_{max} were 9.3mg/ml and 0.65U/mg respectively for E1, it was 5.9mg/ml and 0.82U/mg protein for K_m and V_{max} respectively for 5H over a CMC concentration of 0.2-1.0mg/ml (Figure 4.47 and Figure 4.48)

4.2.11 **Inhibition Studies**

The end-product inhibition of *Roseomonas* sp. and *Anoxybacillus rubiensis* E1 and 5H was studied with CMC as substrate and the sugar D-glucose as inhibitor. Glucose was found to inhibit the cellulase competitively and the inhibition constant (K_i) was determined from a Lineweaver-Burk plot (Figure 4.49) and was found to be 1.35

The inhibition effect of glucose on the activity of *Anoxybacillus rupiensis* E1 and 5H on the substrate was also examined. Glucose competitively inhibited the activity of the enzyme. K_i values were estimated from the graph and it was 1.29 and 1.53 for E1 and 5H respectively (Figures 4.50 and 4.51)

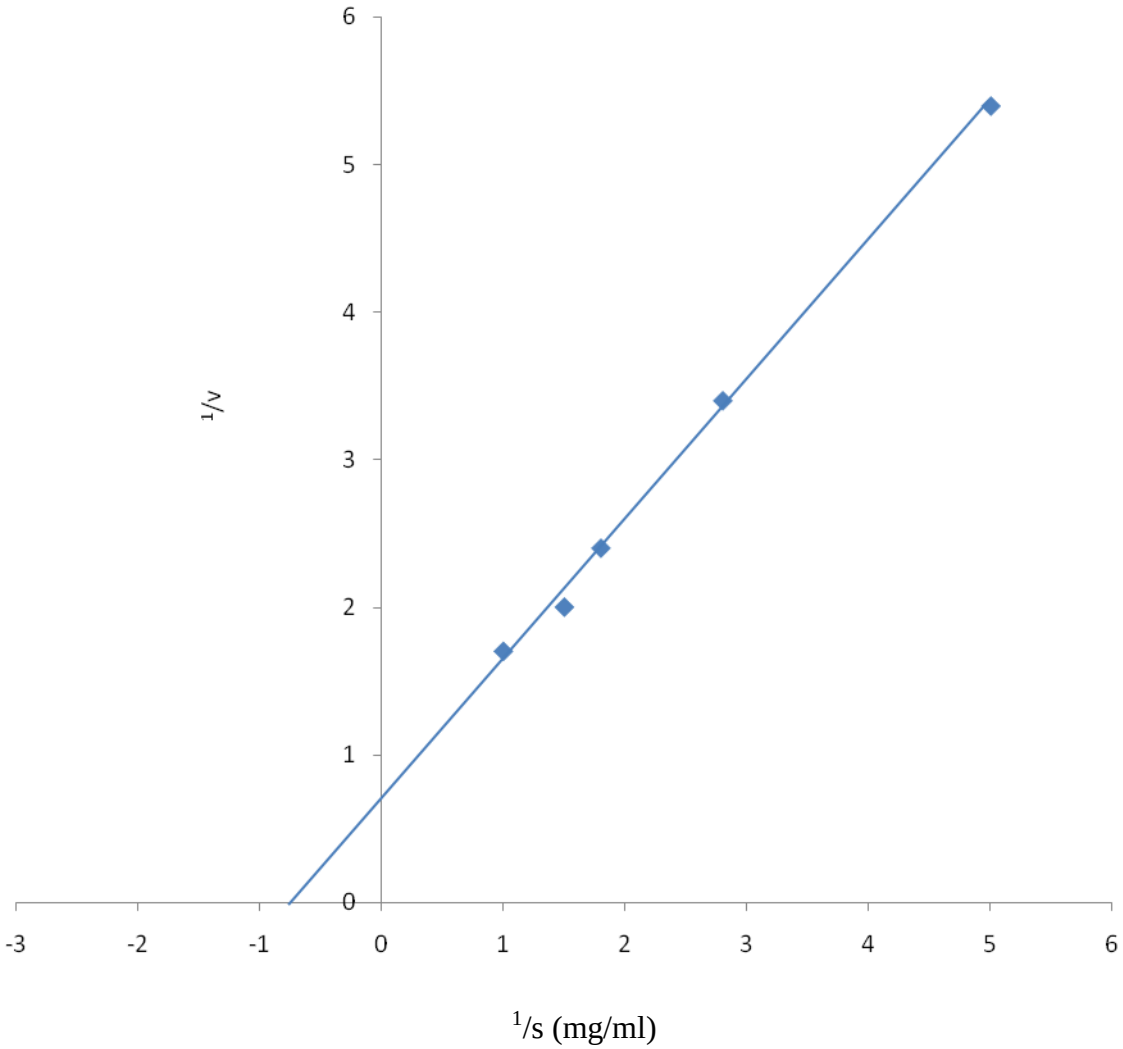


Figure 4. 46. Lineweaver-Burk plot for hydrolysis of carb-oxymethylcellulose by cellulase from 72h old culture supernatant of *Roseomonas* sp. (A1).

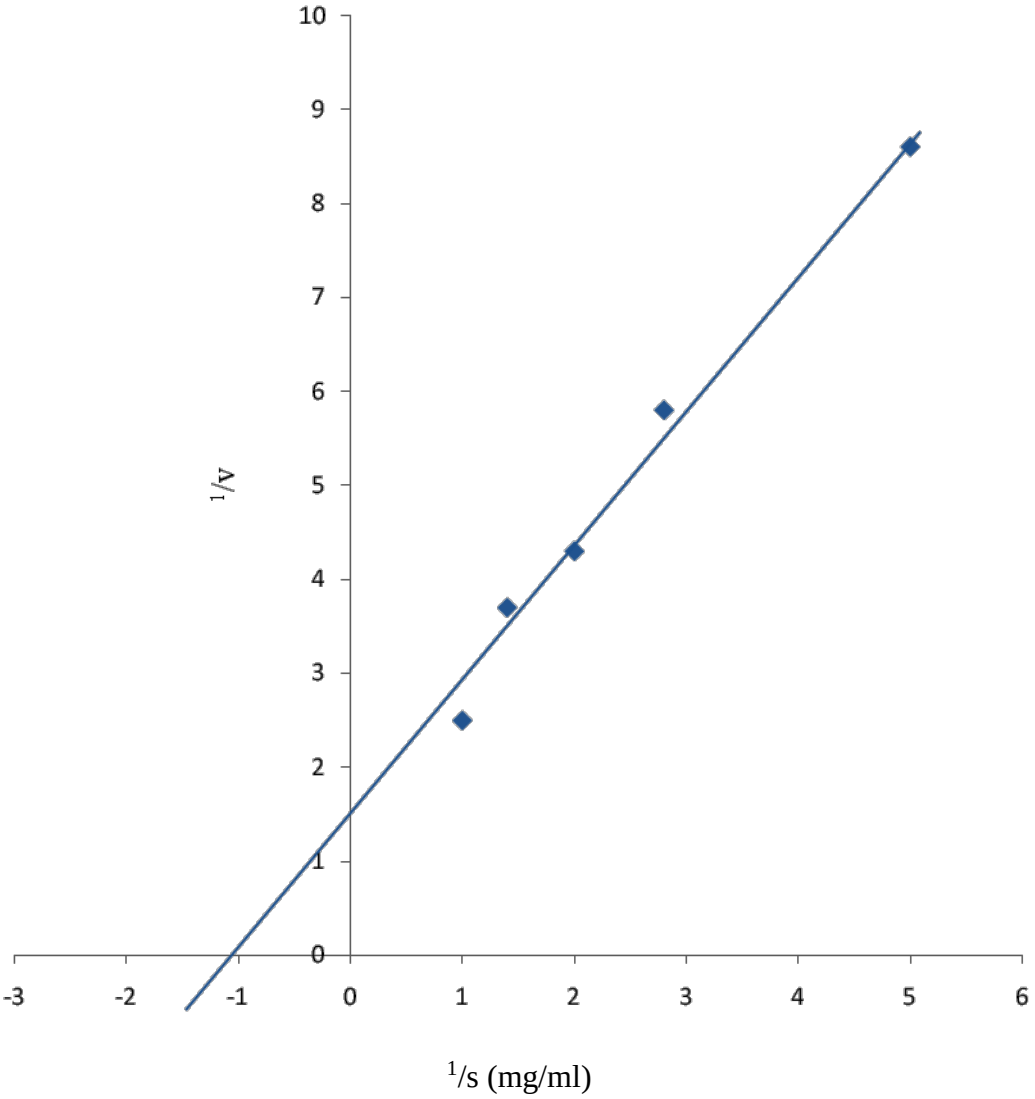


Figure 4. 47. Lineweaver-Burk plot for hydrolysis of carboxymethyl-cellulose by cellulase from 48h old culture supernatant of *Anoxybacillus rupiensis* (E1).

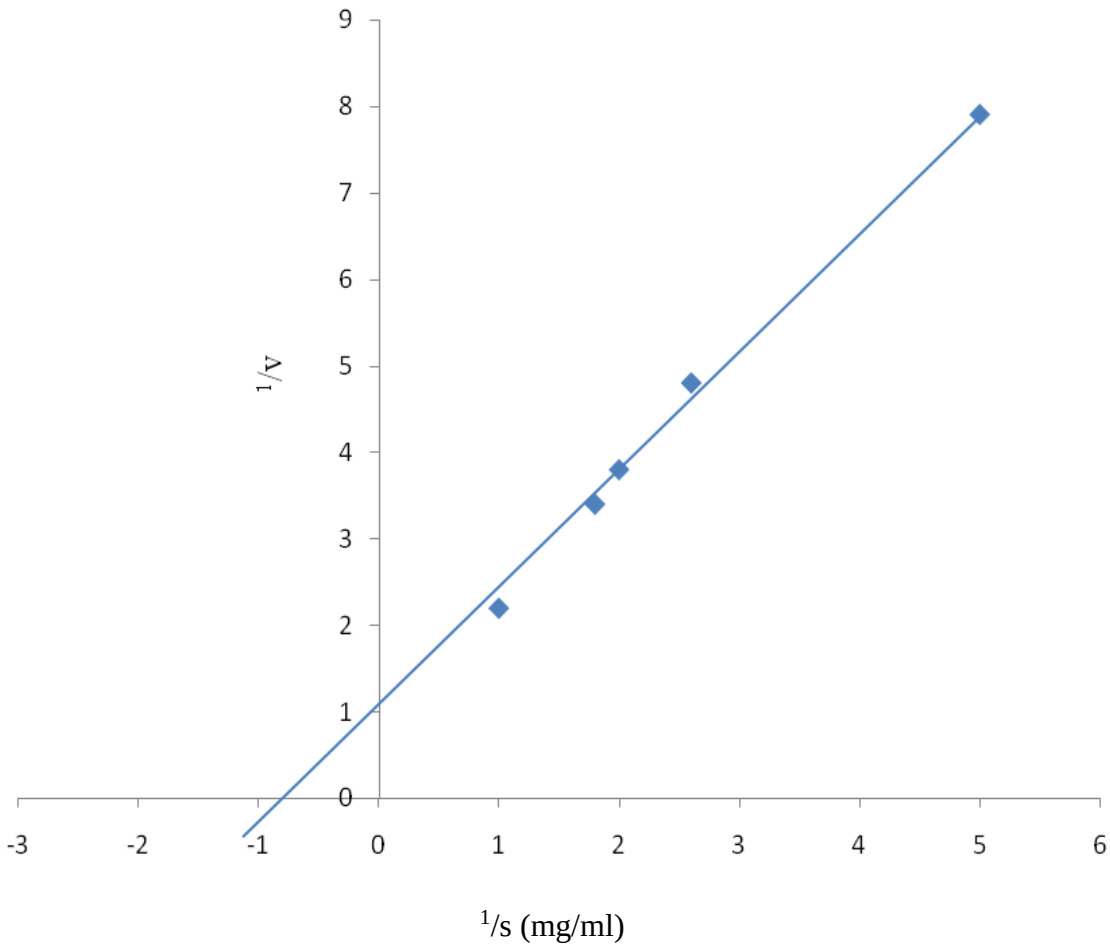


Figure 4.48. Lineweaver-Burk plot for hydrolysis of carboxyme- hylcellulose by cellulase from 72h old culture supernatant of *Anoxybacillus rupiensis* (5H).

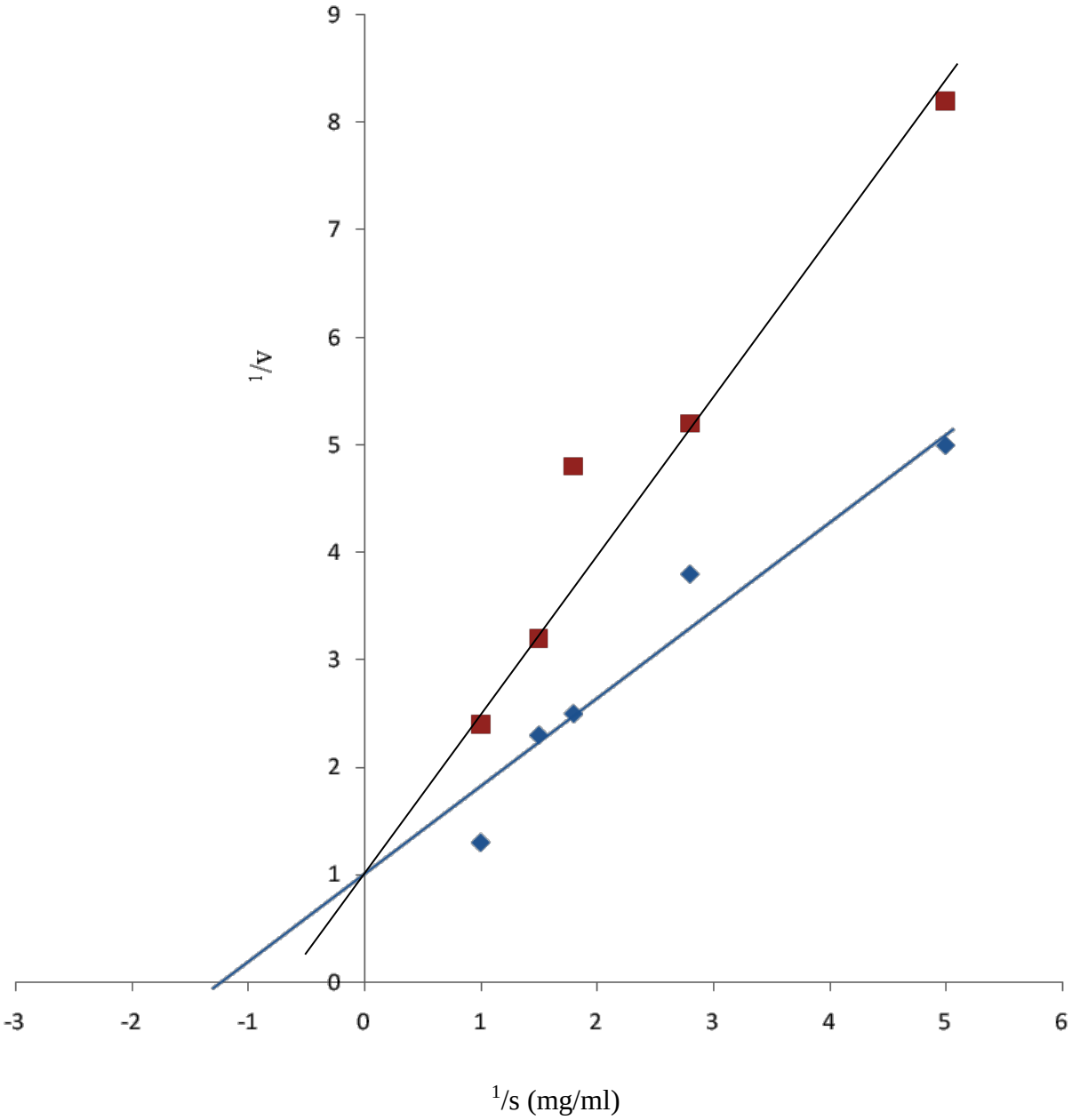


Figure 4.49. Lineweaver-Burk plot of the inhibitory effect of glucose on carboxymethylcellulose hydrolysis by cellulase from *Roseomonas* sp. (A1).

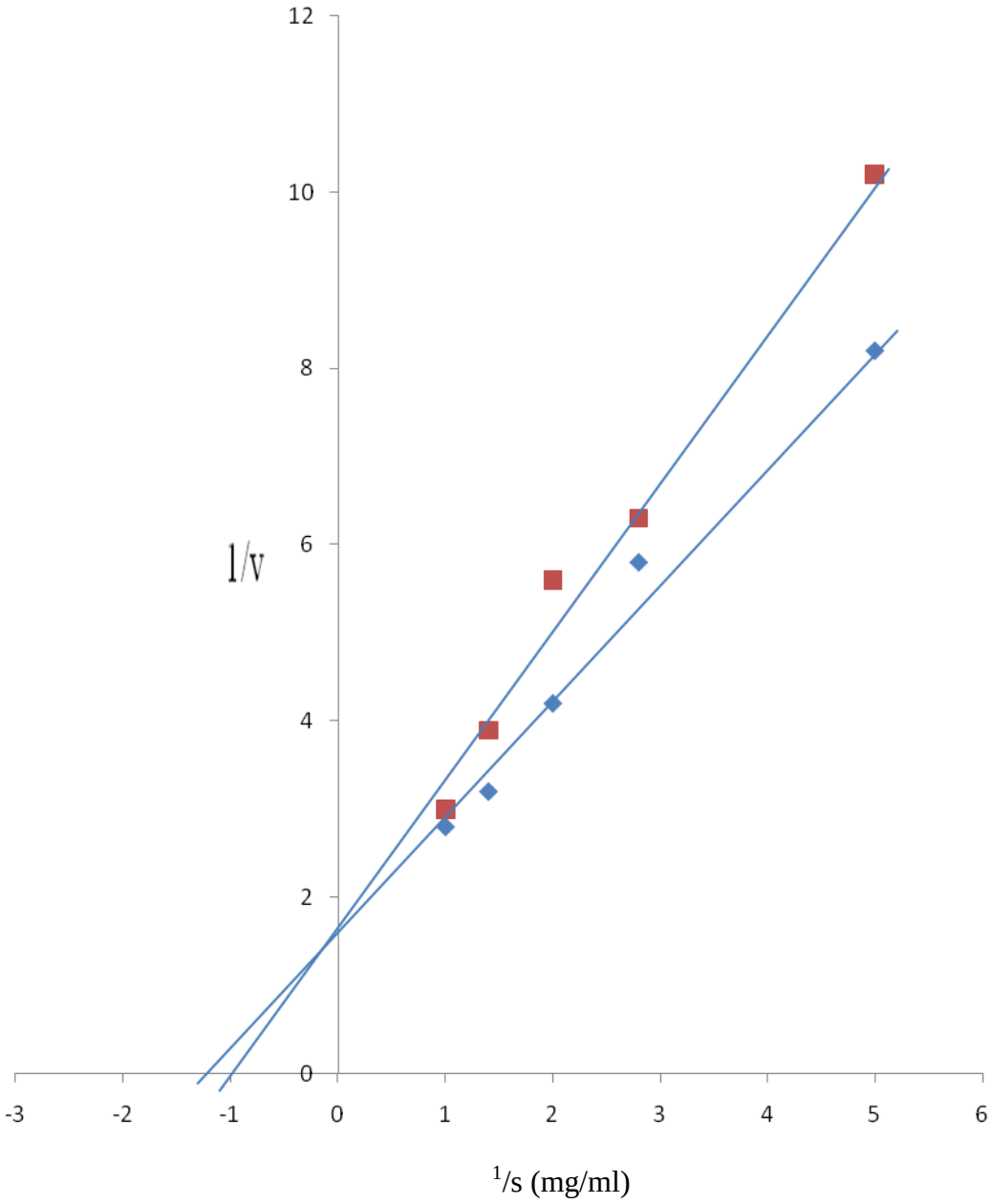


Figure 4.50: Lineweaver-Burk plot of the inhibitory effect of glucose on carboxymethyl- cellulose hydrolysis by cellulase from *Anoxybacillus rupiensis* (E1).

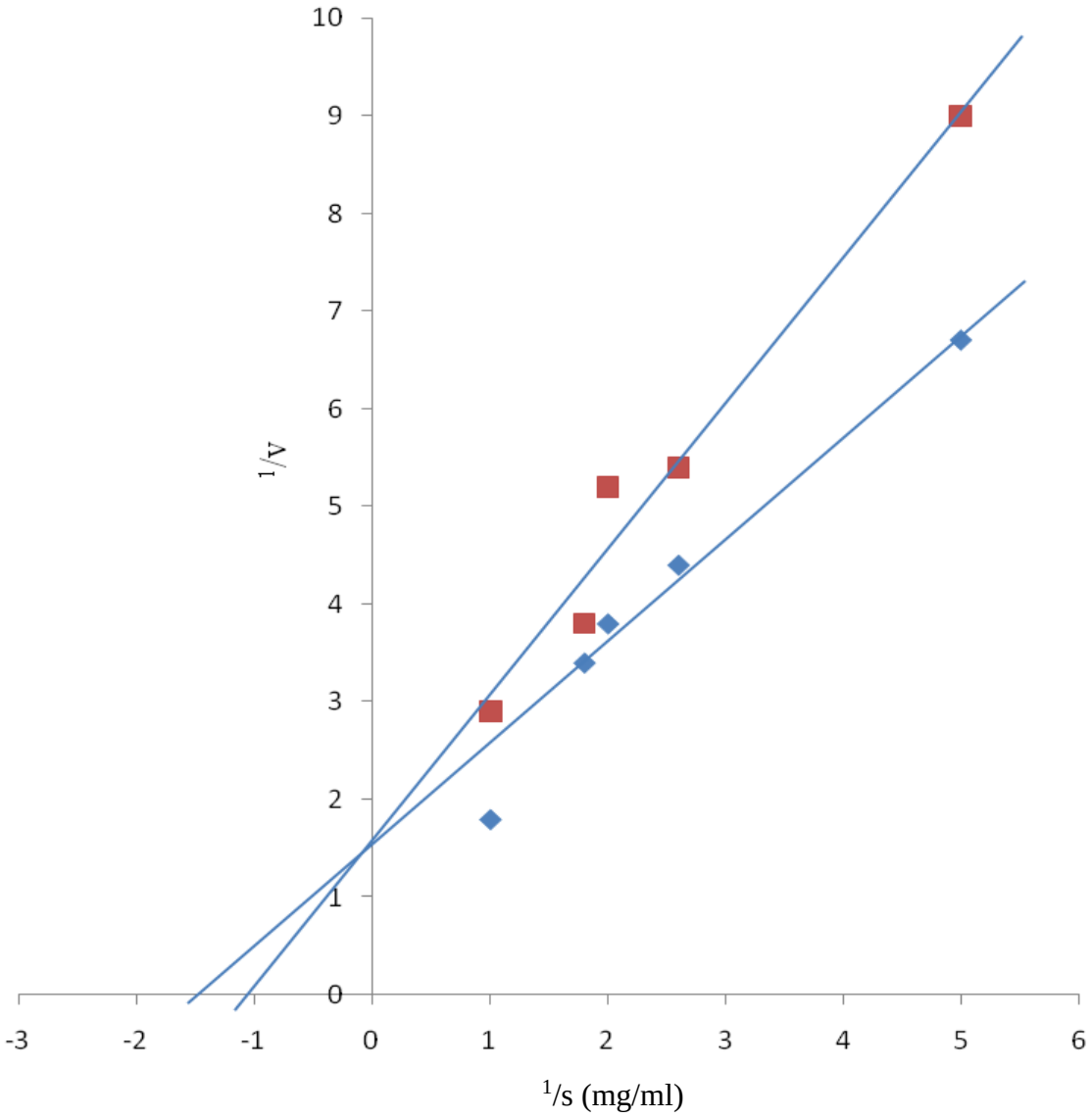


Figure 4.51. Lineweaver-Burk plot of the inhibitory effect of glucose on carboxymethylcellulose hydrolysis by cellulase from *Anoxybacillus rupiensis* (5H).

4.2.12 **Hydrolysis of Substrates**

Hydrolysis of different lignocellulosics substrates was carried out at 50^0C for 48hr in 50mM phosphate buffer pH 7.0. The substrates were used at initial concentration 10mg/ml. Tables 4.26 and 4.27 show the hydrolysis of these substrates. The results indicate that sugarcane bagasse had the highest rate of hydrolysis using enzymes produced by both *Roseomonas* sp. A1 and *Anoxybacillus rupiensis*, E1 while corn cob produced highest hydrolysis in the presence of enzyme produced by *Anoxybacillus rupiensis*, 5H (Tables 4.28). Alkali treated sugarcane bagasse produced a hydrolysis of 21.6 %, and 29.2% when treated with cellulase from *Roseomonas* sp. A1and *Anoxybacillus rupiensis* E1 respectively after 48hrs. This was followed by alkali treated corn cob with 9.2% and 19.1% rate for *Roseomonas* sp. and *Anoxybacillus rupiensis* respectively. The hydrolysis of sugar cane bagasse was significantly higher ($p \leq 0.05$) than that of other substrates treated with cellulase from the two organisms. For *Anoxybacillus rupiensis* 5H alkali treated corn cob produced hydrolysis of 38.9% when treated with the cellulase from other organisms. This was immediately followed by alkali treated sugar cane bagasse with 16.4% hydrolysis rate. Sawdust from all the different woody plants were hydrolyzed to a lesser extent.

When compared to alkali treated corn cob, sugar cane bagasse and sawdust, untreated lignocellulosic materials were hydrolyzed at low rate. Hydrolysis values of corn cob increased from 2.1% to 9.2% and 7.7% to 19.1% for *Roseomonas* sp. A1 and *Anoxybacillus rupiensis* E1culture filtrates upon alkali treatment (Tables 4.26, 4.27 and 4.28). Sawdust gave low hydrolysis values 1.1% to 3.2% for A1 and 1.4 to 3.2% for E1. However, hydrolysis value of sugar cane bagasse increased from 10.2% to 16.4% for *Anoxybacillus rupiensis* 5H culture filtrate upon alkali treatment (Table 4.28), while sugar cane bagasse gave low value 1.9% to 4.3%. There was an increase in the rate of hydrolysis with time for all the substrates.

The cellulase hydrolysis of alkali treated sugar cane bagasse by *Roseomonas* sp. A1 and *Anoxybacillus rupiensis* E1 as well as hydrolysis of alkali treated corn cob by cellulase from *Anoxybacillus rupiensis* 5H at different values of pH from 5.0 to 10.0 is shown in (Figure 4.52). The best hydrolysis was observed at pH 7 for all the isolates as 21%, 23% and 25% for *Roseomonas* sp. A1, *Anoxybacillus rupiensis* E1 and *Anoxybacillus rupiensis* 5H respectively. A decrease in the hydrolysis of sugar cane bagasse at pH values above 7 was recorded for A1 and E1. For 5H after the

decrease of hydrolysis at pH 8.0, an increase of hydrolysis was noticed at pH 9.0 after which there was sharp decrease in the hydrolysis. Hydrolysis at pH 7.0 was significantly higher ($p \leq 0.05$) when compared to other pH values.

Culture filtrates from *Roseomonas* sp. A1 and *Anoxybacillus rupiensis* E1 produced highest hydrolysis with alkali treated sugar cane bagasse at temperature of 60^0C yielding 15% A1 and 17.2% E1 (Figure 4.53). However, the optimum temperature for the hydrolysis of alkali treated corn cob by cellulase from 5H was 65^0C yielding 22.3% (Figure 4.53).The rate of hydrolysis decreased at temperatures above 60^0C for cellulase from isolates A1 and E1 giving 2% and 3% respectively while hydrolysis fell at temperatures above 65^0C for cellulase from isolate 5H producing 6% hydrolysis. The hydrolysis of sugarcane bagasse was significantly ($P \leq 0.05$) higher than the other substrates for culture filtrates from the isolates A1 and E1 while hydrolysis of corn cob was significantly higher in the case of 5H as compared to the other substrates. Sawdust from different plants were hydrolyzed to a lesser extent, hence they are resistant to enzymatic hydrolysis.

Culture filtrates from *Roseomonas* sp. A1, *Anoxybacillus rupiensis* E1 and *Anoxybacillus rupiensis* 5H hydrolyzed untreated corncob, sugarcane bagasse and sawdust at a low rates when compared to alkali treated substrates. The hydrolysis value of corncob increased from 2.1-9.2, 7.7-19.1, 19.6-38.9 for *Roseomonas* sp., *Anoxybacillus rupiensis* and *Anoxybacillus rupiensis* culture filtrates respectively(Table 4.26, 4.27, 4.28). Sawdust gave very low hydrolysis values (1.1-3.2% for *Roseomonas* sp. A1,1.4-4.4% for *Anoxybacillus rupiensis* E1 and 1.9-5.9% for *Anoxybacillus rupiensis* 5H).However, there was an increase in the hydrolysis rate with time for the substrates.

The effect of alkali-treated sugarcane bagasse and corncob concentration on percentage of hydrolysis of organisms' culture filtrate is shown in Figure 4.54. The percentage of hydrolysis increased with increase in concentration of substrates and then became level as the concentration continued. The best concentration of sugarcane bagasse for hydrolysis for the culture filtrates of A1and E1 3% (16 and 20% hydrolysis) while it was 4% for 5H (22% hydrolysis). In Figure 4.55, the hydrolysis of alkali-treated sugarcane bagasse with different concentration of culture filtrates of A1, E1 and 5H is shown. The rate of hydrolysis increased with in increase in concentration of culture filtrates for the three isolates. The percentage hydrolysis increased from

15.2% at concentration of 100% buffer to 34.7% at 20% buffer for A1. That of E1 was increased from 18.6 to 39.8 at 100% to 20% buffer while it was increased from 21.5% to 42.7% at 100% and 20% buffer respectively for 5H. Using 20% buffer of culture filtrate of A1, E1 and 5H respectively produced a significant different in the rate hydrolysis compared to other concentrations.

The length of incubation also has effect on the rate of hydrolysis of alkali-treated sugarcane bagasse and corn cob by cellulase from *Roseomonas* sp. A1 *Anoxybacillus rupiensis* E1 and *Anoxybacillus rupiensis* 5H as shown in Figure 4.53. As time of hydrolysis increased there was an increase in the rate of hydrolysis. Hydrolysis increased significantly from 8.5%, 8.8%, and 7.6% for A1, E1 and 5H to 12.8%, 17.2% and 18.4 at 30hr, 30h and 24hr respectively.

Table 4.26. Hydrolysis (%) of different lignocellulosic substrates by cellulase from *Roseomonas* sp.

Substrate	Treatment/ Time of Hydrolysis (hr)/ Percentage hydrolysis							
	Untreated				Alkali treated			
	1	6	24	48	1	6	24	48
Corn cob	0.4±0.00^{c}	0.8±1.41^{c}	1.3±0.71^{c}	2.1±0.00^{c}	2.0±0.03^{a}	4.6±0.71^{b}	4.9±0.07^{b}	9.2±0.57^{b}
Sugarcane bagasse	5.3±0.00^{a}	6.2±0.00^{a}	12.2± 1.41^{a}	19.7±0.57^{a}	3.3±1,41^{a}	9.9±0.80^{a}	12.6±0.57^{a}	21.1±1.41^{a}
Sawdust (Iroko) *Chlorophora excelsa*	0.1±0.71^{d}	0.2±0.07^{c}	0.6±0.14+	1.6±1.41^{cd}	0.2±0.00^{c}	0.4±0.00^{c}	0.7±0.00^{d}	2.6±0.07^{c}
Sawdust (Ire) *Funtumia africana*	0.1±1.41^{d}	0.7± 0.00^{c}	1.3±0.07^{c}	1.8±0.07^{c}	0.4±0.00^{b}	0.6±0.00^{c}	1.8±0.00^{c}	2.6±0.03^{c}
Sawdust (Gedu) *Tectona grandis*	0.1±0.07^{d}	0.4±0.57^{c}	0.5±0.00^{d}	1.1±0.00^{d}	0.1±0.00^{c}	0.5±0.00^{c}	1.6±0.07^{c}	3.2±0.35^{c}
Sawdust (Oro) *Irvingia* sp.	0.4±0.03^{c}	0.5±0.00^{c}	0.7±0.00^{d}	2.2±0.00^{c}	0.5±0.00^{b}	1.0±0.00^{d}	1.3±0.00^{c}	3.1± 0.00^{c}
CMC	3.2±0.00^{b}	4.6±1.41^{b}	6.1±0.35^{b}	10.4±0.57^{b}				

Each value is a mean of two replicates; ± stands for standard deviation among replicates; Means followed by different superscript within each column differ significantly at $p \leq 0.05$ using Duncan`s Multiple Range Test.

Table 4.27. Hydrolysis (%) of different lignocellulosic substrates by cellulase from *Anoxybacillus rupiensis* E1.

Substrate	Treatment/ Time of Hydrolysis (hr)/ Percentage hydrolysis							
	Untreated				Alkali treated			
	1	6	24	48	1	6	24	48
Corn cob	1.2±0.00^{c}	2.5±0.35^{c}	4.4±0.71^{c}	7.7±1.41^{c}	2.2±0.00^{b}	6.0±0.57^{b}	10.7±2.83^{b}	19.1±0.81^{b}
Sugarcane bagasse	5.9±0.35^{a}	14.4±0.71^{a}	17.4±2.83^{a}	20.0±2.83^{a}	8.7±0.71^{a}	12.2±1.41^{a}	19.1±0.35^{a}	29.2±0.35^{a}
Sawdust (Iroko) *Chlorophora excelsa*	0.5±0.00^{c}	1.2±0.00^{cd}	2.0±0.35^{d}	3.1±0.00^{d}	0.4±0.00^{cd}	1.9±0.35^{c}	2.4±0.71^{c}	3.4±1.41^{c}
Sawdust (Ire) *Funtumia africana*	0.7±0.14^{c}	1.2±1.14^{cd}	2.1±0.00^{d}	3.1±0.71^{d}	1.0±0.00^{c}	2.5±0.14^{c}	3.5±0.35^{c}	4.4±0.00^{c}
Sawdust (Gedu) *Tectona grandis*	0.3±0.00^{d}	0.5±0.00^{d}	0.9±0.00^{e}	1.7±0.00^{e}	0.9±0.00^{c}	1.3±0.05^{d}	1.6±0.00^{d}	4.1±0.21^{c}
Sawdust (Oro) *Irvingia* sp.	0.4±0.71^{cd}	0.5±0.00^{d}	0.8±0.00^{e}	1.4±1.14^{e}	1.0±0.00^{c}	1.3±0.00^{d}	1.6±0.00^{d}	3.2±0.71^{cd}
CMC	4.2±1.14^{b}	9.0±0.21^{b}	13.2±1.14^{b}	16.3±2.83^{b}				

Each value is a mean of two replicates; ± stands for standard deviation among replicates; Means followed by different superscript within each column differ significantly at $p \leq 0.05$ using Duncan`s Multiple Range Test.

Table 4.28. Hydrolysis (%) of different lignocellulosic substrates by cellulase from *Anoxybacillus rupiensis* 5H.

Substrate	Treatment/ Time of Hydrolysis (hr)/ Percentage hydrolysis							
	Untreated				Alkali treated			
	1	6	24	48	1	6	24	48
Corn cob	7.8± 1.17^{a}	19.2±0.71^{a}	23.2±0.85^{a}	36.6± 2.83^{a}	11.6±1.60^{a}	16.3±0.35^{a}	25.4±1.41^{a}	38.9±2.83^{a}
Sugarcane bagasse	1.6±0.19^{c}	3.3±0.15^{c}	5.6±0.03^{c}	10.2±1.28^{c}	3.0±0.00^{b}	8.0±0.35^{b}	14.2±2.13^{b}	16.4±2.13^{b}
Sawdust (Iroko) *Chlorophora excelsa*	0.6±0.03^{d}	1.6±0.35^{c}	2.6±0.35^{d}	4.1±0.71^{d}	0.6±0.00^{d}	2.6±0.19^{c}	3.2±0.00^{c}	4.6±1.41^{cd}
Sawdust (Ire) *Funtumia africana*	0.9±0.00^{c}	1.6±1.14^{c}	2.8±0.00^{d}	4.2±0.00^{d}	1.3±0.00^{c}	3.3±0.71^{c}	4.6±0.15^{c}	5.9±0.71^{c}
Sawdust (Gedu) *Tectona grandis*	0.4±0.00^{d}	0.7±0.00^{d}	1.2±0.03^{d}	2.4±0.19^{e}	1.2± 0.00^{c}	1.7±0.08^{d}	2.3±0.00^{cd}	5.5±0.28^{c}
Sawdust (Oro) *Irvingia* sp.	0.5±0.03^{d}	0.7±0.00^{d}	1.0±0.00^{d}	1.9±0.28^{e}	1.4±0.12^{c}	1.8±0.07^{d}	2.0±0.28^{d}	4.3±0.71^{d}
CMC	4.3±0.43^{b}	9.3±1.35^{b}	12.1±1.41^{b}	18.0±0.35^{b}				

Each value is a mean of two replicates; ± stands for standard deviation among replicates; Means followed by different superscript within each column differ significantly at $p \leq 0.05$ using Duncan`s Multiple Range Test.

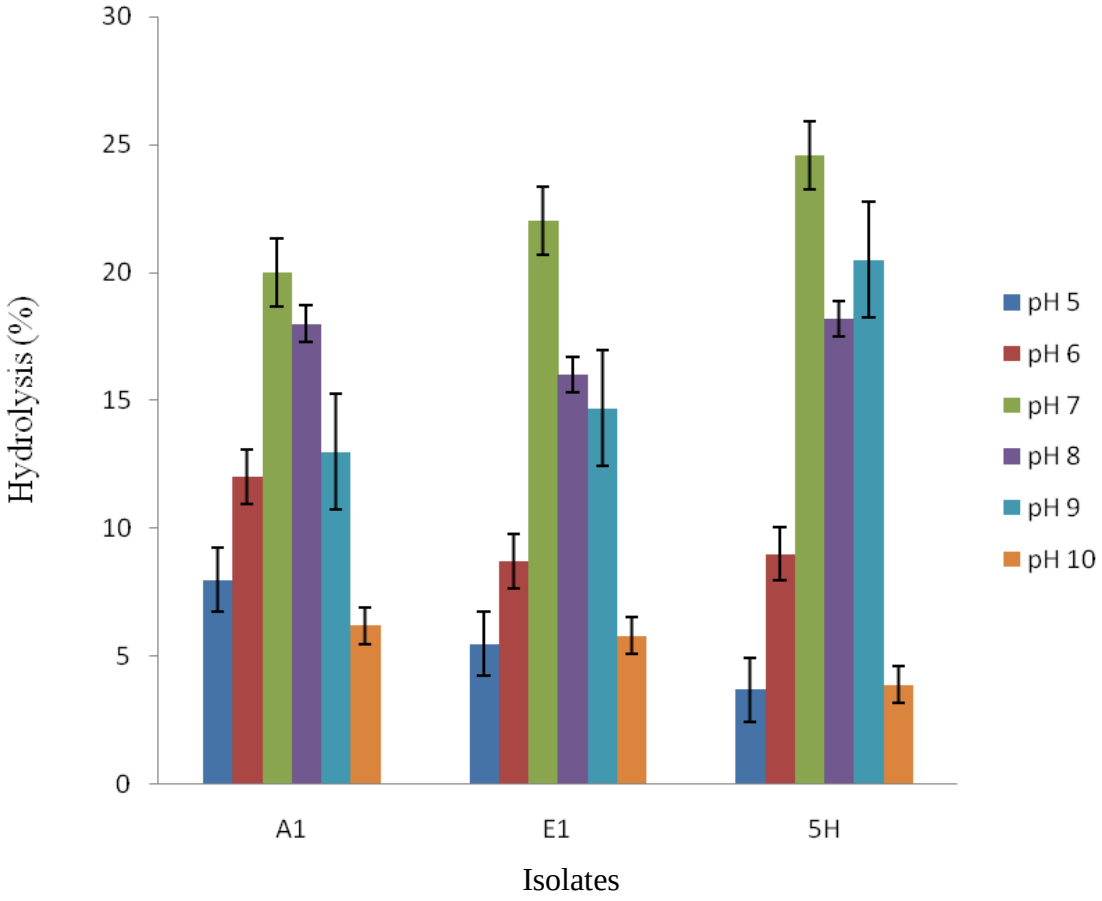


Figure 4. 52: Effect of pH on hydrolysis of alkali-treated sugarcane bagasse by cellulase from *Roseomonas* sp. A1, *Anoxybacillus rupiensis* E1 and corncob by cellulase from *Anoxybacillus rupiensis* 5H. Data are presented as a mean 2 replicates. Standard deviations were less than 10% in all cases.

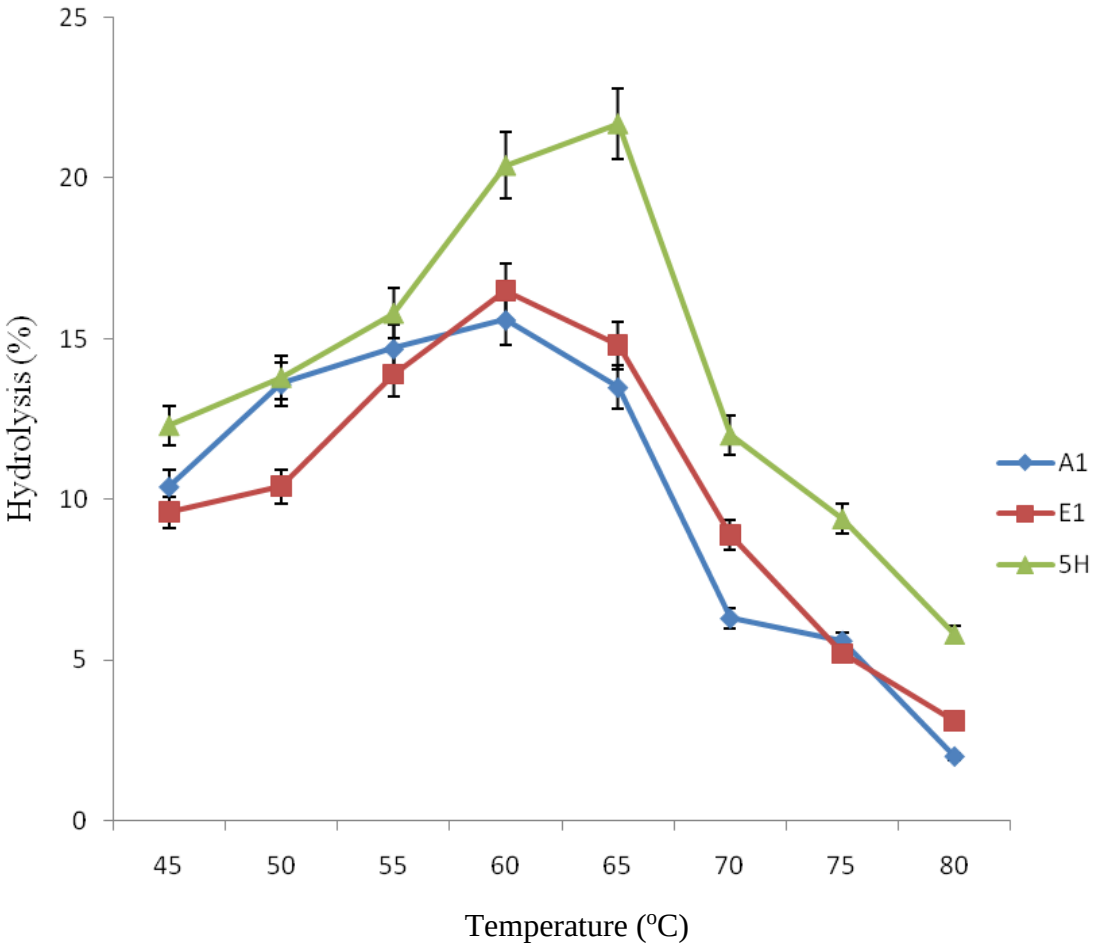


Figure 4.53: Effect of temperature on hydrolysis of alkali-treated sugarcane bagasse by cellulase from *Roseomonas* sp. A1, *Anoxybacillus rupiensis* E1 and corncob by cellulase from *Anoxybacillus rupiensis* 5H. Data are presented as a mean 2 replicates. Standard deviations were less than 10% in all cases.

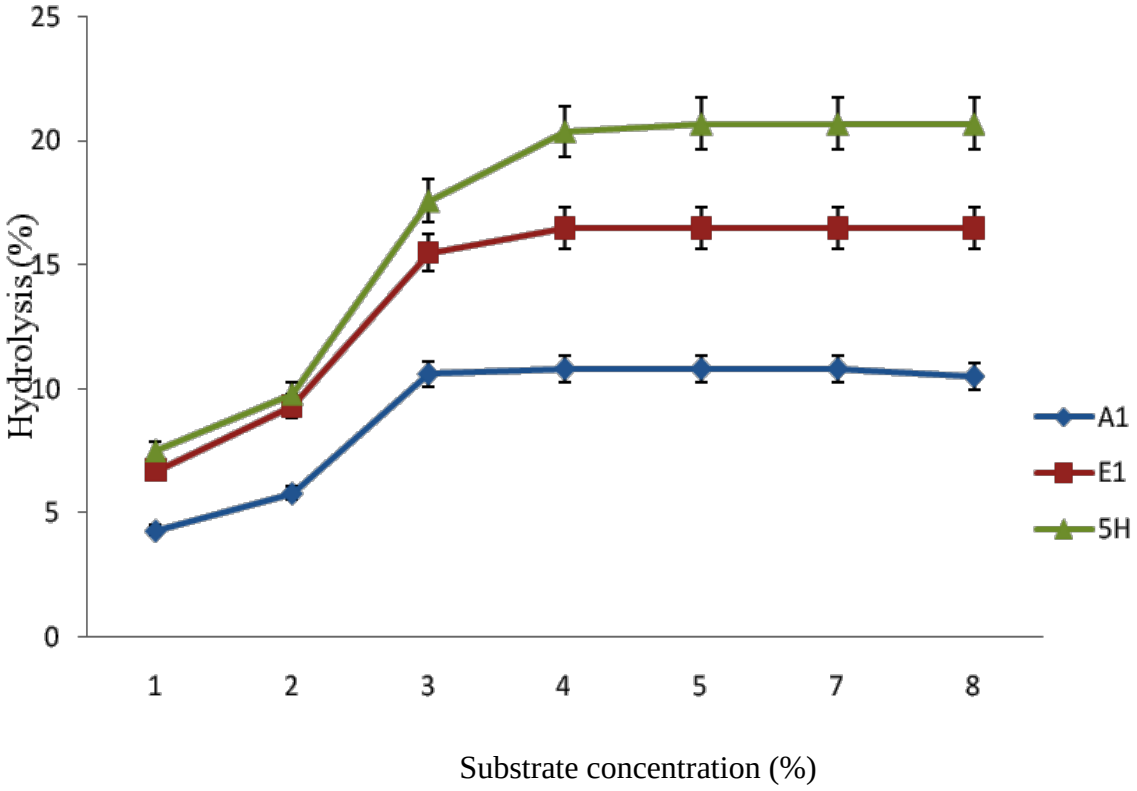


Figure 4.54: Effect of substrate concentration of hydrolysis of alkali-treated sugarcane bagasse by cellulose from *Roseomonas* sp. A1, *Anoxybacillus rupiensis* E1 and corncob by cellulase from *Anoxybacillus rupiensis* 5H. Data are presented as a mean 2 replicates. Standard deviations were less than 10% in all cases.

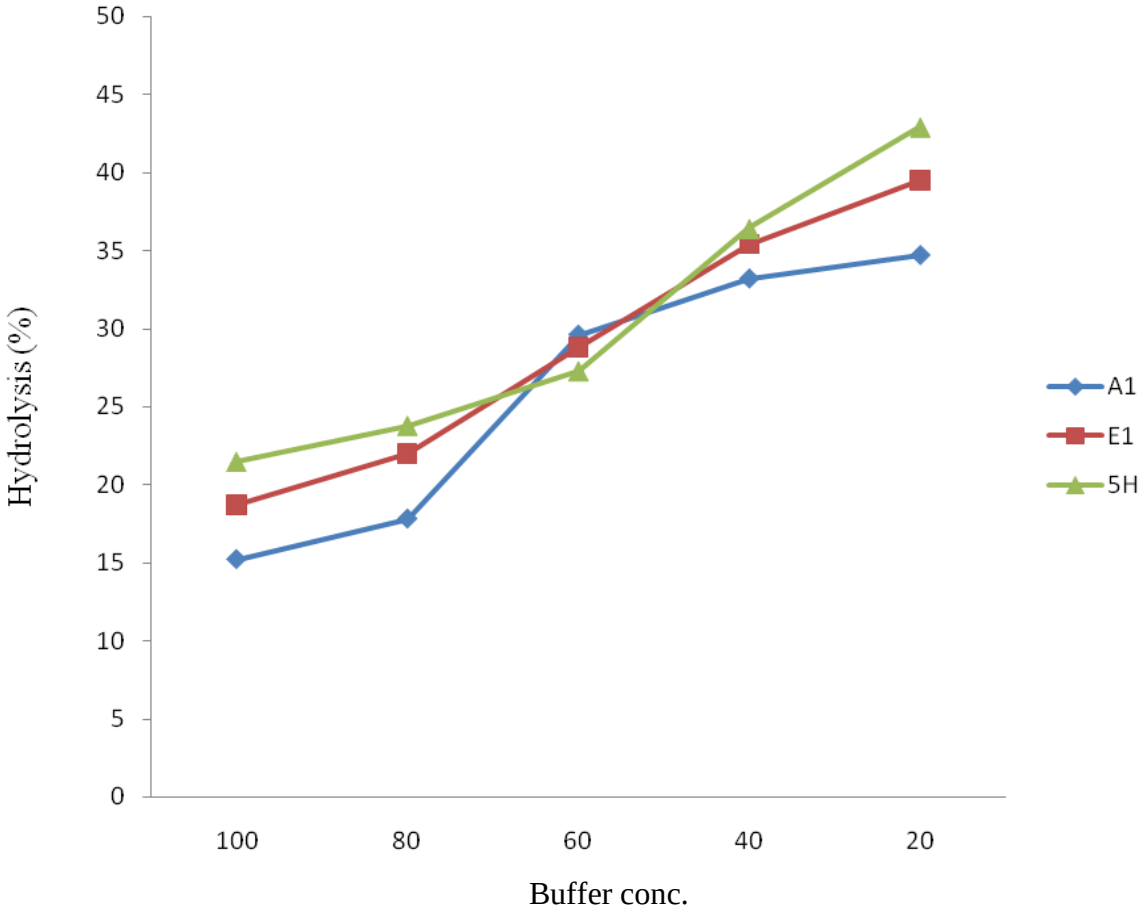


Figure 4.55 Effect of concentration of enzyme on the hydrolysis of alkal-treated sugarcane bagasse by cellulase from *Roseomonas* sp. A1, *Anoxybacillus rupiensis* E1 and corncob by cellulase from *Anoxybacillus ruiensis* 5H. Data are presneted as a mean of 2 replicates. Standard deviations were less than 10% in all cases.

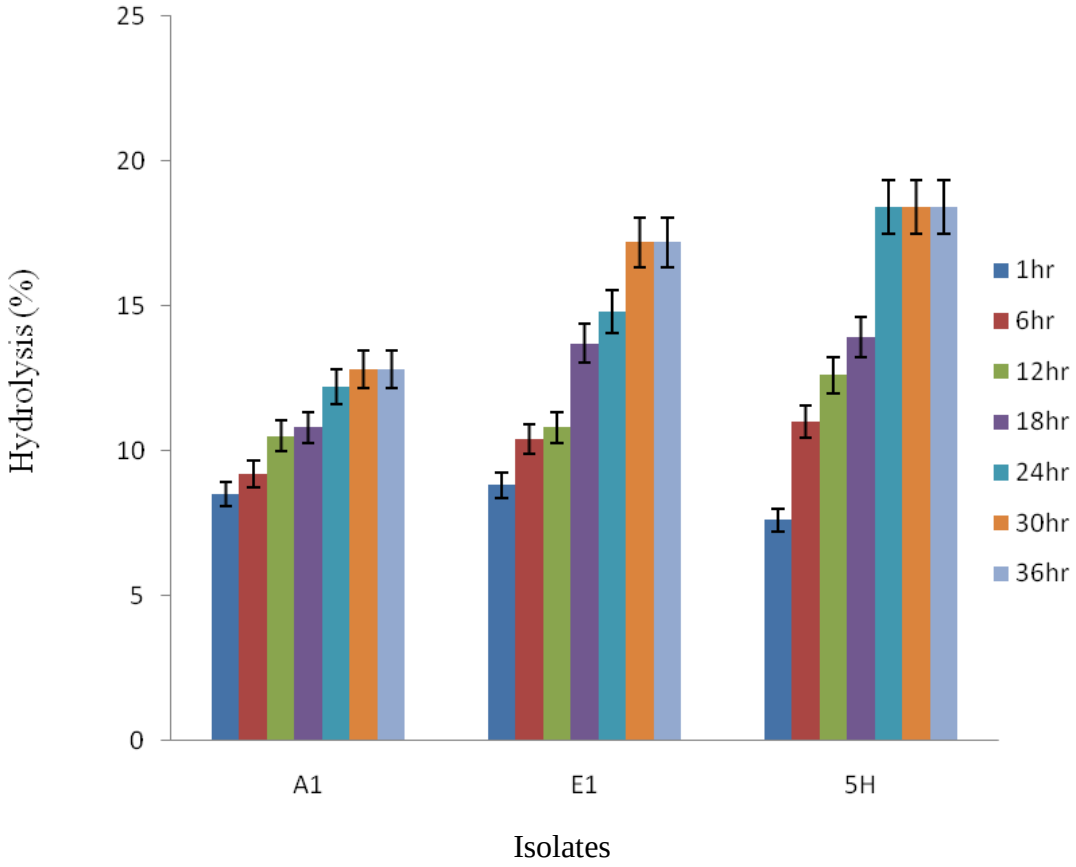


Figure 4. 56: Effect of time of hydrolysis (hrs) of alkali-treated sugarcane bagasse by cellulase from *Roseomonas* sp. A1, *Anoxybacillus rupiensis* E1 and corncob by cellulase from *Anoxybacillus rupiensis* 5H. Data are presented as a mean of 2 replicates.Standard deviations were less than 10% in all cases.

CHARPTER FIVE

5.0 DISCUSSION

The ability of some microorganisms to metabolize lignin, hemicelluloses and cellulose makes them potentially important to take advantage of plant residues. The polysaccharide hydrolysis is one of the most important enzymatic processes on earth and the cellulose synthesis and hydrolysis is a great part of carbon cycle (Solomon *et al*., 1990; Ahmed *et al.,* 2001; Roberto *et al.,* 2005). Thermophilic bacteria are a group of bacteria that prefer high temperature (50^0C-80^0C) for their growth (Claire and Gregory, 2001). These groups of bacteria are found in different environments including soil, hot spring and rotting materials (Natalei, 2002; Po-Jui *et al*., 2004; Abdelneser *et al*., 2007). Thermophilic cellulase-producing bacteria, because of their ability to grow at relatively higher temperatures make them important due to the probable stability of their enzymes at higher temperatures.

Different genera of cellulase-producing bacteria were isolated from sawdust samples examined. They include *Pseudomonas*, *Klebsiella*, *Actinomyces*, *Campylobacter*, *Aerococcus*, *Citrobacter*, *Proteus, Thermoactinomyces, Roseomonas and Anoxybacillus*. Most of these bacteria have been isolated from wood material and plants-eating organisms (Wenzel *et al*., 2002; Roberto *et al*., 2005; Shabnam *et al*., 2011). The observed presence of *Pseudomonas* species in this work may be as a result of the ability of the organism to degrade cellulolytic materials. *Pseudomonads* have been identified to be of importance in bioremediation as a result of their high capacity for biodegradation (Kiyohara *et al*., 1992). *Pseudomonas* species have been used by Kiyohara *et al*. (1992), Premeletha and Rajakumar (1994), Farrell and Quilty (2002) for the biodegradation of a variety of chlorophenolic compounds.

Occurrence of *Klebsiella*, *Anoxybacillus* and *Campylobacter* in the samples might not be unconnected with the fact that microorganisms are ubiquitous in nature and therefore strive on a suitable medium. *Klebsiella* sp. and *Pseudomonas* sp. were in the majority of the isolates from wood-eating insects (Italo *et al*., 2005). Similarly, *Actinomycetes* and *Pseudomonas* were isolated from termites (*Zootermopsis anqusticollis*) by Wenzel *et al*. (2002). Ability of sawdust to support high population of

Pseudomonas has also been reported (Arora *et al*., 2008). Gbolagade (2006) also reported the occurrence of *Citrobacter freundii* and *Pseudomonas aeruginosa* from the fermenting agricultural waste. Similarly, Uguru *et al*. (1997) reported the occurrence of *Thermoactinomyces* sp. in the soil and waste materials of plant origin.

The importance of microorganisms in the process of composting may be attributed to the presence of these bacteria in sawdust. Beffa *et al*. (1996) reported that microbial activity is a prerequisite for a satisfactory composting process. The authors defined composting as intense microbial activity leading to decomposition of most biodegradable materials. The involvement of *Anoxybacillus* in composting process on municipal waste had also been reported (*Shabnam et al*., 2011)

Although, Gram negative bacteria are the major bacteria most studied in relation to biodegradation of organic maters, some Gram positive bacteria that are known to also degrade complex organic matters also exist in actinomycetes group. The reason for this is their nutritional versatility. However, work on Gram positive bacteria in relation to biodegadation is scanty because of the slow rate at which they grow (Zeyaullah *et al*., 2009).

The depth of an environment at which microorganisms are isolated depends on the behaviour of that organisms to factors like temperature, moisture content and oxygen concentration at that depth (www.ehow.com/list7665226 bacteria-soil-different-depth.html#ixzzzuKyqzW9G) Retrived February 21, 2014. *Pseudomonas*, *Roseomonas* and *Cirtobacter* spp. are aerobic bacteria and this may account for why they are found at top layer of sawdust. The isolation of three (3) out of four (4) *Actinomyces nueslundii* at the depth of 60cm may be because of facultative anaerobic nature of the organism (Tortora *et al*., 2002). Occurrence of *Anoxybacillus rupiensis* at 15cm and 50cm depths may be because the organism is both aerobic and facultative anaerobic in nature (Kadriye *et al*., 2011).

Glucose elicited good growth for almost all the isolates. This might not be unconnected with the fact that apart from enhancing the growth of the isolate, glucose has also been found to be responsible for the production of polyhydroxybutyrate which will normally increase the mass of bacteria beyond 30hrs (Marangoni *et al*., 2001). Glucose is one the best carbon source for the growth of the isolates due to its readiness to enter many major metabolic pathways in the cells (Narumol and Jirapa, 2007). The usefulness of glucose to *Pseudomonas* species has been reported (Whiting *et al*., 1976; John and John, 2002). The fact that glucose-containing media gave the highest growth

of mycelia was also expressed by Nwodo-Chinedu *et al.* (2007). It is well documented that most *Pseudomonas* spp. readily oxidize glucose to gluconate and oxygluconate in synthetic media (Whiting *et al*., 1976; John and John, 2002). This fact may account for the greater utilization of glucose by *Pseudomonas* in this study. *Corynebacterium glutamicum* has also been reported to produce tremendous growth when grown on glucose, fructose, sucrose and maltose while the growth was poor with mannose and acetate as carbon or energy sources (Stephan *et al*., 2001). Glucose, fructose, mannitol, raffinose and sucrose were also said to be the best source of carbon for the growth of actinomycetes. However, glucose may not be good for the production of cellulase enzymes (Nwodo-Chinedu *et al*., 2007). The least growth for isolate *Roseomonas* sp (A1) was recorded with lactose $(0.06\pm0.01)OD_{540}$ at 2.0% and 2.5%, for *Anoxybacillus rupiensis* (E1) glucose $(0.13\pm0.02)OD_{540}$ at 2.5% , 8 (0.36 ± 0.04) OD_{540} at 2.5%, *Anoxybacillus rupiensis* (5H) galactose $(0.23\pm0.01)OD_{540}$ at 0.5% and *Actinomyces nueslundii* (6A1) CMC (0.14 ± 0.03) OD_{540} at 2.5%.

The source of nitrogen incorporated in the media for growth of organisms is as important as that of carbon source and in fact play significant role in propagation of the organisms. When other sources of inorganic nitrogen were compared, ammonium nitrates seemed to be the best of them and even among the nitrates . Ammonium nitrate was reported to have greater effect on the growth and enzymes production stimulation. The results of this work corroborated the findings of Narumol and Jirapa,(2007) as stated above. Okeke and Obi (1993) had earlier reported the ability of NH_4NO_3 in eliciting good cellulase production among other nitrates. Haltrich *et al*. (1993) also reported that among the nitrates used in the growth of *Schizophyllum commune*, NH_4NO_3 was found to be the best of them. In all organic nitrogen sources investigated, yeast extract was most utilized by most isolates. Similar observation has earlier been reported by Okeke and Obi (1993) and Kung and Liza (1998). Narumol and Jirapa (2007) observed that the preference of yeast extract to other organic nitrogen sources might not be unconnected with availability of ready to use amino acids present in yeast extract as compared to others. According to these authors the ratio of amino nitrogen (AN) and total nitrogen in the yeast extract were reported to be 40-50% whereas others have less. Tryptone was also good for propagation of some of the isolates to a greater extent. Different concentrations of nitrogen sources produce different amount of growth and enzyme. The production of maximum value of

cellulase by media supplemented with one percent tryptone or yeast extract is in conformity with that observed by Okeke and Obi (1993).

Effect of pH on the growth and enzymes production by different microorganisms have been reported by various workers. With exception of *Actinomyces nueslundii* (3B), all others can be grown in slightly acidic, neutral and alkaline medium, even though each of the isolates has its optimum pH of growth. The results of this study indicates *Actinomyces* spp have wider range of growth. This is in conformity with the finding of Vybornykh and Egorov (1997) where proteolytic enzyme from *Actinomyces thermovulgaris* was found to be stable at acidic, neutral and alkaline environments and only inactivated in acidic environment. The enzyme was also observed to be thermostable. The high activity of cellulase from the majority of the isolates at pH 7 might account for the habitation of lignocellulolytic waste by these organisms from where they were isolated. Armenante *et al*. (1993) observed that the highest rate of biodegradation of waste was at pH of 7.The poor production of bacterial enzymes at low and high pH values might not be unconnected with the fact that acidic and alkaline pHs have inhibitory effect on the growth and enzyme production by bacteria (Archana and Satyanarayana, 2003; Immanuel *et al*., 2006; Abdulnasser *et al*., 2007) The high growth of the *Thermoactinomyces* sp. at pH of 6 in this study is in conformity with the finding of Uguru *et al*., (1997) in which the optimum growth pH for *Thermoactinomyces* isolated from acid soil was 6

The environment from which microorganisms are isolated determines to certain extent the behaviours of those organisms in the laboratory culture media. The isolates in this study were able to grow at high temperature especially those Actinomyces isolated from the depth of 60cm. Therefore, the ability of *Actinomyces* spp. to grow at temperature of 55^0C as compared to others might not be unconnected with the depth from which these organisms were isolated. Four thermophilic *Campylobacter jejuni* were isolated from the samples. Occurrence of thermophilic *Campylobacter* species have been described earlier. John *et al*. (1999) reported the shedding of thermophilic spp. of the organisms by sheep at pasture. This view was also reported by Umen and Okpokwasili (2008) from farm sheep. According to Tsuji *et al*.(1982), *Pseudomonas aeruginosa* was reported to have the widest range of growth temperature among the *Pseudomonas* species. Similarly, the growth of the isolates in this study at the temperature between 50^0C-60^0C is in agreement with finding of Golueke (1982) that composting of a well-managed operation are in the range of 50^0C-65^0C Apart from

this all others did not grow beyond temperature of 50^0C. The ability of microorganisms to grow at a particular temperature is a function of heat tolerance of enzymes contained in such organisms.

Microbial enzyme production is a function of a lot of factors including nutrition and cultural parameters like pH, temperature, carbon and nitrogen sources as well as duration of incubation of the concerned microorganisms. These parameters were studied in order to optimize the production of cellulase by selected bacterial isolates.

The use of CMC for cellulase synthesis is in conformity with the results of Narasimha *et al*. (2006) and Abdul-Taleb *et al*. (2009) who reported high level of cellulase production by using cellulose as carbon source in basal medium for enzyme production by *Aspergillus niger* and *Bacillus* spp. CMC was also used to produce cellulase in *Bacillus* sp. (Khyami-Horani, (1996). More cellulase was produced by cellulose as substrate for cultivation of *Caldibacillus cellulovorans* than when grown on cellobiose or xylan. In studying substrate specificity for the three isolates that were finally selected (*Roseomonas* sp. A1, *Anoxybacillus rupiensis* E1 and *Anoxybacillus rupiensis* 5H) CMC was observed to be the best substrate. This result agreed with that of Abdulnasser *et al*. (2007) that reported highest activity with CMC for *Anoxybacillus flavithermus.*

The nitrogen used in a growth medium is one the major factors affecting enzyme production by microorganisms (Moussa and Tharwat, 2007). In this study, each of the nitrogen source used had different effect on the individual isolate. While the least cellulase induction was recorded for isolates *Roseomonas* sp. (A1), *Pseudomonas aeruginosa* (D, 3, 8, S14), *Anoxybacillus rupiensis* (E1) and *Actinomyces nueslundii* (6B) by $NaNO_3$, it was NH_4Cl for *Pseudomonas aeruginosa* (I, J, L), *Thermoactinomyces vulgaris* (P3), *Actinomyces nueslundii* (3B) and *Anoxybacillus rupiensis* (5H). These observations are in agreement with the work of Spiridonov and Wilson, (1998); Rajoka, (2004) who reported ammonium compounds being the most favorable nitrogen compounds for protein and enzyme synthesis among the inorganic source of nitrogen. The highest production of cellulase by *Pseudomonas aeruginosa* (S14) in the presence of yeast extract is in agreement with the finding of Abdul-Taleb *et al*., (2009). The best nitrogen source for most of the isolates is tryptone since sixteen of the isolates preferred it more than the other nitrogen sources. This observation is at variance with the results of Abdul-Taleb *et al*. (2009) However, in this study, the best cellulase production was achieved in the

medium with combination of 1% (w/v) tryptone and 0.1% (w/v) yeast extract for all the isolates. This result shows that organic nitrogen stimulated more cellulase production when compared to inorganic nitrogen sources. This is supported by the finding of Ray *et al*. (2007).

Incubation temperature plays a very significant role in the metabolic activity of microorganisms. This was reflected in this work as all the isolates produced cellulase maximally at 50^0C except isolate 8 whose optimum temperature for cellulase production was 55^0C. It was observed that any increase or decrease in temperature resulted in gradual, in some cases, decrease in enzyme synthesis. This might be due to inhibition of the isolate growth at this temperature leading to decrease in enzyme synthesis. It has been reported that a higher temperature will alter cell membrane composition due to thermal denaturation of enzyme of metabolic pathway (Ikral-ul-Haq and Khan, 2006). Similarly at lower temperature, transport of substances across the cell is suppress and that lower yield of products are attained (Rajoka, 2004). The influence of temperature on the activity of cellulase is so important because of its application in industries. The activities of the cellulase were also determined. The crude enzyme was active between 45^0C to 70^0C with their optimum at 50^0C to 60^0C. The values recorded in this study are in agreement with that reported for thermophilic cellulase (Vasudeo *et al*., 2011). However, this result is different from the reported case with *Bacillus* sp. CH43 and *Bacillus* sp. HR68 isolated from hot spring with optimum temperature of 70^0C and 60^0C respectively (Mawadza *et al*., 2000). The purified cellulase was active at higher temperature of between 60^0C and 75^0C with maximum activity of enzyme from *Roseomonas* sp. A1 at 70^0C and *Anoxybacillus rupiensis* E1 and 5H at 60^0C. The optimum temperature for cellulase activity produced by thermophilic *Bacillus* sp. CH43 *Bacillus* HR68 isolated from hot spring were 70^0C and 65^0C respectively (Mawadza *et al*., 2000). Roberto *et al*. (2005) had also reported the production of cellulase with optimum activity at 70^0C by *Thermoascus aurantiacus.* These temperatures have been reported for enzymes produced by thermophilic microorganisms. Activity temperature ranges for enzymes from thermophilic microbes were reported to be between 60^0C and 80^0C (Cleraire Gregory, 2001). The 60^0C recorded in this study for cellulase from isolates E1 and 5H is in conformity with the report of Dongyang *et al*.(2011) for thermostable lignocellulase produced by plant decomposing *Aspergillus fumigatus*. Temperature optima here were also higher than 35^0C for cellulases from *Sinorhobium fredii* and *Pseudomonas*

fluorescene (Po-Jui *et al*., 2004; Bakare *et al*., 2005). Similarly the result showed higher thermostability when compared to the activity of cellulase produced by *Streptomyces ruber* (Nermeen *et al*., 2012).

Medium pH has been found to affect many enzyme systems and to have a direct influence on the uptake of mineral nutrient present in the medium of cultivation (Moon and Parulekar, 1999; Ikram-ul-Haq and Khan, 2006). Composition of cell wall and plasma membrane of microorganisms is known to be affected by the medium pH (Elliwood and Tempest,1972). Due to this change in the nature of cell wall and plasma membrane, the growth parameter may vary, especially temperature , and the provided temperature may not remain suitable for the growth of the organisms (Stutzenberger and Jenkins, 1995). This may be the reason for the decrease in the enzyme production at pHs 4.0 and 9.0 for some isolates pH 4.0, 5.0 and 9.0 for others in this study.

The effect of pH on the activity of the enzyme in this study was investigated between pH 4.0 to 9.0. The enzyme has a broad range of pH activity (pH 4-9) with optima pH at 7.0 for all the isolates. The optimum pH in the range of 6-9 for most *Bacillus* spp. cellulases have earlier been reported (Fakumori *et al.*,1985). Similar reports were made by Abdelnasser and Ahmed, (2007). Immanuel *et al*. (2006); and Abdul-Taleb *et al*. (2009) also reported the increase in cellulase activity from pH 4, 5 to 7 and decreased down to 9.0 for *Cellulomonas* sp., *Bacillus* sp. and *Micrococcus* sp.

The results of this study showed a stepwise increase in enzyme activity for isolates *Roseomonas* sp. (A1) and *Pseudomonas aeruginosa* (8) with optimum after 48hr beyond which a prolong decrease was observed. The decrease in enzyme activity after optima might be due to cumulative effect of reducing sugars produced during growth which are known to inhibit cellulases (Ojumu *et al*., 2003).The reduction in enzyme production with time might also be as a result of depletion of micronutrients and macronutrients in the growth medium with passage of time due to their utilization during the logarithm phase of the isolates, which may stress the isolate physiology thereby resulting in the inactivation of secretory machinery of the enzyme (Nochure *et al*., 1993). Abdelnasser *et al*.(2007) also reported the pick production of cellulase enzyme by *Anoxybacillus flavithermus* EHP1 at 36hr but prolong up to 48hr of cultivation. For *Roseomonas* sp. A1 and *Anoxybacillus rupiensis* 5H with fluctuations of enzyme production with passage of time, there is likelihood that these isolates utilized the sugars in the medium before more enzyme is synthesized.

Cellulose and hemicelluloses are found in association with lignin in the lignocellulosic materials. This makes it very difficult for cellulolytic enzymes to break down cellulose due to difficulty encountered in breaking down the lignin (Singh *et al*., 1991). Therfore, in this study, pretreatment was done to break down the lignin component and open up cellulose for enzyme attack. Alkaline pretreatment breaks down the bonds linking lignin, cellulose and hemicelluloses, thereby making the cellulose more accessible to the organisms thus resulting in enhanced susceptibility and substrate utilization and therefore higher enzyme production by the organisms (Singh *et al*., 1991; Onilude, 1996; Maheswaris and Chandra, 2000)

The purification of cellulase showed that cellulase of *Roseomonas* sp. A1, *Anoxybacillus rubiensis* E1, and *Anoxybacillus rubiensis* 5H were purified with recovery yield of 10.7% , 13.1% and 7.6% respectively and specific activities of 20.0U/mg, 26.7U/mg and 22.0 U/mg protein respectively while the cellulase purification fold for three isolates *Roseomonas* sp. (A1), *Anoxybacillus rupiensis* (E1) and *Anoxybacillus rupiensis* (5H) was 4.67, 6.3 and 4.5 respectively. These specific activities reported here are higher than the observed value 3.822U/mg of *Sinorhizobium fredii* CCRC15769 (Po-Tui *et al*., 2004) and 6.8U/mg from cellulase by *Pseudomonas fluorescens* (Bakare *et al*., 2005) but lower than the cellulase from *Streptomyces ruber* of 846.752U/mg (Nermeen *et al*.,2010).

The difference in the production of cellulolytic enzyme on variety of lignocellulosic materials by different organisms might be due to various factors including variable cellulose content in the lignocellulose obtained from different plant sources, heterogeneity of structure and cellulolytic abilities of the organisms at different degree as well as culture conditions (Jecu, 2000; Ogel *et al*.,2001; Kang *et al*., 2004; Roberto *et al*., 2005;Narashimha *et al*.,2006; Ja`afaru and Fagade, 2007; Chinedu *et al*., 2011). While Roberto *et al*.(2005) reported the highest production of cellulase and xylanase by *Thermoascus auranhticus* on corncob. Ojumu *et al*.(2003) observed that sawdust gave the best cellulase activity with using *Aspergillus niger* while corncob was the least cellulase producer. In current study production of enzyme was highest in most cases, within the range of 1.0% to 2.0% of lignocellulosic materials. Higher concentration of lignocelluloses resulted in decrease in enzyme production. This might be due to the fact that at higher concentrations, the medium became more viscous resulting in lower oxygen pressure and poor condition of growth. Catabolic repression can be due to high concentration of catabolite and presence of

rapidly metabolised carbon source (Singh *et al*., 1991; Palma *et al*., 1996; Ninawe and Kuhad, 2005; Chinedu *et al*., 2007; Bakri *et al*., 2008) In this study sugar cane bagasse was the best cellulase producer for both *Roseomonas* sp.A1 and *Anoxybacillus rubiensis* E1 while corncob was observed to the best enzyme producer for *Anoxybacillus rubiensis* 5H, this might be due to degree of polymerisation, crytalinity, structural composition, particle size and available surface area of substrates and mechanisms and interaction of isolate enzyme (Converse, 1993; Mansfied *et al*., 1999).

Effect of metal ions on the cellulase activity was investigated. Na^{+}, Ca^{2+} and Fe^{2+} were stimulatory at varying concentrations and Mn^{2+}, Cu^{2+} Mg^{2+} Hg^{2+} Zn^{2+} Urea, SDS, EDTA and Tween-80 were inhibitory to enzyme activity from A1; for cellulase from E1, Na^{+}, Ca^{2+}, Mg^{2+} Fe^{2+} and tween80 were all stimulatory others were inhibitory whereas Na^{+}, Ca^{2+}, Mg^{2+} Fe^{2+} Cu^{2+} SDS and Tween-80 were stimulatory to cellulase from 5H. The stimulatory effect of Na^{+} Ca^{2+} and Tween80 has been reported (Aygan *et al*.,2010; Shankar and Isaiarasu, 2011). Similarly, the inhibitory effect of SDS, Urea and Zn^{2+} was reported (Aygan *et al*.,2010; Ja`afaru and Fagade, 2010). The strong inhibition of cellulase by Hg^{2+} is well documented. Hg^{2+} is known to react with protein sulphydryl group so also is histidine and tryptophan residues (Volkin and Klibanov, 1989, do Nascimento and Martins, 2004). The stimulatory effect of Ca^{2+} had been reported (Cesar and Mars, 1996; Ghanem *et al*., 2000). Similarly the enhancement of the activity of enzyme by Fe^{2+} and Cu^{2+} was reported (Cesar and Mars, 1996; Gupta *et al*., 2000). Cellulase activity was also observed to be inhibited by Cu^{2+}, SDS and Zn^{2+} (Ishihara *et al*., 1997; Damaso *et al*.,2002; Kimura *et al*., 2000;Aygan *et al*., 2010; Ja`afaru and Fagade,2010). The explanation for the antagonistic behavior has been associated with the alteration of enzyme conformation (Lin *et al*., 1999). Enzymes can be modulated by interaction of cations with acid residues involved in their active sites. Such interaction can either be positively modulated to increase the enzyme activity or negatively modulated to reduce the enzyme activity (Breccia *et al*., 1998; Batallon *et al*., 2000; Dutta *et al*., 2007).

Purified cellulase from the three isolates had hydrolytic activity against CMC, microcrystaline cellulose and salicin but have no xylanase and amylase. The combination of these enzymes will allow synergistic breakdown of many lignocellulosic materials. Eriksen and Goksgyr (1977) reported the improvement in hydrolytic activity of cellulases when acted synergistically compared to individual action.

K_m values of *Roseomonas* sp. A1, *Anoxybacillus rupiensis* E1 and *Anoxybacillus rupiensis* 5H cellulase on CMC were found to be 12.5mg/ml, 9.3mg/ml and 5.9 respectively from the Lineweaver-Burk plot. These values are in agreement with those earlier reported but higher than 1.7mg/ml reported by Singh and Kumar, (1998) however less than 37.88mg/ml by endoglucanase form *Aspergillus fumigates* (Dongyang *et al*., 2011).

V_{max} values of 1.5U/mg, 0.65U/mg and 0.82U/mg were obtained for *Roseomonas* sp. A1, *Anoxybacillus rupiensis* E1 and *Anoxybacillus rupiensis* 5H respectively. However , the values are lower than the 437U/mg reported for *Aspergillus fumigatus* (Dongyang *et al*., 2011). High V_{max} values of purified enzyme showed that the enzyme can hydrolyze cellulose faster than those from other organisms. Kinetics parameters of microbial cellulases is known to be variable. The values are dependent on the detailed structure of the substrates (Basaran *et al*., 2000).

Cellulose is the most abundant of the lignocelluloses in plants and can be hydrolyzed by acid, alkali or enzymatic action (Gawande and Kamat, 1999). Enzymatic action is a more specific process. Some features of natural cellulosic materials are known to inhibit their degradation/bioconversion (Solomon *et al*., 1990, 1999). These include the degree of crystallinity and lignifications and the capillary structure of cellulose. The crystallinity and lignifications limit the accessibility and susceptibility of cellulose to cellulolytic enzyme and other hydrolytic agents (Fan *et al*., 1987). Out of all the lignocellulosic materials used in the study, Corncob was most susceptible to enzymatic hydrolysis from *Anoxybacillus rupiensis* 5H while sugarcane was the most readily hydrolyzed by enzyme from *Roseomonas* sp. A1 and *Anoxybacillus rupiensis* E1. Treatment of alkali-treated corncob with cellulase from 5H produced 38.9% while alkali-treated sugarcane bagasse treated with cellulase from A1 and E1 produced 21.15 and 29.2% hydrolysis respectively. However, alkali-treated sawdust from all the plants used in this study was found to be more resistant to enzymatic hydrolysis. The high rate of hydrolysis of corncob and sugarcane bagasse might be due to high cellulose and low lignin content when compared to that of sawdust. Lignin is recalcitrant to both chemical and enzymatic attack and therefore may limit access of lignocelluloses to hydrolytic enzymes. Other important factors which affect relative susceptibility of lignocellulosic materials to cellulase action are crystallinity, available surface mixture with impurity and affinity of substrate for the enzyme (Lakshmikant and Mathur, 1990). The differences in resistance of these

agricultural residues to enzymatic hydrolysis is attributed to lignin content (Singh *et al*., 1988).

Alkali-treated procedures have been reported to reduce the recalcitrant of lignocellulosic materials to enzymatic hydrolysis (Singh *et al*., 1988; Onilude 1996; Abu, 2004; Ja'afaru and Fagade, 2007). Pretreatment of cellulose had earlier been reported to open up the structure and remove the secondary interaction between glucose chains (Fan *et al*., 1987; Tang *et al*., 1996). Alkali-pretreatment of lignocelluloses in this work led to higher rate of hydrolysis. Bateman and Basham (1976) and Onilude (1996) had observed that alkali-pretreatment of natural plant cell wall residue is the last step leading to the exposure of the lignocellulosic components, thereby aiding degradation of such component.

It was observed in this study that by optimizing the conditions of hydrolysis of corncob and sugarcane bagasse by *Roseomonas* sp. A1, *Anoxybacillus rubiensis* E1 and *Anoxybacillus rubiensis* 5H cellulases, the alkali-treated sugarcane bagasse and corncob were hydrolyzed best at pH 7.0, temperatures of 60^{o}C for cellulase from A1 and E1 while it was 65^{o}C for cellulase from 5H, substrate concentration of 3% for A1 and 4% for both E1 and 5H. The decrease in the rate of hydrolysis as reaction proceeds might be due to the fact that the enzyme was becoming inactivated such that the readily susceptible regions of the substrate are hydrolyzed leaving the more resistant sites. It could also result from accumulation of reducing sugars formed as end products. However, there was increment in the degree of hydrolysis as the water concentration reduced from the enzyme. Although a lot of work have been done on cellulase from bacteria, majority of these studies were on mesophilic ones. Little was done on thermostable cellulase from thermophilic bacteria. Again there is scanty information on the ability of *Anoxybacillus* sp to partake in the production of thermostable extracellulase enzymes and application of enzyme so produced. This study has therefore brought to fore the potential of cellulase enzyme from these organisms in hydrolysis of lignocelluloses.

CHAPTER SIX

6.0 CONCLUSION AND SUMMARY

Substantial part of municipal wastes are made of lignocellulosic materials which consititute both health hazard and environmental problem. The use of microoganisms and their enzymes in recycling cellulose-containing wastes could be of importance in reducing the problem of environmental pollution. The best thermostable cellulase producers among the bacterial isolates in this work are *Roseomonas* sp. A1 and *Anoxybacillus rupiensis* E1 and 5H.

From this work, *Anoxybacillus rupiensis* and *Roseomonas* sp. were found to produce substantial amount of cellulase. This study has been able to add to the list of bacteria that can produce lignocellulosic materials-degrading enzymes. The ability of these isolates to use waste materials like corncob, sugarcane bagasse and sawdust is an asset since this will lead to reduction in cellulase production cost, hence the application of both the isolates and their enzymes will be easy especially in an agrarian country like ours.

Similarly whole cells of the organisms can be applied in environmental degradation of municipal wastes because the wastes are lignocellulosic in nature. It is also an avenue for combating environmental pollution problem.

The thermostability of the organisms and their enzymes is an added advantage over other organisms that might have been used in this as they can withstand the composting temperature of the wastes.

Further studies need to be done in understanding the safe nature of the enzymes from these organisms as regard their application in food and feed processing industries as well as grant them the GRAS status.

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APPENDICES

APPENDIX 1: Sterilization of Laboratory Materials

All the glasswares used in the experiments such as Petri dishes, conical flasks, pipettes, beakers, test-tubes, McCartney bottles and measuring cylinders were washed with detergent and thoroughly rinsed with tap water and then allowed to air dry on the laboratory bench. Petri dishes, test-tubes and pipettes were placed inside canisters and sterilized in the hot air oven (OHAS DHG-9101) at 180^0C for 1hr. The conical flasks and beakers were also sterilized in the oven and at the same temperature while the filter papers were wrapped with aluminum foil and sterilized in the autoclave at 121^0C for 15minutes. Inoculating wire loops, scapels and forceps were sterilized by dipping in 95% ethanol and flaming till red hot and allowed to cool before used. Inoculating hood was sterilized with UV light while working benches were sterilized by swabbing them with cotton wool soaked in 95% ethanol.

APPENDIX 2: Culture media

a) Berg`s medium

The composition is as follows:

$NaNO_3$	2g
$MgSO_4$	0.5g
K_2HPO_4	0.05g
$FeSO_4$	0.01g
$CaCl_2$	0.02g
$MnSO_4$	0.02g
Water	1000L

b Natalie`s medium

The composition is follows:

Yeast extract	1g
Tryptone	10g
Cellulose	10g
K_2HPO_4	0.5g
Water	1000L

APPENDIX 3: Buffer preparation

a) Acetate Buffer

Stock solutions

A: 0.2M solution of acetic acid (11.55ml in 1000ml)

B: 0.2M solution of sodium acetate (16.4g of $C_2H_3O_2Na$ or 27.2g of $C_2H_3O_2Na.3H_2O$ in 1000ml)

xml of A + yml of B, diluted to a total of 100ml.

b) Phosphate Buffer

Stock Solutions

A: 0.2M solution of monobasic sodium phosphate (31.2g $NaH_2PO_4.2H_2O$ in 1000ml).

B: 0.2M solution of dibasic sodium phosphate (28.39g of Na_2HPO_4 or 71.7g of $Na_2HPO_4.12H_2O$ in 1000ml).

xml of A+ yml of B, diluted to a total of 200ml.

APPENDIX 4: Dinitrosalicylic Acid Reagent preparation

Dinitrosalicylic acid reagent was prepared by combining 1.0 g DNSA with 20 ml 2 N NaOH and 20 g potassium tartrate in 100 ml distilled water. The absorbance of standard aqueous solutions of D-glucose of various concentrations (0-10mg/ml) was determined and used to construct a graph of % absorbance as related to mg of glucose per ml.