Growth Characteristics, Enzyme Activity and Production of Secondary Metabolites from Selected Novel *Streptomyces* Isolates from Kenyan Soils

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other University.

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DEDICATION

This work is dedicated to my beloved parents Mr. and Mrs. Karanja, my wife Anne Kelly Kambura, son Shawn Karanja and daughter Melissa Wangithi. Thanks to all of you for the support you have and are still giving me. Without your encouragement and support this journey would be long and tough.

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LIST OF ABBREVIATIONS AND ACRONYMS

ALiP	T7A Lignin Peroxidase
DB	Differential Broth
DCM	Dichloromethane
DNA	Deoxyribonucleic Acid
EI	Enzymatic Index
G+C	Guanine and Cytosine
GC – MS	Gas Chromatography – Mass Spectrophotometer
IPG	Isopropylidene Glycerol
ISP	International Streptomyces Project
MR	Methyl red
NA	Nutrient Agar
NCTC	National Culture Type Collection
OD	Optical Density
PAI	Pathogenicity Island
SAM	*S*-Adenosyl Methionine
SIM	Sulfur-Indole Mortility
SM	Sierra Media
T20	Tween 20
T80	Tween 80
VP	Voges Proskauer

ABSTRACT

There is growing interest in evaluating the potential usefulness of enzymes in various industrial processes such as waste management, detergent manufacturing and food processing. This has led to the need in having knowledge of the spatial and temporal variation of the organisms producing the different enzymes and factors affecting enzyme activity. Besides this, multiple drug resistance has recently been on increase especially on antibiotics and chemotherapeutic drugs leading to the need in discovering more drugs to counteract these effects. The high toxicity usually associated with cancer chemotherapy drugs and their undesirable side effects increase the demand for novel antitumor drugs active against untreatable tumors, with fewer side effects and/or with greater therapeutic efficiency. Due to these challenges, scientists have expounded search for solutions to include even the prokaryotes and especially the Actinobacteria which are widely distributed in a variety of natural and man-made environments.

This study was geared towards screening for production of protease, esterase, amylase and lipase enzymes as well as secondary metabolites from selected novel Streptomyces species from Kenyan soils that could be of benefit to mankind and the environment. Four Streptomyces isolates from Chyulu National Park (Chy 4-10, Chy 15-10, Chy 15-5 and Chy 2-3) and one from Ruma National Park (Ruj 7-1) were studied. Morphological, physiochemical and biochemical characterization of the isolates was carried out. All the isolates produced amylase, lipase, protease and

esterase enzymes apart from isolate Chy 4-10 that did not produce esterase enzyme as indicated by the enzymatic index. The isolates also showed a range of antagonistic activity against *Staphylococcus aureus* (NCTC 10788) and *Escherichia coli* (NCTC 10418). The isolates grew well at pH 6, 7, 9 and temperatures of 27.5 ^{o}C, 30 ^{o}C, and 32.5 ^{o}C. They preferentially utilized glucose and xylose and also required sodium chloride (0 g/l – 17.5 g/l) for growth.

Antimicrobial products were also extracted and analyzed using Gas Chromatography - Mass Spectrophotometer (GC-MS). 0.54 g/l, 0.62 g/l, 0.41 g/l, 0.3 g/l and 0.14 g/l were yields of the crude secondary metabolites extracted from the isolates. The crude secondary metabolites had different levels of activity against *Staphylococcus aureus* (NCTC 10788) and *Escherichia coli* (NCTC 10418) test organism. Further characterization of the crude secondary metabolites was carried out to detect the chemical compounds present. Chemical compounds ranging from amides, amines, acids, pyrrolizidines, butenolides, alcohols and hydrocarbons were detected. These results confirmed that the isolates were capable of producing extracellular enzymes and secondary metabolites that would have potential for industrial applications.

1.0 CHAPTER ONE: INTRODUCTION

1.1 Background information

The phylum Actinobacteria is made up of gram-positive organisms with a high mole % G+C composition (> 55 % G+C). This group is comprised of 39 families and 130 genera making it one of the largest phyla within bacteria. They encompass a wide range of morphology from coccoid (e.g. *Micrococcus*) or rod-coccoid (e.g. *Arthrobacter*), fragmenting hyphal forms (e.g. *Nocardia*) to those with permanent and highly differentiated branched mycelium (e.g. *Streptomyces*) (Stackebrandt *et al.,* 1981).

Many of the Actinobacteria are spore forming which range from motile zoospores to specialized propagules (Stackebrandt and Schumann**,** 2000). They are also physiologically very diverse as evidenced by their production of numerous extracellular enzymes and by the thousands of metabolic products they synthesize and excrete. Actinobacteria especially members of *Streptomycetaceae* are the major antibiotic producers in the pharmaceutical industry. However, a few Actinobacteria are important human, animal and plant pathogens. For example, *Mycobacterium tuberculosis* infection result in tuberculosis; *Corynebacterium diphtheriae* causes diphtheria; *Propionibacteirum acnes* is the causative agent of acne (Ryan and Ray, 2004).

Although Actinobacteria form a distinct cluster in the 16S rRNA trees, no other reliable biochemical or molecular characteristics that are unique to this group are presently known **(**Ludwig and Klenk, 2001). However, recent analyses of genomic sequences have led to identification of many conserved indels and unique proteins that are shared by either all

Actinobacteria, or different subgroups of them, and provide important means for elucidating their taxonomy, phylogeny and unique biochemical and physiological characteristics **(**Gao and Gupta, 2005; Gao *et al.*, 2006).

Actinobacterial species are widely distributed in both terrestrial and aquatic ecosystems and they play an important role in decomposition and recycling of biomaterials (Watve *et al*., 2001, Singh and Agrawal, 2003). These bacteria are primarily saprophytic and contribute significantly to the turnover of complex biopolymers, such as lignocellulose, hemicellulose, pectin, keratin, and chitin (Williams *et al*., 1984).

Ecologically, Actinobacteria particularly, the genus *Actinomyces* consist of a heterogeneous group of Gram-positive, mainly facultative anaerobic or microaerophilic rods with various degrees of branching. They are frequently found as members of the normal microflora, especially in the mouth but they are also found to be etiologic agents in infections, such as in classical Actinomyces, human bite wounds and abscesses at different body sites, eye infections, oral, genital and urinary tract infections (Sarkonen *et al.,* 2001).

Streptomyces spp. are generally soil-dwelling organisms that exist as semi-dormant spores (Mayfield *et al.,* 1972). They are the most important group of the Actinobacteria with high G+C content of the DNA. Morphological characteristic of *Streptomyces* include growth by vegetative hyphae measuring between 0.5 – 2.0 µm in diameter and production of an extensive mycelia that is fragmented. Members of the group produce aerial hyphae bearing chains of conidiospores on their tips when growing on agar resulting to dull, powdery or

velvety appearance of the colonies that are difficult to pick from the surface of the agar plates (Kieser *et al.,* 2000).

Further studies have demonstrated that Actinobacteria also inhabit the rhizosphere of many plant species, including cereal crops such as wheat (Miller *et al.,* 1989, Crawford *et al.,* 1993, El-Tarabily *et al.,* 1997 ; Siciliano *et al.,* 1998). Sardi *et al.,* (1992) reported the presence of Actinobacteria in root samples of a maize crop (*Zea mays* L.) variety called Piranão widely cultivated in Brazil, with the majority of the isolates belonging to the genus *Streptomyces*.

de Araujo *et al*. (2000) found that Actinobacteria could be isolated from the roots and leaves of maize (*Zea mays* L.), with the most commonly isolated genus being *Microbispora*, although *Streptomyces* and *Streptosporangium spp*. were also represented.

Okazaki *et al*. (1995) were also able to isolate *Microbispora* spp. at a much higher frequency from wheat plant (*Triticum aestivum* L.) leaves than from the soil. Some Actinobacteria are also known to form more intimate associations with plants and colonize their internal tissues.

Within the order *Actinomycetales* there are examples of both endophytic and plant-pathogenic species. The best-characterized examples of the plant-pathogenic Actinobacteria are the potato scab causing *Streptomyces scabies*, *Streptomyces acidiscabies*, and *Streptomyces turgidiscabies.* Pathogenicity has been associated with the presence of a conserved and transmissible pathogenicity island (PAI) in their genomes

(Bukhalid and Loria, 1997 and Healy *et al.*, 1999), which encodes for phytotoxin, thaxtomin, and also contains plant virulence factor genes such as *nec1* (Loria *et al.*, 1995, Bukhalid *et al.*, 1998 ; Healy *et al.*, 2000).

Besides acting as organic matter decomposers, Actinobacteria have great potential as agents for control of plant pathogens (Thirup *et al.*, 2001; Hoster *et al.*, 2005) and/or for plant growth promotion (Nassar *et al.*, 2003). This is due to their capacity to produce antibiotics, siderophores, enzymes that have antimicrobial activity, substances that promote plant growth, solubilization of phosphates and competition with plant pathogens for substratum and nutrients (Crawford *et al.*, 1993; Cattelan & Hartel, 2000). Among the studies conducted *in vitro* aiming the selection of agents for biological control and plant growth, characteristics such as antagonistic activity against pathogens, capacity of colonizing the root system, production of siderophores, hydrolytic enzymes, and plant growth regulating substances are of fundamental importance (Cattelan, 1999).

The demand for new antibiotics continues to grow due to the rapid spread of antibiotic-resistant pathogens causing life-threatening infections. Although considerable progress is being made within the fields of chemical synthesis and engineered biosynthesis of antimicrobial compounds, nature still remains the richest and the most versatile source for new antibiotics (Koehn, 2005; Baltz, 2006; Pelaez, 2006).

Bacteria belonging to the family *Actinomycetaceae* are well known for their ability to produce secondary metabolites, many of which are active against pathogenic microorganisms. These bacteria have been isolated from terrestrial sources although the

first report of mycelium-forming Actinomycetes being recovered from marine sediments appeared several decades ago (Weyland, 1969). Many microbiologists believe that free-living bacteria are cosmopolitan due to their easy dispersal (Finlay, 2004). However, chemical and physical factors contribute to selection of species and strains that are best adapted to that particular environment. Due to the broad bacterial species definition one may find members of one species in two very different environments (Staley, 2004; Dolan, 2006). However, comprehensive analysis of the recent studies strongly suggests that free-living microbial taxa exhibit biogeographic patterns (Martiny, 2006).

1.2 Justification of the study

The use of biological systems or agents to catalyze chemical transformations on industrial scale is well established and includes both free enzymes and whole cells (Faber, 1992). The enantio-selectivity of enzymes makes them suited for resolving racemic mixtures by means of kinetic resolution (Chen *et al.,* 1982; Sih and Wu, 1989). Microbial carboxylesterases which include lipases and esterases catalyze the hydrolysis of a broad range of natural and unnatural esters, often showing high enantio-selectivity and region-selectivity hence there is need to discover new sources so as to meet their demand in industrial uses

Knowledge of the spatial and temporal variation of enzymes in such ecosystems, the organisms producing the different enzymes and factors affecting enzyme activity are important to understand and optimize organic matter removal in wastewater treatment plants. For instance up to 60–70 % of the organic matter in the incoming wastewater of

domestic wastewater treatment plants is accounted for by proteins and lipids (Martinez *et al*., 1996). Removal of these compounds through mechanical methods is expensive and therefore enzymes provide a cheap way to remove them from these wastes. Besides this, industrially useful enzymes with novel applications, or which improve upon the activities of ones being currently used, are frequently being sought (Marrs *et al*., 1999).

Around 23,000 bioactive secondary metabolites produced by microorganisms have been reported and over 10,000 of these compounds are produced by Actinomycetes, representing 45 % of all bioactive microbial metabolites discovered (Berdy, 2005). Among Actinomycetes, around 7,600 compounds are produced by *Streptomyces* species (Berdy, 2005). Many of these secondary metabolites are potent antibiotics, which has made Streptomycetes the primary antibiotic-producing organisms exploited by the pharmaceutical industry (Berdy, 2005). Members of this group are producers, in addition, of clinically useful antitumor drugs such as anthracyclines (aclarubicin, daunomycin and doxorubicin), peptides (bleomycin and actinomycin D), aureolic acids (mithramycin), enediynes (neocarzinostatin), antimetabolites (pentostatin), carzinophilin, mitomycins and others (Newman and Cragg, 2007; Olano *et al*., 2009). However, the search for novel drugs is still a priority goal for cancer therapy, due to the rapid development of resistance to multiple chemotherapeutic drugs. In addition, the high toxicity usually associated with cancer chemotherapy drugs and their undesirable side effects increase the demand for novel antitumor drugs active against untreatable tumors, with fewer side effects and/or with greater therapeutic efficiency.

This study was therefore geared towards unveiling and assessing novel enzymes and secondary metabolites that could be of commercial importance hence improving industrial and pharmaceutical applications as well as other sectors where they may be of use.

1.3 Hypotheses

The isolated Actinobacteria strains produce novel enzymes and secondary metabolites.

1.4 Objectives

1.4.1 General objective

Characterization of enzymes and secondary metabolites from Actinobacteria isolates obtained from soil samples collected from Chyulu and Ruma National Parks of Kenya.

1.4.2 Specific objectives

1. To characterize Actinobacteria isolates morphologically and biochemically.
2. To determine the effect of different physiochemical conditions on growth of the various isolates.
3. To assess enzymatic activities of different types of enzymes in the crude extracts from the isolates.
4. To screen for the presence of useful secondary metabolites from the isolates.
5. To carryout characterization of the secondary metabolites from the isolates.

2.0 CHAPTER TWO: LITERATURE REVIEW

2.1 Literature review

2.1.1 Actinobacteria as a source of antibiotics

Despite their importance in soil ecology, Actinomycetes are best known as a source of antibiotics. This became apparent in 1940, following Selman Waxman's seminal discovery of actinomycin (Waksman and Woodruff, 1940), and by the 1980s, Actinomycetes were known to account for almost 70% of the world's naturally occurring antibiotics (Okami and Hotta, 1988) some of these are shown in (**Table 1)**.

The numbers of antimicrobial compounds that have been isolated and reported from these species have increased exponentially in the two decades as indicated from reports (Watve *et al.*, 2001).

Table 1: Examples of antibiotics produced by *Streptomyces sp.* (Kieser *et al.,* 2000)

Antibiotic	**Producer**	**Chemical Class**	**Target**	**Application**
Actinomycin D	*Streptomyces* sp.	Peptide	Transcription	Antitumor
Actinomycin A	*Streptomyces* sp.	Macrolide	Cytochrome system	Telocidal
Avermectin	*S. avermetilis*	Macrolide (PK)	Chloride ion channels	Antiparasitic
Daptomycin	*S. roseosporus*	Lipopeptide	Lipoteichoic acid	Antibacterial
Nystatin	*S. noursei*	Polyene	Membrane (pore former)	Antifungal
Nikkomycin	*S. tendae*	Nucleoside	Chitin biosynthesis	Antifungal; insecticidal
Neomycin	*S. fradiae*	Aminoglycoside	Protein synthesis	Antibacterial
Phleomycin	*S. verticillus*	Glycopeptide	DNA strand breakage	Antitumor
Polyoxins	*S. cacaoi var asoensis*	Nucleoside-peptide	Chitin biosynthesis	Antifungal

2.1.2 Biodegradation of organic materials

Actinobacteria are involved in the decomposition of organic materials in soil. These materials include lignin and other recalcitrant polymers **(Table 2)** as well as agricultural and urban wastes (McCarthy, 1987 and Crawford, 1988). The mycolic acid-containing

Actinomycetes are involved in filamentous foaming in activated sludge systems (Reyes *et al.,* 1997).

In addition to production of antibiotics and degradation of compounds, Actinomycetes are an important source of enzymes and bioactive products (Bull *et al*., 1992). Most produce secondary metabolites that have antibacterial, anti-fungal, anti-tumor or antiprotozoal activities making them a target in large-scale industrial screening programs.

Table 2: Actinobacteria reported from different ecological habitats and their biochemical role.

Actinobacteria isolates	**Source/ habitat**	**Activities**	**References**
Streptomyces venezuelae	Coastal soil	Chitinase	Mukherjee and Sen, 2006
Marine Actinomycetes	Sediments	Chitinolytic	Pisano *et al*., 1986, 1992
Streptomyces spp.	Sediments	Fatty acid	Das *et al*., 2007
Marine Actinomycetes	Sediments	Cellulolytic	Veiga *et al*., 1983
Streptomyces sp.	Sediments	Cellulolytic	Chandramohan *et al*., 1972; Balasubramanian *et al*., 1979
Rhodococus spp.	Sediments	Nitrile hydrolyzing	Heald *et al*., 2001; Brando and Bull, 2003
Streptomyces spp.	Sediments	Amylolytic, proteolytic and Lipolytic	Ellaiah *et al*., 2002, 2004; Das, 2007

2.1.3 Enzymes from Actinobacteria

2.1.3.1 Proteases

Proteases are the most important class of industrial enzymes as they account for up to 25 % of all commercial enzymes used in the world. It is estimated that two thirds of the industrially produced proteases are from a microbial source (Gerhartz, 1990; Moon and Parulekar, 1991). The majority of these proteases are used in food, pharmaceutical and detergent industries. For example alkaline proteases are used in detergent powders, and in food processing, e.g. in production of protein hydrolysate (Phadatare *et al.,* 1993). Acid proteases are used extensively in meat tenderization and in the production of fermented foods by moulds from soybean, rice and others cereals (Nout and Rombouts, 1990). They are also used in the baking industry for the modification of wheat proteins whereas in the dairy industry they are used in the manufacture of cheese (Boing, 1982). Porto *et al.* (1996) studied *Streptomyces clavuligerus* cultures for protease production. They reported that the amount of enzyme produced varies greatly with the culture media used. An important advantage of these proteases produced from *Streptomyces* is they are secreted into the media and hence can easily be extracted and purified by filtration (Phadatare *et al.,* 1993).

2.1.3.2 Lipases

Microbial carboxylesterases that include lipases (EC 3.1.1.3) and esterases (EC 3.1.1.1); catalyze the hydrolysis of a broad range of natural and unnatural esters, often showing high enantio-selectivity and region-selectivity. To date there are over 70 commercially available

lipases that are extracellularly produced by various micro-organisms (Bornscheuer and Kazlauskas, 1999).

Lipids are commonly used as carbon sources in *Streptomyces* fermentation but studies on their esterase and lipolytic activities are largely indescribed (Tesch *et al.,* 1996; Sommer *et al.,* 1997; Abramic *et al.,* 1999). For example cell-bound lipases have been described in only five well-known Streptomycetes such as *Streptomyces clavuligerus, Streptomyces lividans, Streptomyces coelicolor, and Streptomyces rimosus* and in the related *Saccharopolyspora erythraea* (Large *et al.,* 1999).

2.1.3.3 Carboxylesterases

Distribution of cell-bound and extracellular carboxylesterases have been investigated among the genus *Streptomyces* (Gandolfi *et al.,* 2000) and reports show that carboxylesterase activities are often cell-bound and thus useful for catalyzing esterification in organic solvent. The potential of cell bound carboxylesterases of *Streptomyces* as enantioselective biocatalysts has been evaluated in the hydrolysis of various racemic esters of isopropylidene glycerol and two streptomycetes strains have been selected: strain 90852 able to preferentially furnish (*S*)-IPG and strain 90930 giving the (*R*)-enantiomer.

2.1.3.4 Methyltransferases

SAM-dependent methyltransferases are well known in Streptomycetes, other bacteria, and fungi where they are involved in antibiotic biosynthesis (Chen *et al.,* 1992) as well as in the methylation of other compounds (Kreuzman *et al.,* 1988). Previously, cultures of *Streptomyces griseus* have sequentially converted 7-methoxycoumarin to a mixture of 6-

hydroxy-7-methoxycoumarin and 7-hydroxy-6-methoxycoumarin (Sariaslani and Rosazza, 1983) by a pathway involving O deethylation, aromatic hydroxylation to a 6,7-catechol, and subsequent methylation. The intermediary of 6, 7-dihydroxycoumarin has been established through incubation of *S. griseus* cultures with the catechol as a substrate to accumulate both monomethyl-ether isomers. The formation of 7-hydroxy-6-methoxycoumarin and 6-hydroxy-7-methoxycoumarin has indicated presence of a methyltransferase enzyme system in *S. griseus*. The incorporation of the ^{14}C-labeled methyl group from ^{14}C-methylmethionine demonstrated that an *S*-adenosyl methionine (SAM)-dependent transmethylating system was involved in this reaction.

2.1.3.5 Lignin peroxidase

Lignin is the second most abundant biopolymer, comprising 15 % of the Earth's biomass (Hammel, 1992) and consists of an apparently random complex of phenolic and non-phenolic compounds (Crawford, 1981). The majority of bonds within a typical lignin structure consist of arylglycerol β-aryl ether (β-O-4) and di-aryl propane (β- 1) bonds (Crawford, 1981). Since lignin also contains molecules of different chirality, lignin-degrading enzymes must be non-stereoselective (Crawford, 1981). Because of the irregularity of the lignin structure, relatively few micro-organisms are capable of degrading lignin. The most-studied, lignin-degrading system is that of the white rot fungus, *Phanerochaete chrysosporium*. *P. chrysosporium* secretes both lignin peroxidase and manganese peroxidase for lignin degradation (Kirk and Farrell, 1987).

The Gram-positive bacterium, *Streptomyces viridosporus* T7A, also produces a variety of enzymes that play a role in lignin degradation. These extracellular enzymes include various peroxidases, cellulases, esterases, and endoglucanases (Crawford *et al*., 1983; Borgmeyer and Crawford, 1985; Adhi *et al*., 1989). *S. viridosporus* T7A produces four extracellular lignin peroxidase isoforms, and although all require hydrogen peroxide as an electron donor, each isoform has a different substrate range. Lignin peroxidase of *S. viridosporus* T7A has been studied in the degradation of Kraft indulin lignin (Giroux *et al*., 1988), acetovanillone, vanillyl alcohol, guaiacol and humic acid model compounds (Kontchou and Blondeau, 1991). Because *S. viridosporus* T7A lignin peroxidase (ALiP) is known to degrade lignin and various phenolic compounds, *Streptomyces* may be used to degrade or mineralize recalcitrant waste compounds such as azo dyes and pesticides (Gauger *et al*., 1986)

2.1.3.6 Amylases

Starch hydrolyzing enzymes are widely distributed in *Streptomyces* species (Goldberg and Edwards, 1990), and some of them can attack and hydrolyze raw starch granules (Fairbarn *et al.*, 1986), with the release of maltose as the predominant product. Such enzymes are useful for the industrial conversion of raw starch into sugars for fermentation (Norman, 1978). α-Amylase from *Streptomyces praecox* NA-273 (Takaya *et al*, 1979) has been purified and shown to contain three isoenzymes that convert starch to maltose, without formation of glucose (Suganuma *et al.*, 1980). On the other hand, the purified amylase from a chlorotetracycline-producing strain of *Streptomyces aureofaciens* cleaves starch

through an endo-mechanism, producing glucose, maltose and maltotriose in α-configuration as main products (Hostinova and Zelinka, 1978).

2.1.4 Secondary metabolites from Streptomyces

Actinomycetes are a major source of secondary metabolites, and the vast majority of these compounds are derived from the single genus *Streptomyces*. *Streptomyces* produce chemically diverse secondary metabolites that are structurally related, as well as structurally unrelated types of secondary metabolites from the same culture. Production of these secondary metabolites is usually during the stationary or slower stages of growth (Alexandra, 1997). Their production however is increased by inducing a biosynthetic enzyme (synthase) or increasing the limiting factor. Much of the published data indicates that the most important environmental signal triggering secondary metabolism is nutrient starvation, particularly that of phosphate (Sola-Landa *et al.*, 2003). The signaling networks behind the regulation of secondary metabolism in Streptomyces have recently been reviewed by (Bibb, 2005). Synthesis of secondary metabolites is normally coded by clustered genes on chromosomal DNA of 20 kb to more than 100 kb and infrequently on plasmid DNA.

Many of these secondary metabolites are potent antibiotics, which has made Streptomycetes the primary antibiotic-producing organisms exploited by the pharmaceutical industry (Berdy, 2005). However, besides antibiotics which present the largest group of bioactive secondary metabolites, the Streptomycetes compounds show several other biological activities. These are: firstly, antagonistic agents that include;

antibacterials, antifungals, antiprotozoans as well as antivirals: secondly, pharmacological agents that shows the following activities; antitumor, immunomodulators, neurological agents and enzyme inhibitors: thirdly, agrobiologicals comprising of; insecticides, pesticides and herbicides: fourthly, compounds with regulatory activities such as; growth factors, siderophores or morphogenic agents (Sanglier *et al.*, 1996; Berdy, 1995; 2005).

Due to the ability in synthesizing numerous compounds that exhibit extreme chemical diversity, *Streptomyces* strains have become a major part of industrial strain collection used in screening for new bioactive molecules (Demain and Davies, 1999).

3.0 CHAPTER THREE: MATERIALS AND METHODS

3.1 Isolates

The Actinobacteria isolates used in this study were isolated previously (Nonoh *et al.,* 2010). They comprised Chy 15–10, Chy 4–10, Chy 15–5, Chy 2–3 (from Coastal province - low altitude zone) and Ruj 7–1 (Western province - medium altitude zone). In each province, samples were collected from three locations. Soil samples from Coastal province were collected from Chyulu Hills National Parks whereas the samples from Western province were collected from Ruma National Park. Isolation was carried out and the isolates characterized at molecular level to determine their identity as well as their usefulness in producing secondary metabolites with antifungal potential (Nonoh *et al.,* 2010).

3.2 Growth of Actinobacteria isolates

The isolates were revived in a differential growth media **(Appendix 1)** in a shaker incubator (Gallen Kamp, Germany) (200 rpm, 28 ºC) for 96 h (hours). The original stocks of the isolates from which the working stocks were prepared were kept in a freezer (Sanyo MDF-594 AT, Japan) at – 80 °C.

3.3 Morphological characterization of the Actinobacteria isolates

Morphological characterization of the isolates was done under a compound microscope (Leitz, Japan) and a dissecting microscope (Leica Zoom, United States of America) (×16). Simple and Gram staining techniques were used to characterize cell morphology (Cappuccino and Sherman, 2002).

3.3.1 Simple staining

Smears of Actinobacteria isolates grown for 24 h were prepared on glass slides and heat fixed. The smears were stained using crystal violet as described by Murray and Robinow (1994) and viewed under a compound microscope (Leitz, Japan) (× 100) to determine cell shape.

3.3.2 Gram staining

Hucker's staining method as described by Hendrickson and Krenz (1991) was used to stain the isolates. Gram positive cells would appear purple while Gram negative cells would be pink in colour under a compound microscope (Leitz, Japan) (×100).

3.4 Biochemical characterization of the Actinobacteria isolates

3.4.1 Starch hydrolysis

Starch hydrolysis test was carried out to detect the presence of amylase-like enzyme activity through hydrolysis of starch. Starch agar containing 0.2 % soluble starch that served as the polysaccharide substrate was used. After the inoculation of the starch agar with the Actinobacteria isolates, incubation was done in a flat bed incubator (Carbolite 301 Controller, Jencons, United Kingdom) at 30 °C for 48 h. Determination of the hydrolytic activity was carried out by flooding the plates with iodine solution Visual presence of clear zones of hydrolysis around the colonies indicated positive results (Cappuccino and Sherman, 2002; Harold, 2002).

3.4.2 Sugar utilization tests

Different sugars were used to test the ability of the various isolates to utilize different carbon sources. Basal broth media **(Appendix 2)** containing the various sugars was inoculated with the isolates and incubated in a shaker incubator (Gallen Kamp, Germany) (30 °C for 96 h at 100 rpm). Utilization of the various sugars was ascertained by growth of the isolates. This was determined by measuring the optical densities of the broth cultures using a UV spectral photometer (Shimadzu UV 240, Japan) at 600 nm (Williams *et al*., 1989).

3.4.3 Methyl Red (MR) test

During energy production, hexose monosaccharide glucose is the major substrate oxidized by all enteric organisms. Variations of the end product of this process occur due to the enzymatic pathways present in the bacteria. Therefore the test was to determine the ability of the isolates to oxidize glucose with production and stabilization of high concentrations of acid end products according Harold's (2002) protocol. Test tubes containing MR broth (Difco ^{TM}) were inoculated with each of the isolates and incubated in flat bed incubator (Carbolite 301 Controller, Jencons, United Kingdom) at 30 °C for 48 h. Methyl red indicator was added to aliquots of each culture. For positive culture, methyl red appeared yellow (Cappuccino and Sherman, 2002).

3.4.4 Voges- Proskauer (MR-VP) test

Voges-Proskauer test was carried out to determine the capability of the isolates to produce nonacidic or neutral end products, such as acetylmethylcarbinol from the organic acids that resulted from glucose metabolism. MR-VP broth (Difco ^{TM}) was inoculated with each of the isolates and incubated in a flat bed incubator (Carbolite 301 Controller, Jencons, United Kingdom) at 30 ℃ for 48 h. Barrit's reagent (Fluka Biochemika, Germany) was added to aliquots of each culture where VP positive culture gave a rose coloration (Cappuccino and Sherman, 2002).

3.4.5 Gelatin liquefaction

The test was to determine the ability of the isolates to secrete hydrolytic extracellular enzyme (gelatinase) capable of hydrolyzing gelatin into amino acids. Nutrient Broth supplemented with 12 % gelatin was used to demonstrate the hydrolytic activity of gelatinase (Harold, 2002). After incubation in a flat bed incubator (Carbolite 301 Controller, Jencons, United Kingdom) at 28 ℃, cultures were placed in the refrigerator at 4 ℃ for 30 min (minutes). Cultures that remained liquefied were considered positive for gelatin hydrolysis (Cappuccino and Sherman, 2002).

3.4.6 Indole production and Hydrogen sulfide production

Sulfur-Indole Mortility (SIM) agar media was used to test for the production of Tryptophanase enzyme and the ability to produce hydrogen sulfide from substrates such as sulfur containing amino acids and organic sulfur. Presence of indole was detected by addition of Kovac's reagent (Fluka, Germany) to 48 h cultures of each

isolates (Harold, 2002). Positive results were indicated by production of a cherry red layer. Absence of black coloration in the media following incubation indicated absence of hydrogen sulfide (Cappuccino and Sherman, 2002).

3.4.7 Catalase test

This test was to determine the ability of the isolates to degrade hydrogen peroxide by producing an enzyme catalase. Tryptic Soy Agar slants containing 3 % hydrogen peroxide were used as outlined by Cappuccino and Sherman (2002). A positive reaction was indicated by bubbles of free oxygen gas (O_2), which indicated presence of catalase enzyme.

3.4.8 Nitrate reduction test

The ability of the isolates to reduce nitrates to nitrites or beyond was carried out using nitrate broth medium containing 1 % potassium nitrate according to the methods of Harold (2002) and that of Cappuccino and Sherman (2002). Following inoculation and incubation, addition of sulfanilic acid and alpha-naphthylamine produced a cherry red coloration, which was indicative of positive results (Cappuccino and Sherman, 2002).

3.4.9 Urease test

The test was done to determine the ability of the isolates to degrade urea by means of the enzyme urease. Urea broth media containing phenol red indicator was used according to the methods of Harold (2002). A positive reaction was indicated by phenol red turning into deep pink color (Cappuccino and Sherman, 2002).

3.5 Physiochemical characterization of the Actinobacteria isolates

3.5.1 Effect of pH on growth of the Actinobacteria isolates

Growth and survival of microorganisms is greatly influenced by the pH of the environment, and all bacteria and other microorganisms differ as to their requirements. Each species has the ability to grow within a specific pH range, which may be broad or limited, with the most rapid growth occurring within a narrow optimum range (Cappuccino and Sherman, 2002). The aim of the experiment was therefore to determine the optimum pH requirements for the isolates. 100 µl of each bacteria inoculum was transferred into International Streptomyces Project (ISP_2) broth media **(Appendix 3)** adjusted to varying pH ranges of 3, 6, 7 and 9, using 1N sodium hydroxide and 1N hydrochloric acid. The cultures were incubated in a shaker incubator (Gallen Kamp, Germany) (30 ºC for 48 h at 100 rpm) and optical density readings read at 600 nm using a UV spectrophotometer (Shimadzu UV 240, Japan).

3.5.2 Effect of temperature on growth of the Actinobacteria isolates

Bacteria, as a group of organisms, exist over a wide range of temperatures. However, individual species can only exist within a narrower spectrum of temperatures as it normally influences the rate of chemical reactions through its action on cellular enzymes (Cappuccino and Sherman, 2002). The aim of the experiment was therefore to determine the optimum temperature requirements for growth of the isolates.

Growth of the five isolates was monitored by spectrophotometric measurement of the optical density at 600 nm. Experiments were performed at 15, 20, 25, 27.5, 30, 32.5,

35, 36, 37, and 38 °C. 100 µl of each bacterium inoculum was transferred into International Streptomyces Project (ISP_2) broth media **(Appendix 3)**. Prior to the experiments, bacteria were acclimatized to the temperature conditions in the growth experiments. All cultures were incubated on a rotary shaker incubator (Gallen Kamp, Germany) (15, 20, 25, 27.5, 30, 32.5, 35, 36, 37, and 38 °C for 12 h at 100 rpm) in the dark. Precultures of acclimatized strains that were used for setting up growth experiments were grown overnight. Experiments were performed in 100-ml Erlenmeyer flasks in triplicate for each isolate. The medium used for the experiments was preincubated in a flat bed incubator for 6 h under the same temperature conditions as the temperature conditions in the experiment. Measurement of the optical density was started 72 h after inoculation. The optimal growth temperature was determined graphically.

3.5.3 Sodium Chloride tolerance test

For this test, NaCl broth **(Appendix 4)** of Hayward (1964) was used. 50 ml of the medium was autoclaved at 121 °C for 15 min in clean 100 ml conical flasks. 100 µl of test strain inoculums were inoculated into the medium contained in conical flasks and incubated on rotary shaker (Gallen Kamp, Germany) (30 °C for 96 h at 100 rpm). Growth of the five isolates was monitored spectrophotometrically by measuring the optical density at 600 nm.

3.6 Screening of the isolates for enzymes

In order to carryout screening of enzymes and secondary metabolites from the isolates, optimization of growth conditions and media composition was done to achieve good results. This was achieved through analysis and compilation of physiochemical results. Results were incorporated in the screening procedures **(Table 3)**.

Table 3: Modified media compositions to optimize production of enzymes

Isolates	Media			
	Amylolytic activity	**Lipolytic activity**	**Esterasic activity**	**Proteolytic activity**
Chy 4-10	NA, 0.2% starch, pH 7, 5g/l NaCl at 30°C	SM, 1%T20, pH 7, 5g/l NaCl at 30°C	SM, 1%T80, pH 7, 5g/l NaCl at 30°C	DB, 6ml/l skim milk, Glucose, pH 7, 5g/l NaCl at 30°C
Chy 15-10	NA, 0.2% starch, pH 9, 5g/l NaCl at 32.5°C	SM, 1%T20, pH 9, 5g/l NaCl at 32.5°C	SM, 1%T80, pH 9, 5g/l NaCl at 32.5°C	DB, 6ml/l skim milk, Xylose, pH 9, 5g/l NaCl at 32.5°C
Chy 15-5	NA, 0.2% starch, pH 9, 10g/l NaCl at 30°C	SM, 1%T20, pH 9, 10g/l NaCl at 30°C	SM, 1%T80, pH 9, 10g/l NaCl at 30°C	DB, 6ml/l skim milk, Glucose, pH 9, 10g/l NaCl at 30°C
Chy 2-3	NA, 0.2% starch, pH 9, 5g/l NaCl at 32.5°C	SM, 1%T20, pH 9, 5g/l NaCl at 32.5°C	SM, 1%T80, pH 9, 5g/l NaCl at 32.5°C	DB, 6ml/l skim milk, Glucose, pH 9, 5g/l NaCl at 32.5°C
Ruj 7-1	NA, 0.2% starch, pH 6, 17.5g/l NaCl at 27.5°C	SM, 1%T20, pH6, 17.5g/l NaCl at 27.5°C	SM, 1%T80, pH 6, 17.5g/l NaCl at 27.5°C	DB, 6ml/l skim milk, Xylose, pH 6, 17.5g/l NaCl at 27.5°C

Key:

NA = Nutrient Agar

SM = Sierra Media

DB = Differential Broth

T20 = Tween 20

T80 = Tween 80

3.6.1 Determination of amylolytic activity

Amylolytic activity was determined using the methodology described by Hankin and Anagnostakis (1975). Isolates were inoculated in modified nutrient agar with 0.2 % of soluble starch (Sigma Aldrich, Germany). After incubation in a flat bed incubator (Carbolite 301 Controller, Jencons, United Kingdom) (at optimum temperatures for each isolate for 96 h), the cultures were treated under iodine vapours, which allowed the visualization of clear halos around the colonies.

3.6.2 Determination of the esterasic activity

The media used was as described by Sierra (1975), containing (g/l): peptone 10.0, NaCl (optimum concentration for each isolate), $CaCl_2$ $2H_2O$ 0.1, agar 18.0. To the sterilized culture media, previously sterilized Tween 80 was added in a final concentration of 1 % (v/v). The medium was inoculated with the isolate and incubated (at optimum temperatures for each isolate for 96 h). An opaque zone of calcium precipitates was recorded as positive reaction for hydrolysis of Tween 80 (Sands, 1990).

3.6.3 Determination of the lipolytic activity

The media used was as described by Sierra (1975), containing (g/l): peptone 10.0, NaCl (optimum concentration for each isolate), $CaCl_2$ $2H_2O$ 0.1, agar 18.0. To the sterilized culture media, previously sterilized Tween 20 was added in a final concentration of 1 % (v/v). The medium was inoculated with the isolates and the presence of an opaque zone of calcium precipitates after four days of incubation indicated a positive reaction.

3.6.4 Determination of the proteolytic activity

To determine protease activity, a media containing nutrient broth (8 g/l), sugars (glucose-Cy 4-10, Chy 15-5, Chy 2-3; xylose-Chy 15-10, Ruj 7-1) 1 g/l and agar 18 g/l was used according to Vieira (1999). After autoclaving, 15 ml of skimmed milk separately autoclaved was added before cooling and mixed well. 20 ml of media was poured in Petri-dishes and the isolates inoculated. After incubation in a flat bed incubator (Carbolite 301 Controller, Jencons, United Kingdom) (at optimum temperatures for each isolate for 48 h), casein hydrolysis was indicated by the presence of clear halos around the colonies.

3.7 Determination of the enzymatic activity

The isolates were grown in modified differential broth media for 24 h. After that, aliquots of 100 µl for each of the isolates broth were inoculated on the specific culture media for each enzyme to be investigated. The cultures were incubated in a flat bed incubator (Carbolite 301 Controller, Jencons, United Kingdom) (at optimum temperatures for each isolate for 96 h). The enzymatic activity was expressed by the relationship between the average diameters of the degradation halo over time (24, 48, 72 and 96 h) according to (Hankin *et al.*, 1971).

3.8 Degradation assays of the crude extra-cellular proteins

3.8.1 Degradation assay of the crude extra-cellular proteins on starch

Actinobacteria isolates were cultivated in modified differential broth medium containing 0.3 % soluble starch. The cultures (100 mL in 500 ml flasks) were incubated in an orbital shaker (at isolate optimum temperatures for 144 h at 100 rpm). Crude extra-cellular

proteins were extracted from the supernatant after centrifugation (Refrigerated centrifuge, H-2000, Japan) of the culture broth (10,000 × g, 20 min; 4 °C).

500 ml of the culture supernatant was prepared as stated above (2.8.1.1) in each isolate and added to 40 g of soluble starch. The suspensions were stirred gently at 4 °C. After 30 min, the suspensions were centrifuged (Refrigerated centrifuge, H-2000, Japan) (10,000 × *g* for 10 min), and the precipitated starch washed twice with 500 ml of 20 mM acetic acid-potassium acetate buffer at pH 5.5. The adsorbed enzymes were eluted from starch by shaking in 250 ml of 20 mM sodium borate buffer (pH 6.8) (40 °C for 2 h) and the released enzyme solutions was transferred in to a dialysis tubing and dialyzed overnight. The dialyzed enzyme solutions were used as the crude extra-cellular proteins for amylase activities. Paper disc (Whatman® qualitative filter paper, Grade 1, Aldrich chemical co. ltd., United States of America) were prepared using a paper punch and impregnated with the prepared enzyme solutions. This was achieved through soaking of the paper discs in the enzyme solution and thereafter draining the excess solution for 10 s. This was followed by placing the impregnated paper discs in Petri-dishes containing nutrient agar supplemented with 0.2 % soluble starch (g l^{-1}). After incubation in a flat bed incubator (Carbolite 301 Controller, Jencons, United Kingdom) (27.5 °C, 30 °C and 32.5 °C for 72 h), Petri-dishes were flooded with an iodine solution which allowed visualization of clear halos around the colonies (Hankin and Anagnostakis, 1975). Halo diameters were measured and data recorded.

3.8.2 Degradation assay of the crude extra-cellular proteins on Tween 20

Aliquots of Actinobacteria (100 µl) were inoculated into 100 ml portions of sterile 10 % reconstituted skim milk (Kumura *et al*., 1991) and incubated in a flat bed incubator (Carbolite 301 Controller, Jencons, United Kingdom) (27.5 °C, 30 °C and 32.5 °C for 72 h), followed by another incubation (6 °C for 72 h). These portions were centrifuged (Refrigerated centrifuge, H-2000, Japan) (20,000 x *g* at 4 °C for 30 min). The supernatants were filtered using 0.45-µm cellulose acetate filter units (Toyo Roshi Kaisha, Ltd., Japan). The filtrates were used as the crude extra-cellular proteins for lipase activity experiment. Paper discs were prepared, impregnated with the filtrates and placed in Petri-dishes containing media described by Sierra (1975), supplemented with 1 % (v/v) Tween 20. Zones of crystals around the discs were observed, measured and data recorded after incubation (27.5 °C, 30 °C and 32.5 °C for 72 h).

3.8.3 Degradation assay of the crude extra-cellular proteins on Tween 80

Preparation of crude extra-cellular proteins was done similarly to the one of lipases as stated above. However, impregnated paper discs were placed in Petri-dishes containing media described by Sierra (1975), supplemented with 1 % (v/v) Tween 80. Zones of crystals around the discs were observed, measured and data recorded after incubation (27.5 °C, 30 °C and 32.5 °C for 72 h).

3.8.4 Degradation assay of the crude extra-cellular proteins on Skimmed milk

Actinobacteria isolates were grown in 100 ml of differential broth medium supplemented with 1.5 ml skimmed milk in 250 ml shake flasks in an orbital shaker (Gallen Kamp,

Germany) (200 rpm at 27.5 °C, 30 °C and 32.5 °C). Cells were harvested through centrifugation after 9 days of growth (2500 *g* for 30 min). The supernatant fluid was passed over a 0.22 µm filter, concentrated (against polyethylene glycol 4000), and dialyzed overnight at 4 °C against 0·01 mmol l^{-1} Tris-HCl, pH 7·5, containing 5 mmol l^{-1} $CaCl_2$ (Tris-$CaCl_2$ buffer). Dialyzed supernatants were used as the extra-cellular proteins. Paper discs were impregnated with the dialyzed supernatants and placed in Petri-dishes containing media described by Vieira (1999), supplemented with skimmed milk. After incubation (27.5 °C, 30 °C and 32.5 °C for 48 h), clear zones of hydrolysis were observed, measured using a ruler and data recorded.

3.9 Extraction of secondary metabolites

Differential broth cultures of the isolates were prepared in 500 ml conical flasks and incubated in a rotary shaker (Gallen Kamp, Germany) (at isolate's optimum temperature for 240 h at 100 rpm). Filtration of the broth cultures by use of Whatman filter paper no.1 was done after fermentation to remove bacterial cells. The cell free culture filtrates were extracted three times with ethyl acetate at volume ratio of 1:1 by use of a separating funnel. The extract was passed through a pad of anhydrous sodium sulphate to remove excess water and thereafter evaporated to dryness using a rotary vacuum evaporator (RE 100B, Bibby Sterilin, United Kingdom). Yields of the extracts were determined and recorded. The crude extracts were used for biological activity tests as well as Gas Chromatography - Mass Spectrophotometry (GC - MS) analysis of the compounds contained in them.

3.9.1 Bio assay of the crude extracts against Gram –ve and +ve bacteria

Agar diffusion method (Lorian, 1996) was used to determine antibacterial activity of the crude extracts. The bacteria used comprised, *Escherichia coli* (NCTC 10418) and *Staphylococcus aureus* (NCTC 10788). Paper discs were prepared and impregnated with 10 µl of the sample crude extract prepared by dissolving the dry crude extracts in 1 ml ethyl acetate. The impregnated paper discs were allowed to dry in a fume chamber and then placed on agar seeded with the test organisms. The positive control consisted of commercial Kanamycin antibiotic (1 mg/ml) while negative control consisted of un-inoculated plate. Incubation was done in a flat bed incubator (Carbolite 301 Controller, Jencons, United Kingdom) (37 ºC for 24 h) and diameters of zones of inhibitions measured using a ruler and recorded.

3.9.2 GC-MS Analysis of secondary metabolites from the Actinobacteria isolates

Chemical screening of the active compounds present in the crude extracts was done by use of a GC - MS to detect the active compounds as well as their quantity and quality ratios. Each samples was reconstituted using 1 ml DCM (Dichloromethane (≥99.8 %; Aldrich chemical co. ltd., USA.) and passed through a glass wool to remove solid materials. 40 µl of the collection in triplicate was transferred into auto sampler glass vials having Teflon caps and analyzed using GC - MS whose conditions are given below. Agilent Technologies 7890A system was used. Oven conditions set during the analysis were: 1 min for equilibration time; 35 °C for 5 min, 10 °C / min to 280 °C for 10.5 min and 50 °C / min to 285 °C for 9.9 min as the oven program while the running time was 50 min. Injection

was done in splitless mode and the conditions used were as follows: 250 °C for the heater, 8.8271 Psi as the pressure, a total flow of 10.2 ml / min, septum purge flow of 3 ml / min, gas saver at 20 ml / min after 2 min and Purge flow to split vent at 6mL / min at 0.8minutes. The column used was HP-5MS, (5 % methyl silox), (30 m × 250 µm × 0.25 µm). The compounds identified were generated from a computer program that involved calculation by the data system of a similarity index, match factor or purity between the unknown spectrum and library (reference) spectra. For this analysis, NIST/EPA/NIH MASS SPECTRAL LIBRARY (NIST 05) and NIST MASS SPECTRAL SEARCH PROGRAM Version 2.0d were used.

3.10 Data Analysis

All statistical analysis were performed with SAS software package, version 9.1 (SAS Institute, 2003) using two way analysis of variance (ANOVA) for the replicated treatments. Dependent variables were growth of isolates, colony and halo diameters in enzymatic activities. Separation of means was done by use of Tukeys' test and means used to draw graphs and tables.

4.0 CHAPTER FOUR: RESULTS

4.1 Morphological characterization of the isolates

Morphological characterization of the isolates was carried out through microscopy and it revealed that all the isolates were Gram positive. All the isolates formed colored tough, leathery and filamentous colonies that were hard to pick from the culture media **(Figure 1a – 5a)**. Four isolates (Chy 4-10, Chy 15-10, Chy 15-5 and Ruj 7-1) also produced colored pigments, which were secreted into the media contained in Petri dishes after 144 h of incubation.

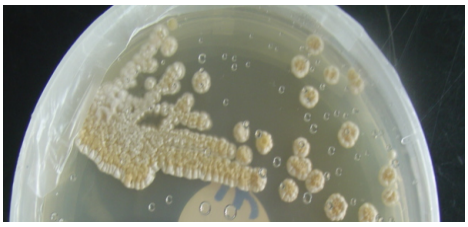

Figure 1a: A pure plate culture showing colonies of isolate Chy 15-10 (x16).

Figure 1b: Cells of a pure Chy 15–10 isolate under a compound microscope (×100).

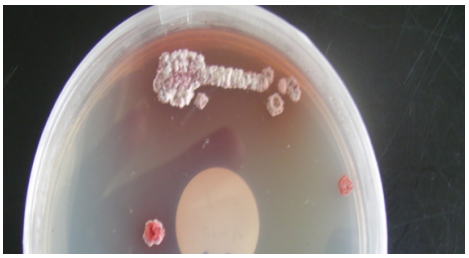

Figure 2a: A pure plate culture showing colonies of isolate Chy 4-10 (x16).

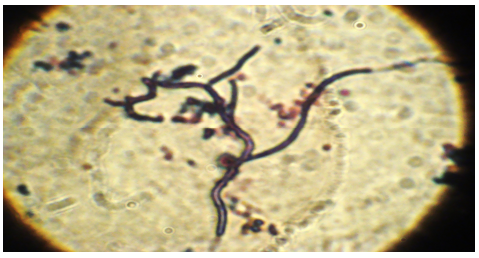

Figure 2b: Cells of a pure Chy 4–10 isolate under a compound microscope (×100).

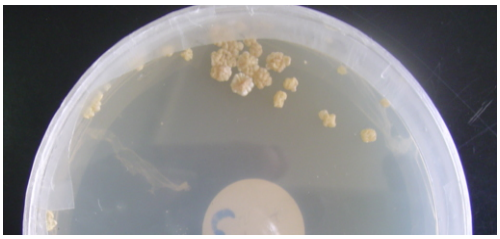

Figure 3a: A pure plate culture showing colonies of isolate Chy 15-5 (x16).

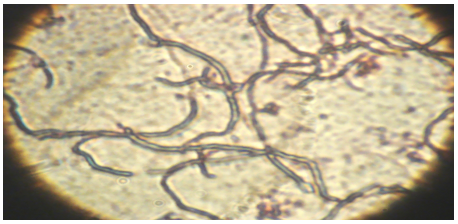

Figure 3b: Cells of a pure Chy 15–5 isolate under a compound microscope (×100).

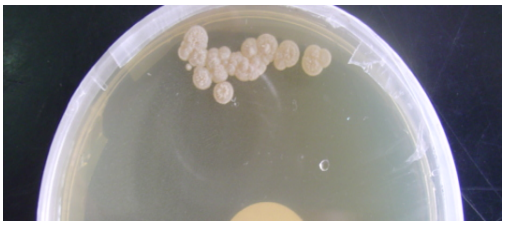

Figure 4a: A pure plate culture showing colonies of isolate Chy 2–3 (x16).

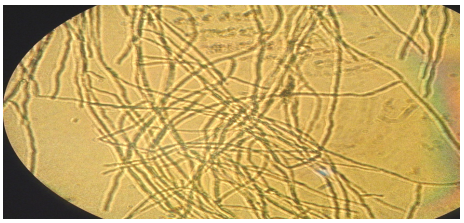

Figure 4b: Cells of a pure Chy 2–3 isolate under a compound microscope (×100).

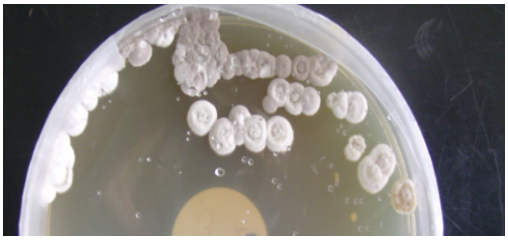

Figure 5a: A pure plate culture showing colonies of isolate Ruj 7-1 (x16).

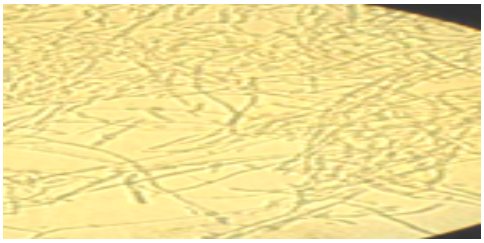

Figure 5b: Cells of a pure Ruj 7-1 isolate under a compound microscope (×100).

Different morphological characteristics were exhibited by the isolates. Colony shape was round in all the isolates as shown below in **(Table 4)**. Some of the isolates had different colony colours; Chy 4-10 colonies were red, Chy 2-3 white, Ruj 7-1 white brown whereas Chy 15-10 and Chy 15-5 were yellow brown **(Table 4)**. The isolates also showed different spore forms; Chy 15-10 were spiral and branched, Chy 15-5 round, Chy 4-10 spiral, Ruj 7-1 chain-like whereas Chy 2-3 were ovoid **(Table 4)**. Secretion of colored pigments into the media was also noted. Isolates Chy 15-10 and Ruj 7-1 produced brown pigments while Chy 15-5 produced yellow and Chy 4-10 produced red pigments. Chy 2-3 did not show signs of producing any colored pigments **(Table 4)**.

Table 4: Morphological characteristics of the isolates as observed under a dissecting microscope (Magnification x16) and a compound microscope (Magnification x100)

Isolates	**Morphological Characteristics**			
	Colony Shape	**Colony Color**	**Spores**	**Pigmentation**
Chy 15-10	Round	Yellowish-Brown	Spiral and branched	Brownish
Chy 15-5	Round	Yellowish-Brown	Round	Yellowish
Chy 4-10	Round	Reddish-white	Spiral	Reddish
Ruj 7-1	Round	Whitish-Brown	Chain-like	Brownish
Chy 2 - 3	Round	White	Ovoid	None

4.2 Biochemical characterization of the isolates

All the isolates hydrolyzed starch and also degraded hydrogen peroxide suggesting that they produced an amylase-like and catalase-like enzyme respectively. None of the isolates liquefied gelatin but all of them hydrolyzed skim milk. On Methyl red test, isolate Chy 15-10 produced acetoin (non-acid end product) during fermentation of glucose whereas there was production of acid end products from the other four isolates. There was no production of tryptophanase enzyme and hence the negative indole production results in all the isolates. However, the ability to produce hydrogen sulfide from substrates such as sulfur containing amino acids and organic sulfur was confirmed positively in isolates Chy 15-10, Chy 4-10, Chy 15-5 and Chy 2-3. Reduction of nitrates to nitrites was only positive in

isolates Chy 2-3 and Ruj 7-1. Degradation of urea through enzyme urease was confirmed positive in isolates Chy 4-10, Chy 15-5 and Ruj 7-1 **(Table 5)**.

Table 5: Biochemical characteristics of the isolates

Biochemical tests	**ISOLATES**				
	Chy 5-10	**Chy 4-10**	**Chy 15-5**	**Chy 2-3**	**Ruj 7-1**
Starch Hydrolysis	+	+	+	+	+
Methyl Red	-	+	+	+	+
Voges- Proskauer	-	-	-	-	-
Gelatin	-	-	-	-	-
Indole	-	-	-	-	-
Hydrogen sulfide	+	+	+	+	-
Catalase	+	+	+	+	+
Nitrate reduction	-	-	-	+	+
Urease	-	+	+	-	+
Skim Milk	+	+	+	+	+

Key:

(+) A positive result for the reaction

(-) A negative result for the reaction

4.2.1 Utilization of sugars by the isolates

Glucose resulted in highest growth of isolate Chy 4-10 (OD_{600}= 0.601 nm). However, sucrose (OD_{600}= 0.473 nm) and xylose (OD_{600}= 0.383 nm) also led to a considerable growth of the isolate **(Figure 6a)**.

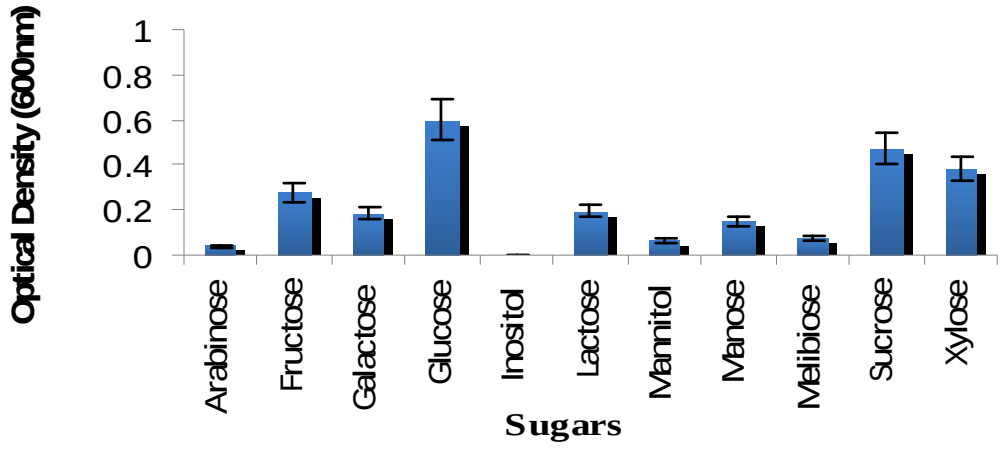

Figure 6a: Utilization of different sugars by isolate Chy 4-10. ($P<0.05$)

For isolate Chy 15-10, xylose led to highest growth (OD_{600} = 0.899 nm). It provided optimum growth whereas mannitol (OD_{600} = 0.010 nm), inositol (OD_{600} = 0.017 nm), lactose (OD_{600} = 0.019 nm) and arabinose (OD_{600} = 0.037 nm) did not support high growth of the isolate. Glucose (OD_{600} = 0.509 nm) and mannose (OD_{600} = 0.499 nm) were also utilized although growth of the isolate was at a lower level from xylose **(Figure 6b)**.

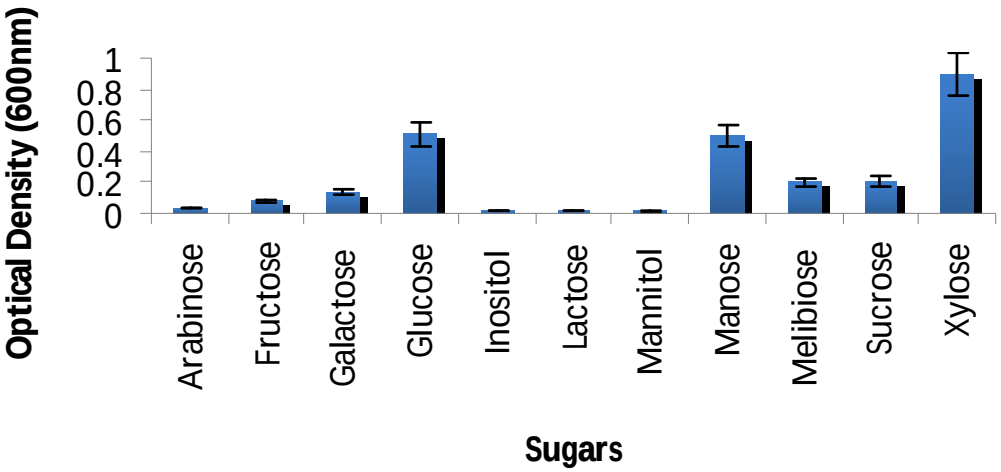

Figure 6b: Utilization of different sugars by isolate Chy 15-10. ($P<0.05$)

For isolate Chy 15-5, glucose had the highest growth (OD_{600} = 0.719 nm) compare to the other sugars. Considering the difference in growth between the various sugars, optimum growth for the isolate would only be realized with glucose as the carbon source **(Figure 6c)**.

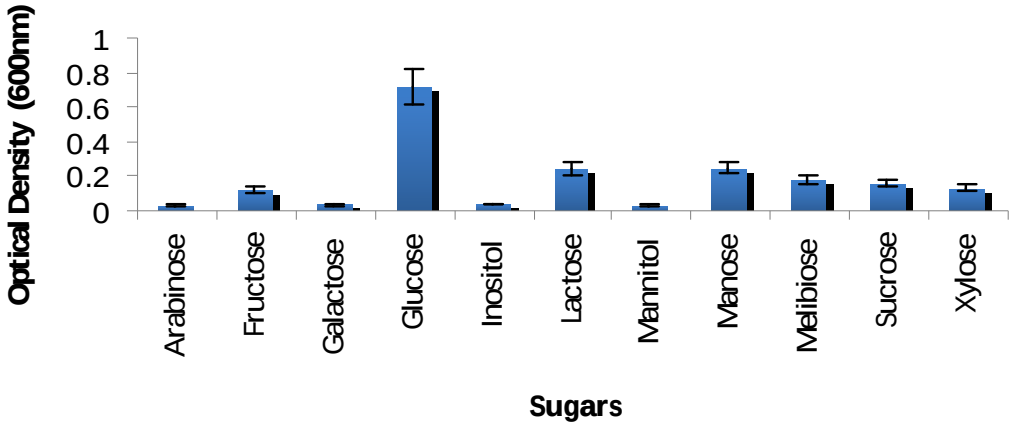

Figure 6c: Utilization of different sugars by isolate Chy 15-5. ($P<0.05$)

For isolate Chy 2-3, glucose (OD_{600} = 0.475 nm) similarly led to high growth whereas mannitol (OD_{600} = 0.021 nm) led to lowest growth of the isolate **(Figure 6d)**.

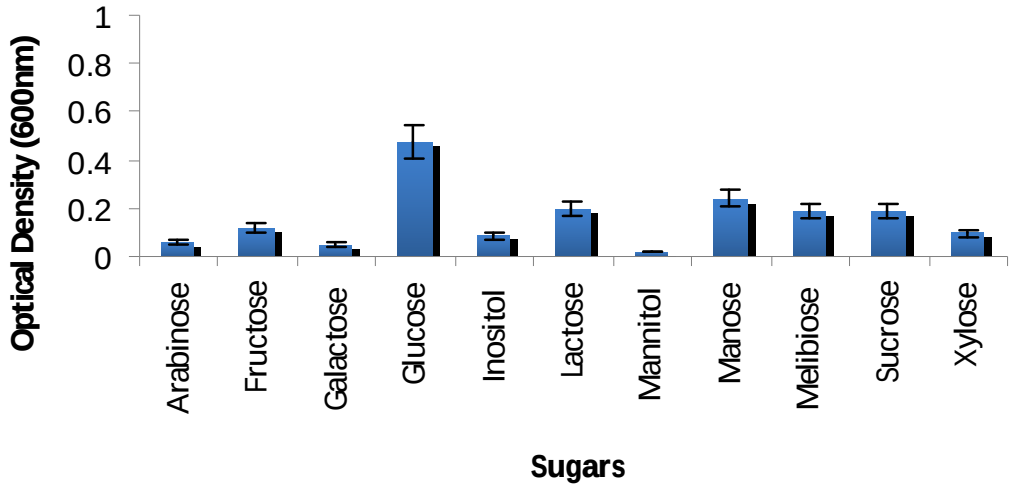

Figure 6d: Utilization of sugars by isolate Chy 2-3. (P<0.05)

For isolate Ruj 7-1, xylose supported high growth (OD_{600} = 0.900 nm) followed by glucose though at a much lower optical density (OD_{600} = 0.325 nm) **(Figure 6e)**.

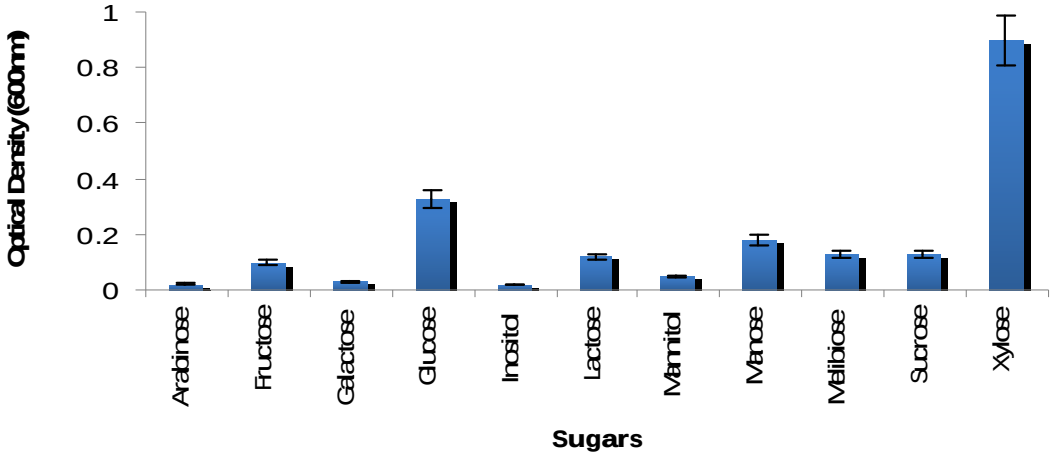

Figure 6e: Utilization of sugars by isolate Ruj 7-1. (P<0.05)

4.3: Physiochemical characterization of the isolates

4.3.1 pH tolerance by the isolates

All the isolates were able to grow at acidic, neutral and alkaline pH conditions. However, the different pH conditions yielded different growth levels for the various isolates. pH 7 led to highest growth of isolate Chy 4-10 with an optical density (OD_{600} = 0.604 nm) **(Figure 7a)**. Lowest growth of the isolate was yielded at pH 3 (OD_{600} = 0.173 nm) **(Figure 7a)**. Highest growth of isolate Chy 15-10 was observed at pH 9 (OD_{600} = 0.610 nm) whereas minimal growth was at pH 3 (OD_{600} = 0.178 nm) **(Figure 7b)**. For isolate Chy 15-5, pH 9 (OD_{600} = 0.591 nm) led to the highest growth of the isolate followed by pH 7 (OD_{600} = 0.549 nm). Minimal growth of the isolate was yielded at pH 3 (OD_{600} = 0.205 nm) **(Figure 7c)**. Growth of isolate Chy 2-3 was highly yielded at pH 9 (OD_{600} = 0.489 nm) whereas pH 3 had the lowest growth with an optical density (OD_{600} = 0.193 nm) **(Figure 7d)**. For isolate Ruj 7-1, highest growth was yielded at pH 6 (OD_{600} = 0.619 nm) whereas minimal growth was at pH 3 (OD_{600} = 0.215 nm) **(Figure 7e)**.

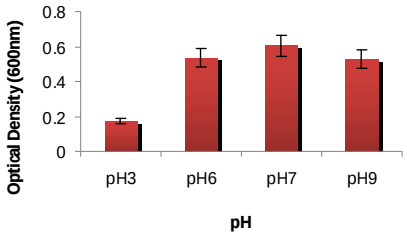

Figure 7a: Effect of pH on growth of the isolate Chy 4-10.

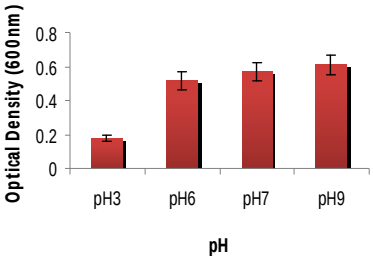

Figure 7b: Effect of pH on growth of the isolate Chy 15-10. ($P<0.05$)

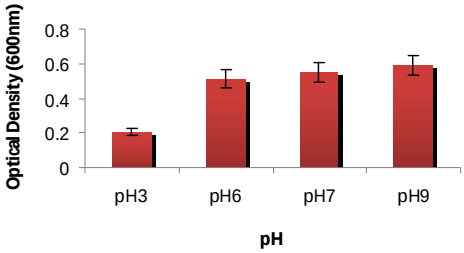

Figure 7c: Effect of pH on growth of the isolate Chy 15-5.

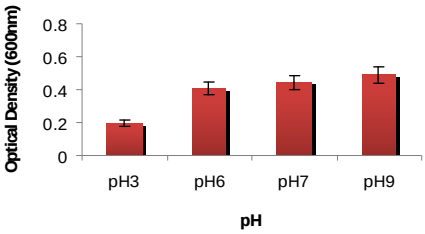

Figure 7d: Effect of pH on growth of the isolate Chy 2-3. ($P<0.05$)

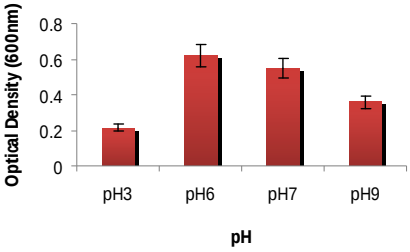

Figure 7e: Effect of pH on growth of the isolate Ruj 7-1.

4.3.2 Effect of temperature on growth of the Actinobacteria isolates.

In all the isolates investigated, there was a linear increase in the temperature-dependent growth curves at the temperature range 15 ℃ to 32.5 ℃. With further increases in temperature, the growth curves showed either a plateau or a linear decrease. The optimal growth temperature had the highest OD at 600 nm **(Figure 8a)**. The optimum growth temperature for isolate Chy 4-10 was 30 ℃ (OD_{600} = 0.610 nm). Beyond 30 ℃, there was a decline in growth of the isolate **(Figure 8a)**. For isolate Chy 15-10, 32.5 ℃ (OD_{600} = 0.602 nm) was the optimum temperature for growth of the isolate **(Figure 8b)** and 30 ℃ (OD_{600} = 0.562 nm) was the optimum temperature for growth of isolate Chy 15-5 **(Figure 8c)**. For isolate Chy 2-3, 32.5 ℃ (OD_{600} = 0.518 nm) was the optimum temperature needed for optimal growth of the isolate **(Figure 8d)**.

For isolate Ruj 7-1, 27.5 ℃ (OD_{600} = 0.653 nm) was the optimum temperature for growth of the isolate when compared with other temperature regimes **(Figure-8e)**.

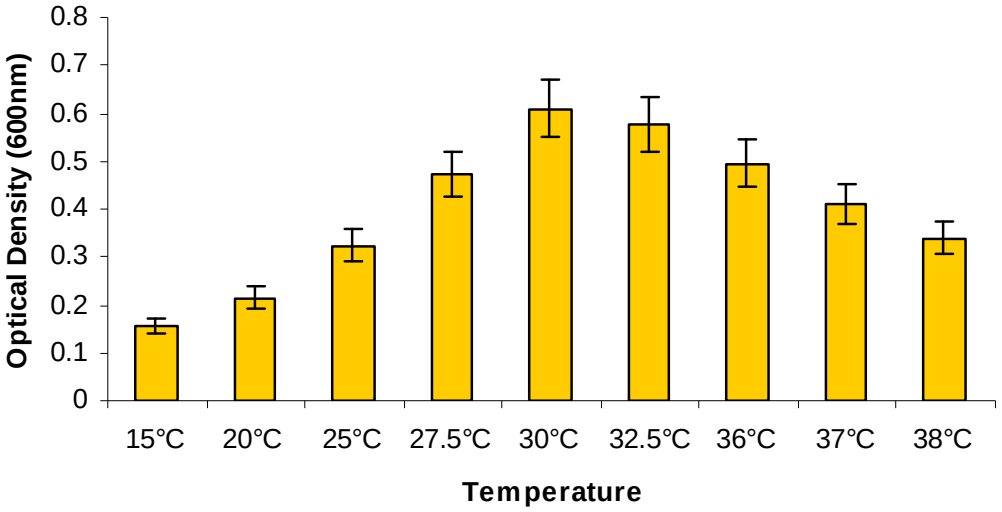

Figure 8a: Growth of isolates Chy 4-10 under different temperature ranges. ($P<0.05$)

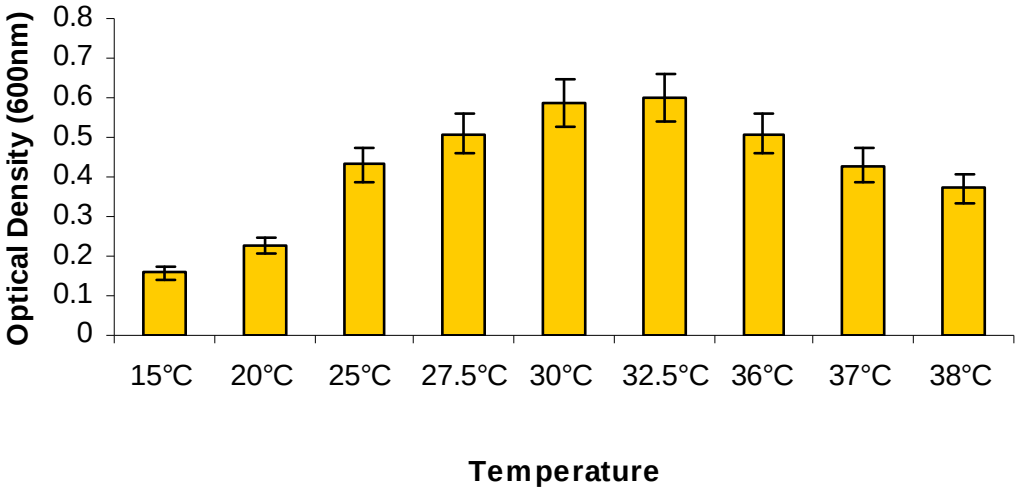

Figure 8b: Growth of isolates Chy 15-10 under different temperature ranges. ($P<0.05$)

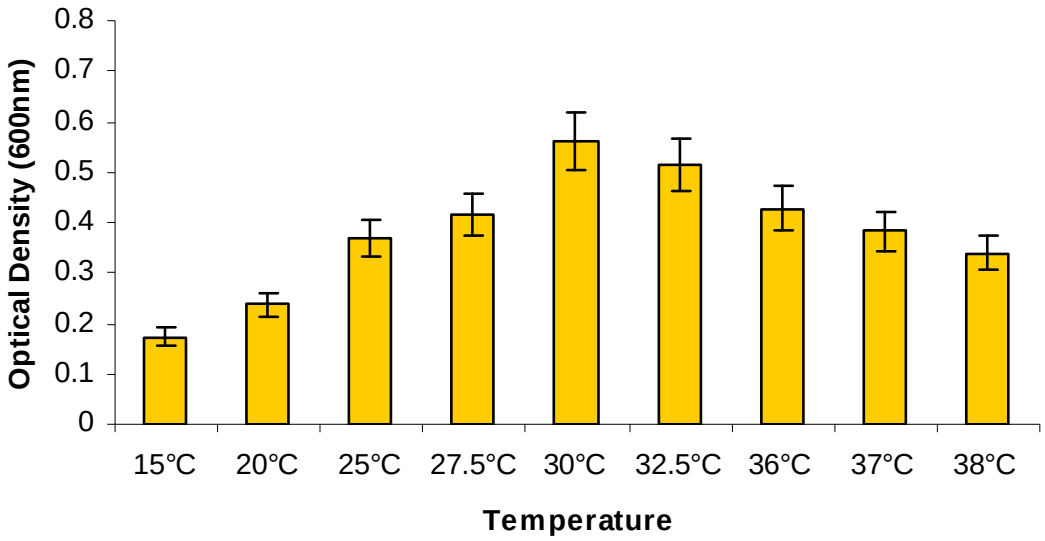

Figure 8c: Growth of isolates Chy 15-5 under different temperature ranges. (P<0.05)

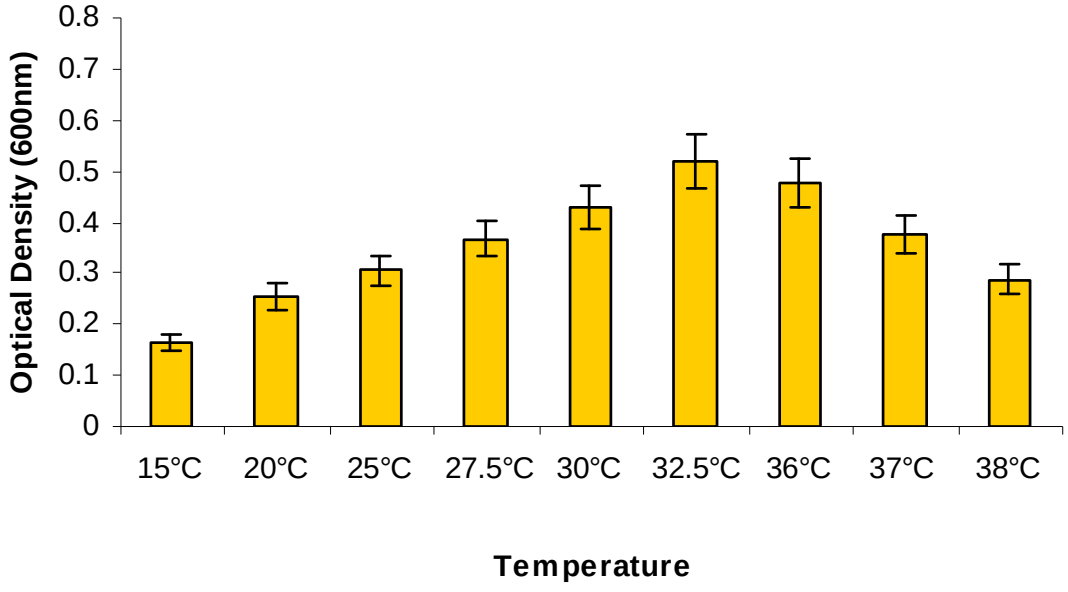

Figure 8d: Growth of isolates Chy 2-3 under different temperature ranges. (P<0.05)

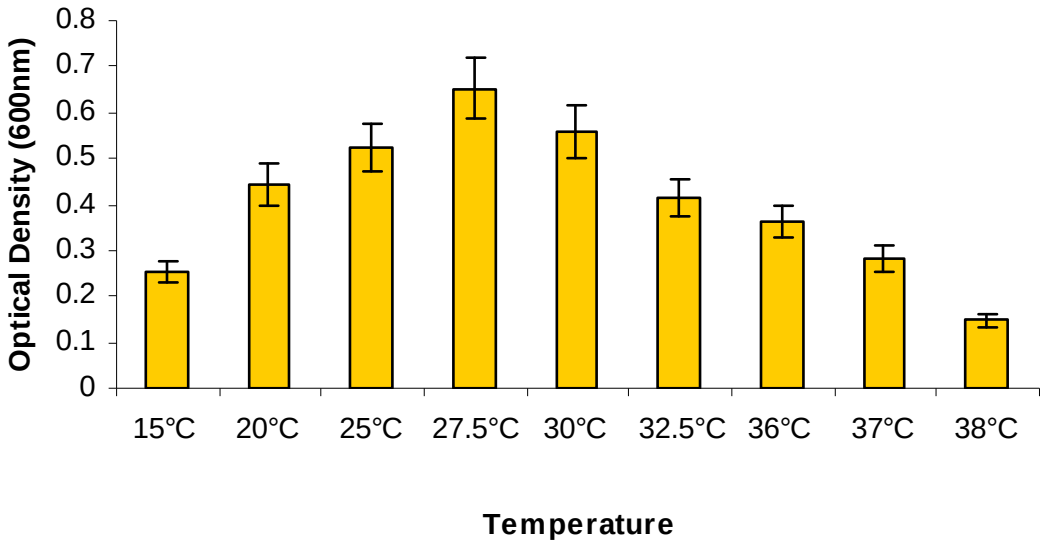

Figure 8e: Growth of isolates Ruj 7-1 under different temperature ranges. ($P<0.05$)

4.3.3 Effect of Sodium chloride concentrations on growth of the isolates.

Isolates were subjected to different sodium chloride concentrations to determine the concentration for optimum growth for each isolate. All the isolates were observed to utilize sodium chloride for growth. Optimum growth for isolate Chy 4-10 was achieved in 5 g/l (OD_{600} = 0.411 nm) whereas the lowest growth was recorded in 32.5 g/l (OD_{600} = 0.026 nm) concentration **(Figure 9a)**. 5 g/l (OD_{600} = 0.423 nm) gave the highest growth for the isolate Chy 15-10. Lowest growth on the other hand was recorded in 32.5 g/l (OD_{600} = 0.010 nm) concentration **(Figure 9b)**. Sodium chloride concentration that recorded highest growth of isolate Chy 15-5 was 10 g/l (OD_{600} = 0.169 nm) whereas 32.5 g/l (OD_{600} = 0.023 nm) was the concentration with the lowest growth of the isolate **(Figure 9c)**. For isolate Chy 2-3, optimum growth was recorded in 5 g/l (OD_{600} = 0.388 nm) whereas lowest

growth was at 32.5 g/l (OD_{600} = 0.009 nm) concentration **(Figure 9d)**. The optimum growth of isolate Ruj 7-1 was recorded in 17.5 g/l (OD_{600} = 0.584 nm) sodium chloride concentration **(Figure 9e)**.

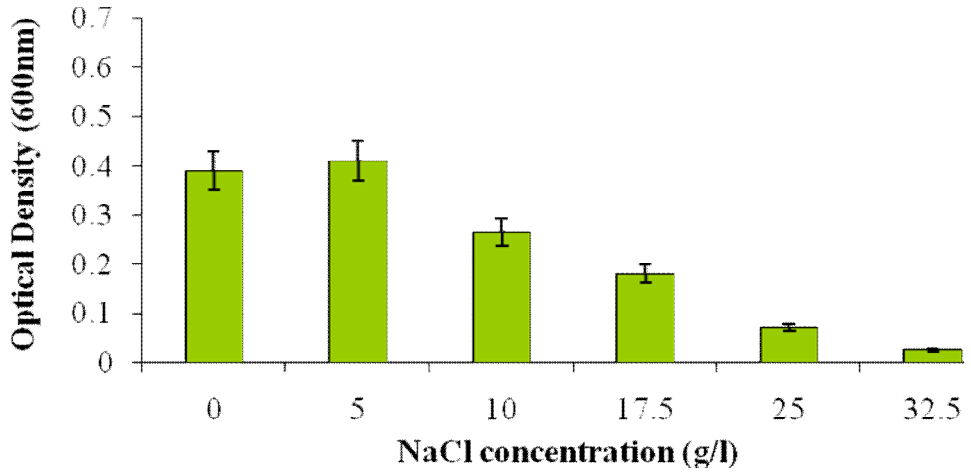

Figure 9a: Growth of isolate Chy 4-10 under different sodium chloride concentrations ($P<0.05$).

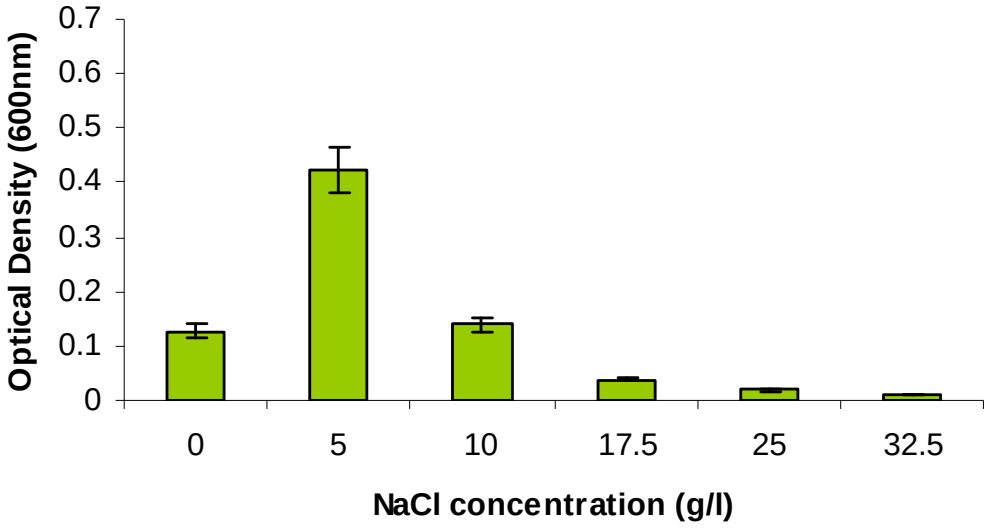

Figure 9b: Growth of isolate Chy 15-10 under different sodium chloride concentrations ($P<0.05$).

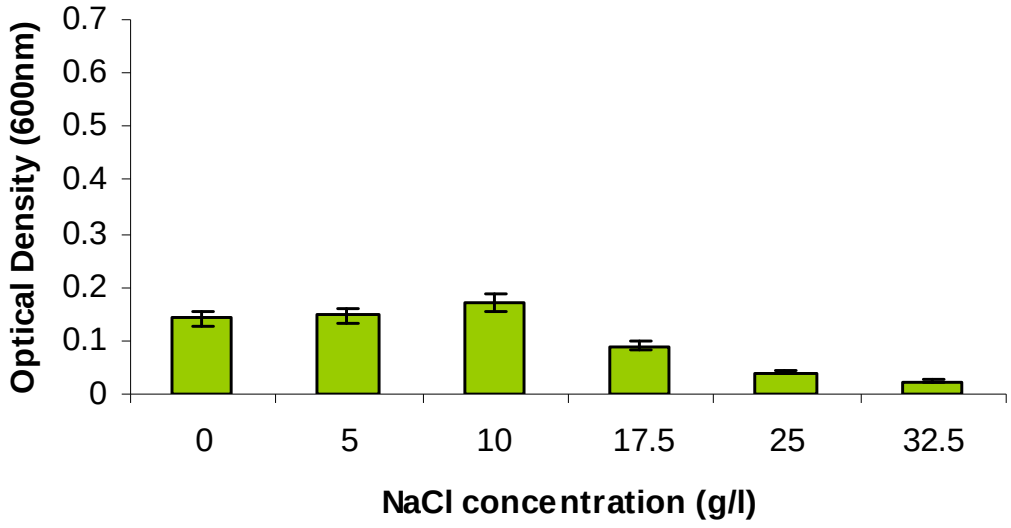

Figure 9c: Growth of isolate Chy 15-5 under different sodium chloride concentrations ($P<0.05$).

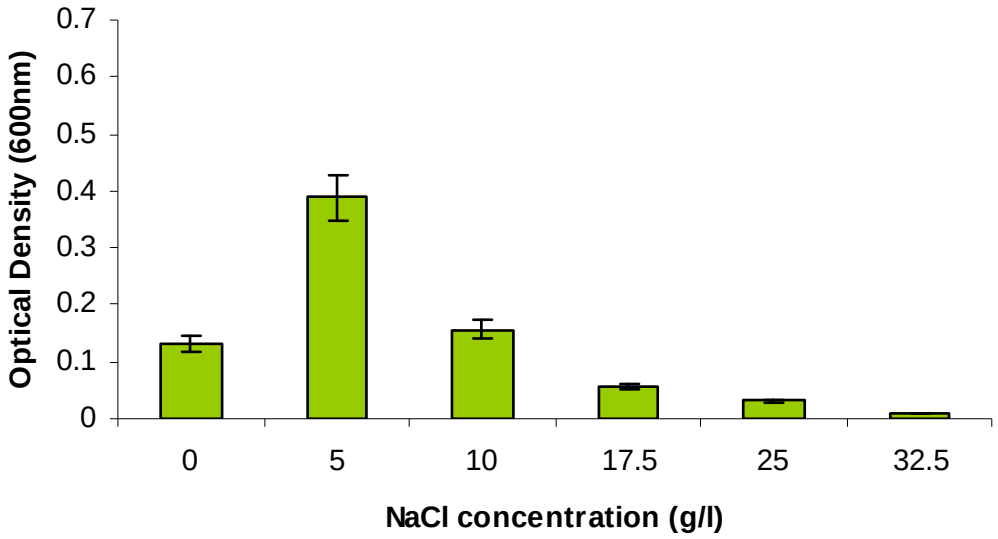

Figure 9d: Growth of isolate Chy 2-3 under different sodium chloride concentrations (P<0.05).

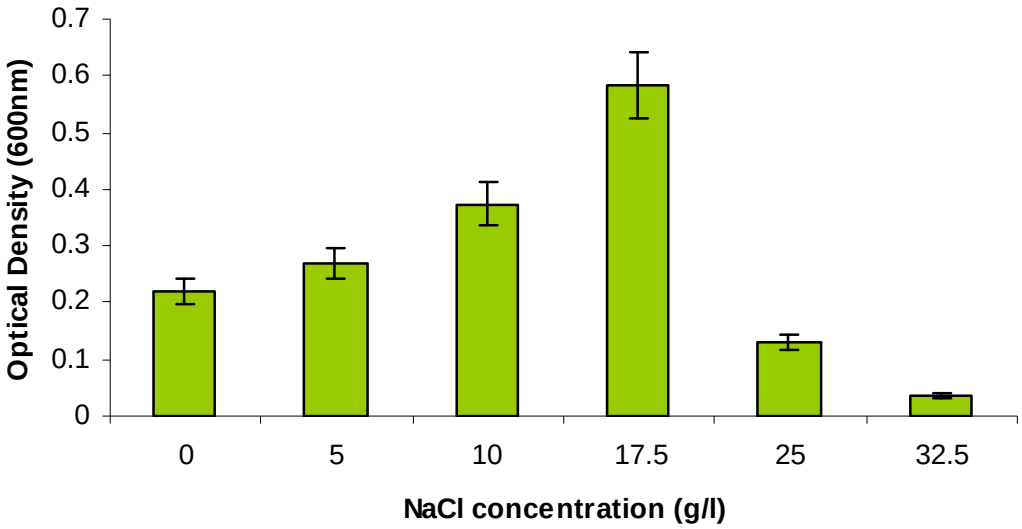

Figure 9e: Growth of isolate Ruj 7-1 under different sodium chloride concentrations (P<0.05).

4.4 Determination of the enzymatic activity

All the studied isolates hydrolyzed lipids, skim milk and starch apart from isolate Chy 4-10 that did not hydrolyze Tween 80 **(Table 6)**. A zone of crystals was recorded as positive reaction for hydrolysis of Tween 80 **(Figure 10)**.

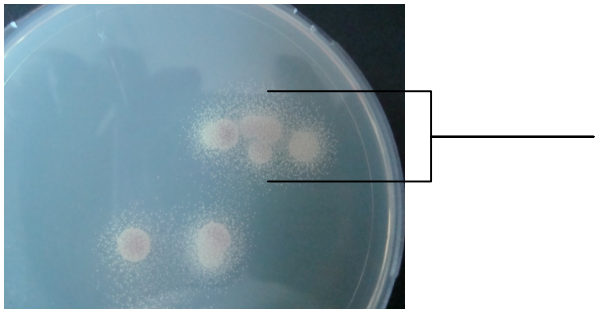

Figure 10: Hydrolysis of Tween 80 by isolate Ruj 7-1.

Table 6: Hydrolysis of substrates by the Actinobacteria isolates

Isolate	**Substrates**			
	Starch	**Tween20**	**Tween80**	**Skimmed milk**
Chy 4-10	+ve	+ve	-ve	+ve
Chy 15-10	+ve	+ve	+ve	+ve
Chy 15-5	+ve	+ve	+ve	+ve
Chy 2-3	+ve	+ve	+ve	+ve
Ruj 7-1	+ve	+ve	+ve	+ve

Key:

(+ve) = A positive result for the hydrolysis of the substrate.

(-ve) = A negative result for the hydrolysis of the substrate.

4.4.1 Enzymatic Activity of the isolates on the various substrates

On amylase-like activity, isolate Chy 4-10 expressed the highest EI (5.2 ± 0.100) followed by isolate Ruj 7-1 with an EI of (4.4 ± 1.500). Low EI was expressed by isolate Chy 2-3 with an EI of (3.4 ± 0.500) **(Table 7)**. EI on esterase-like activity for isolate Ruj 7-1 (5.3 ± 0.500) was the highest followed by isolate Chy 15-5 (3.8 ± 1.000). Isolate Chy 4-10 did not express EI on esterase-like activity **(Table 7)**. EI on lipase-like activity for isolates Ruj 7-1 (4.2 ± 1.500) and Chy 15-5 (4.1 ± 1.500) were higher when compared with other isolates respectively **(Table 7)**. Low EI on lipase-like activity was expressed in isolate Chy 2-3 (3.0 ± 0.500) **(Table 7)**. Isolate Chy 4-10 expressed the highest EI in terms of protease-like activity (7.3 ± 0.000) compared to other isolates **(Table 7)**.

Table 7: Enzymatic activity of the isolates on the various substrates.

Isolate	**Enzymatic Index**			
	Amylase (starch)	**Esterase (T80)**	**Lipase (T20)**	**Protease (skim milk)**
Chy 4-10	5.2 ± 1.000^{a}	0.0 ± 0.000^{d}	3.5 ± 0.000^{bc}	7.3 ± 0.000^{a}
Chy 15-10	4.0 ± 0.500^{c}	3.1 ± 0.500^{bc}	3.5 ± 0.500^{bc}	4.4 ± 0.500^{b}
Chy 15-5	3.7 ± 0.500^{cd}	3.8 ± 1.000^{b}	4.1 ± 1.500^{ab}	4.8 ± 1.000^{b}
Chy 2-3	3.4 ± 0.500^{d}	2.2 ± 0.500^{c}	3.0 ± 0.500^{c}	2.9 ± 0.500^{c}
Ruj 7-1	4.4 ± 1.500^{b}	5.3 ± 0.500^{a}	4.2 ± 1.500^{a}	4.8 ± 0.500^{b}

*The enzymatic index is represented by a ratio of degradation halo in diameter against diameter of colony in cm.

*Averages followed by the same alphabetical letter don't differ among themselves ($\rho < 0.05$).

4.4.2 Determination of enzymatic activities of crude extra-cellular proteins

Enzymatic activities of crude extra-cellular proteins from the isolates were determined using various substrates. Amylase-like activity was recorded highest in isolate Chy 4-10 (27.33 ± 0.33) and lowest in Chy 2-3 (11.00 ± 0.33). Isolates Ruj 7-1 (17.33 ± 0.88) and Chy 4-10 (0.00 ± 0.00) presented highest and lowest esterase-like activity respectively whereas isolate Ruj 7-1 (23.00 ± 0.58) recorded the highest lipolytic activity **(Figure 11)** compared to other isolates. Proteolytic activity was recorded highest in isolate Chy 4-10 (21.33 ± 2.4) **(Table 8)**.

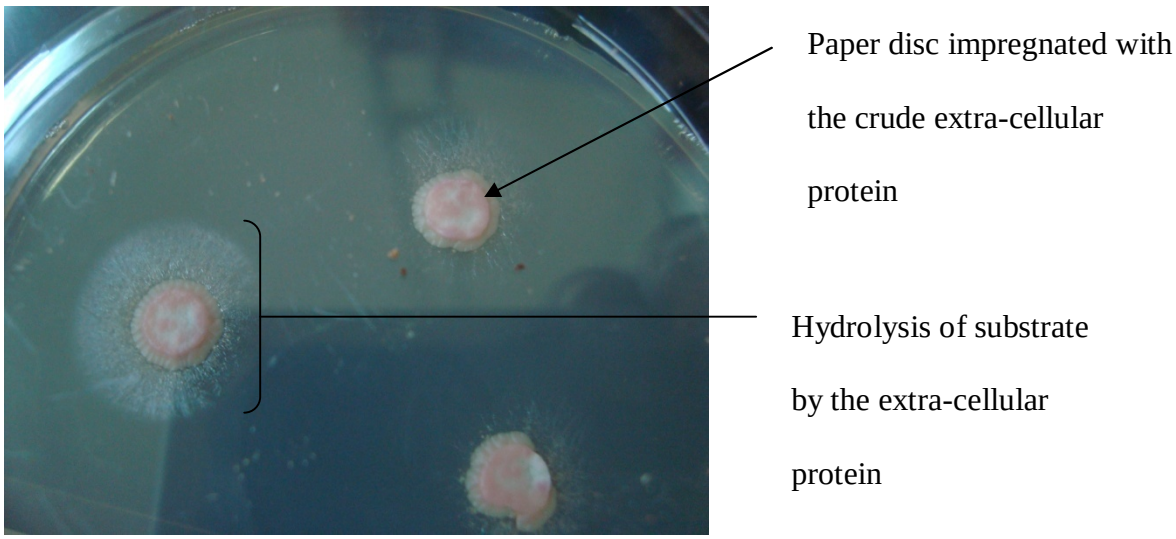

Figure 11: Hydrolysis of Tween 20 by a crude extra-cellular protein from isolate Chy 4-10.

Table 8: Enzymatic activities of crude extra-cellular proteins

Isolate	Enzymatic activities (Diameter in mm + SE)			
	Amylase	**Esterase**	**Lipase**	**Protease**
Chy 4-10	27.33 ± 0.33^{a}	00.00 ± 0.00^{d}	17.33 ± 0.58^{b}	21.33 ± 2.40^{a}
Chy 15-10	21.67 ± 0.58^{c}	11.33 ± 0.33^{b}	13.67 ± 0.33^{c}	17.67 ± 1.20^{b}
Chy 15-5	20.67 ± 0.33^{c}	16.33 ± 0.33^{a}	19.00 ± 0.67^{b}	15.33 ± 0.88^{c}
Chy 2-3	11.00 ± 0.33^{d}	8.33 ± 0.58^{c}	10.33 ± 0.58^{d}	14.33 ± 0.88^{d}
Ruj 7-1	24.00 ± 0.58^{b}	17.33 ± 0.88^{a}	23.00 ± 0.58^{a}	20.33 ± 1.45^{a}

* Mean values with the same alphabetical letter are not significantly different at 95 % confidence level (t- test).

4.5: Extraction and activity of secondary metabolites

Production of secondary metabolites by the isolates was observed in differential broth cultures by a change in colour of the media from colorless to; red (Chy 4-10), dark brown (Chy 15-10), light brown (Chy 15-5), Orange (Chy 2-3) and black (Ruj 7-1) **(Figure 12a – 12e)**.

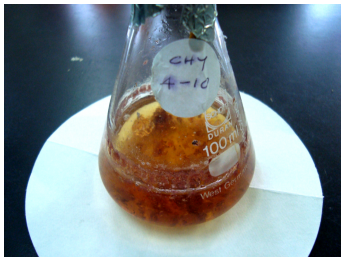

Figure 12a: Chy 4-10.

Figure 12b: Chy 15-10.

Figure 12c: Chy 15-5.

Figure 12d: Chy 2-3.

Figure 12e: Ruj 7-1.

The isolates were tested for inhibition of growth on test organisms *S. aureus* (NCTC 10788) and *E. coli* (NCTC 10418) **(Table 9)**. Isolates Chy 4-10, Chy 15-5 and Ruj 7-1 were found to suppress the growth of the two test bacteria while isolates Chy 15-10 and Chy 2-3 only suppressed growth of *S. aureus* (NCTC 10788) **(Table 9)**. Isolate Chy 4-10 (24 mm) and Chy 2-3 (20 mm) showed stronger inhibition on *S. aureus* (NCTC 10788) while isolates Chy 15-5 (18 mm) and Ruj 7-1 (15 mm) showed stronger inhibition on *E. coli* (NCTC 10418) **(Table 9)**. Isolates Chy 4-10, Chy 15-5 and Ruj 7-1 showed strong inhibition on both test organisms hence broad-spectrum activity **(Table 9)**. This showed the potential of the isolates to produce antibiotics. Yields of the secondary metabolites produced were determined as shown in **(Table 9)**. Isolate Chy 15-10 produced the highest yield (0.62 g/l) whereas Ruj 7-1 produced the least (0.14 g/l).

Table 9: Bio-assays of secondary metabolites crude extracts from the *Streptomyces* isolates on *S. aureus* (NCTC 10788) and *E. coli* (NCTC 10418). The positive control consisted of commercial Kanamycin antibiotic (1 mg/ml) while negative control consisted of un-inoculated plate.

Isolate	Activity		Disc assay (diameter in mm)		Yield (g/l)
	***S. aureus* (NCTC 10788)**	***E. coli* (NCTC 10418)**	***S. aureus* (NCTC 10788)**	***E. coli* (NCTC 10418)**	
Chy 4-10	+	+	24	9	0.54
Chy 15-10	+	-	13	-	0.62
Chy 15-5	+	+	15	18	0.41
Chy 2-3	+	-	20	-	0.3
Ruj 7-1	+	+	19	15	0.14
+ve control	+	+	15	19	
-ve control	-	-	0	0	

Key:

(+) = Inhibition

(-) = No inhibition.

4.5.1: GC - MS analysis of crude extracts from the Actinobacteria isolates.

Mass spectra and retention time analysis were used to determine the chemical compounds present in the respective isolate metabolites. Among the compounds identified comprised of amides, amines, acids, pyrrolizidines, quinones, alcohols and hydrocarbons. Amides identified from isolate Chy 4-10 were: Propanamide, N, N-dimethyl- (17.667 min), Acetamide,2-chloro-N-(2-ethyl-6-methylphenyl)-N-(2-methoxy-1-methylethyl)- (23.715

min) and Formamide, N-(2,4-diamino-1,6-dihydro-6-oxo-5-pyrimidinyl)- (29.000 min). Amines comprised of: Pyrimidine, 2-methoxy-5-methyl- (18.698 min), Pyridine, 2-methoxy-5-nitro- (22.057 min), Pyrimidine-2(1H)-thione, 4,4,6-trimethyl-1-(1-phenylethyl)- (29.023 min) and l-Phenylalanine, N-(2,6-difluorobenzoyl)-, methyl ester (29.291 min). Acids: Benzenepropanoic acid (16.391min), Sulfurous acid, 2-ethylhexyl hexyl ester (17.063 min), 2-Hexenoic acid, 5-hydroxy-3,4,4-trimethyl-, (E)- (21.408 min), 1,2-Benzenedicarboxylic acid, butyl 2-methylpropyl ester (23.468 min), n-Hexadecanoic acid (23.468 min), Phosphonic acid, bis(1-methylethyl) ester (24.028 min), Octadecanoic acid (25.327 min) and 1-Phenanthrenecarboxylic acid, 7-ethenyl-1,2,3,4,4a,4b,5,6,7,8,10,10a-dodecahydro-1,4a,7-trimethyl-, methyl ester, [1R-(1.alpha.,4a. (27.813 min). Pyrrolizidines: Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro- (21.587 min) and Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)- (26.940 min). Quinone: p-Benzoquinone, 2-hydroxy-5-(methylthio)- (25.932 min). Alcohols: Propenylguaethol (17.175 min), Phenol, 3-methoxy- (17.555 min) and Phenol, 3,5-dimethoxy- (22.416 min). Hydrocarbons: Tridecane, 1-iodo- (20.669 min), Tridecane, 5-propyl- (20.736 min), 7-Acetyl-1,7-diazabicyclo[2.2.0]heptane (21.206 min), Heneicosane (21.744 min), Nonadecane (22.774 min), 1,4-Dioxaspiro[4.5]decane, 6-methylene- (23.222 min), Heptadecane (24.678 min), Hexacosane (28.821 min), Triacontane (29.560 min), Nonacosane (31.240 min), and Tetracosane (33.592 min). Other chemical compounds identified were: Caprolactam (15.248 min) and Squalene (30.658 min) **(Figure 13a)**.

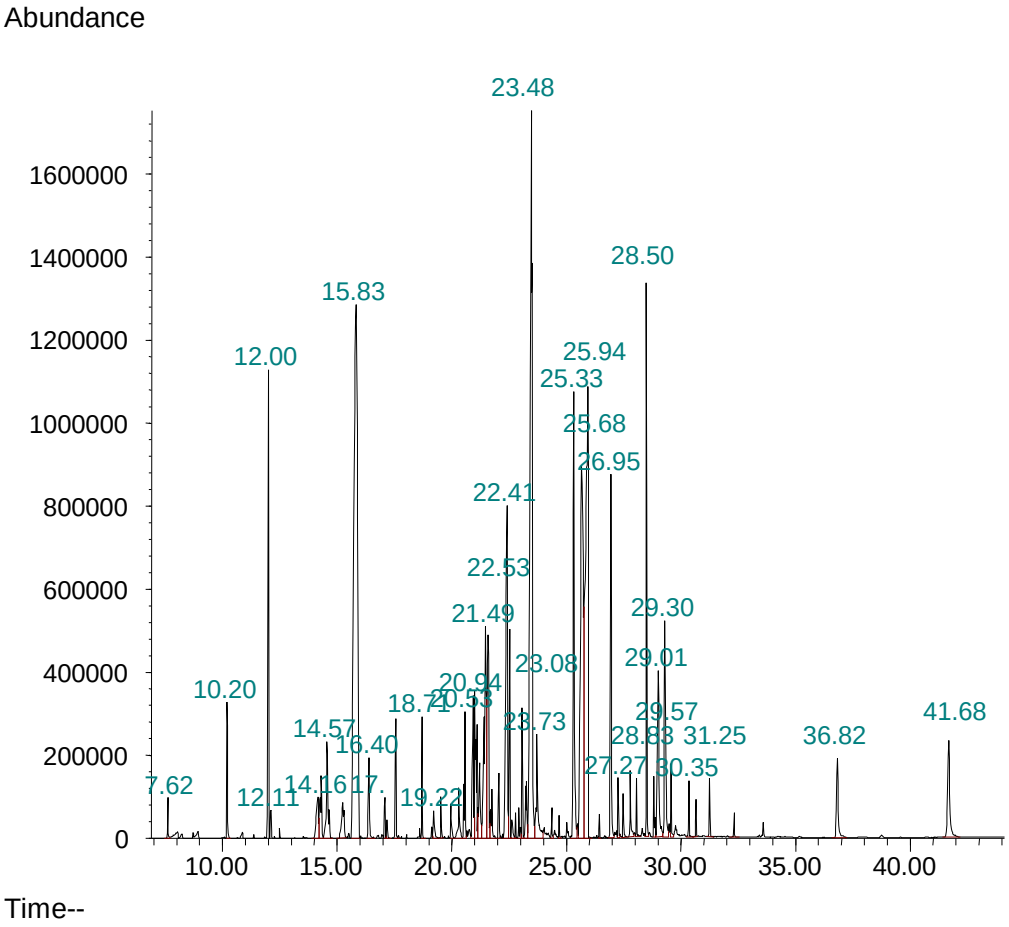

Figure 13a: GC - MS chromatogram for isolate Chy 4–10.

Chemical compounds identified from isolate Ruj 7–1 included: Amide: Acetamide, 2-chloro-N-(2-ethyl-6-methylphenyl)-N-(2-methoxy-1-methylethyl)- (23.715 min). Alcohols: 1, 4-Dioxane-2, 6-dimethanol (15.584 min), 1-Tridecanol (22.707 min) and Phenol, 4, 4'-(1-methylethylidene) bis [2, 6-dichloro- (29.359 min). Acids: Dodecanoic acid (19.101 min), Tetradecanoic acid (21.340 min), Diethyldithiophosphinic acid (22.236 min), Octadecanoic acid (25.260 min), 1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester (28.485 min) and Oxalic acid, monoamide, monohydrazide, N-(2,5-dimethylphenyl)-N2-(4-methylbenzylideno)- (29.919 min). Hydrocarbons: Hexadecane (19.526 min), Heptadecane (27.253 min), Octadecane (28.060 min), Hexacosane (28.821 min), Heptacosane (29.560 min), Octadecane, 1-iodo- (30.031 min), Octacosane (30.882 min), Tetracosane (31.240 min), Nonacosane (31.890 min) and Triacontane (32.315 min) **(Figure 13b)**.

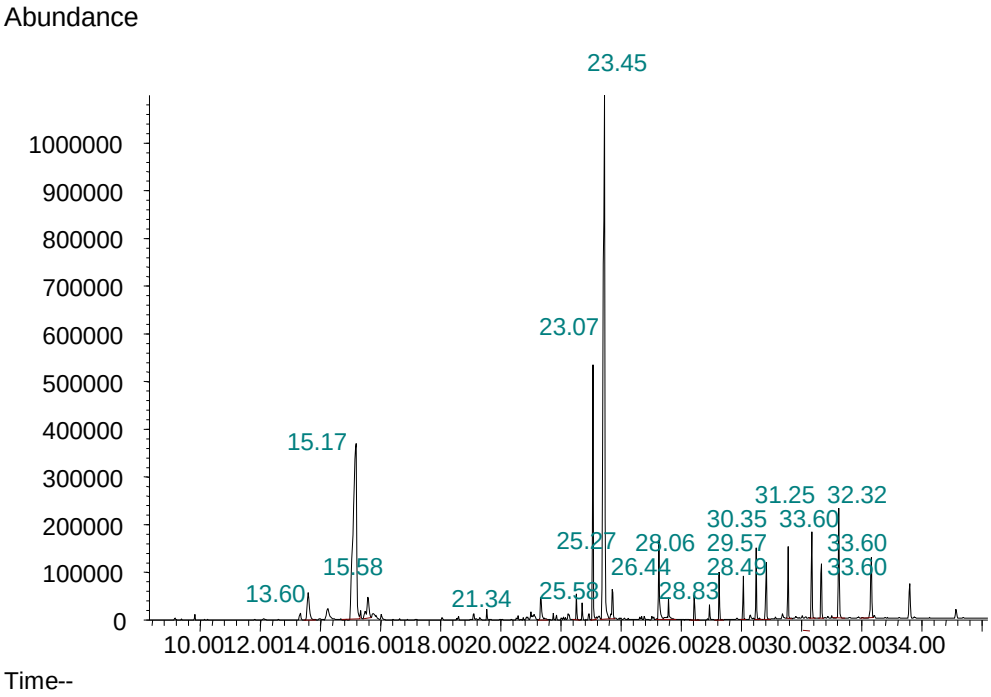

Figure 13b: GC - MS chromatogram for isolate Ruj 7-1.

Chemical compounds identified from isolate Chy 2-3 included: Amides: Propanamide, 2-methyl- (9.694 min), N-Methoxymethyl-N-methylformamide (11.015 min), L-Prolinamide (21.072 min), Acetamide, 2-chloro-N-(2-ethyl-6-methylphenyl)-N-(2-methoxy-1-methylethyl)- (23.737 min) and Formamide, N-(2,4-diamino-1,6-dihydro-6-oxo-5-pyrimidinyl)- 29.157 min). Amines: 2-Ethylpiperidine (12.830 min) and Pyrimidine-2(1H)-thione, 4,4,6-trimethyl-1-(1-phenylethyl)- (29.381 min). Acids: Acetic acid, 2-methylpropyl ester (5.416 min), 2-Propenoic acid, 2-methyl- (8.709 min), Hexanoic acid, 2-methyl- (9.044 min), Pentanoic acid (9.515 min), Benzeneacetic acid (15.450 min), Benzenepropanoic acid (16.480 min), 5-Oxohexanethioic acid, S-t-butyl ester (17.802

min), Dodecanoic acid (19.146 min), n-Hexadecanoic acid (23.535 min), Octadecanoic acid (25.327 min), p- Fluorophenoxyacetic acid (25.574 min), 1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester (28.508 min) and Octadecanoic acid, ethenyl ester (41.700 min). Alcohols: 1,2-Ethanediol, monoacetate (7.678 min), p-Dioxane-2,5-dimethanol (16.032 min), Cyclohexanol, 4-methoxy- (18.272 min) and Phenol, 3,5-dimethoxy- (22.528 min). Ketones: 2-Pyrrolidinone (12.449 min), 2,5-Piperazinedione, 3,6-bis(2-methylpropyl)- (25.910 min) and 16-Hentriacontanone (36.795 min). Hydrocarbons: Cyclohexane (13.681 min), Tetradecane (17.063 min), Hexadecane (19.526 min), Hexacosane (28.821 min) and Tetracosane (30.344 min). Pyrrolizidines: Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro- (21.766 min) and Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl (26.985 min). Caprolactam was also detected at (15.808 min) retention time **(Figure 13c)**.

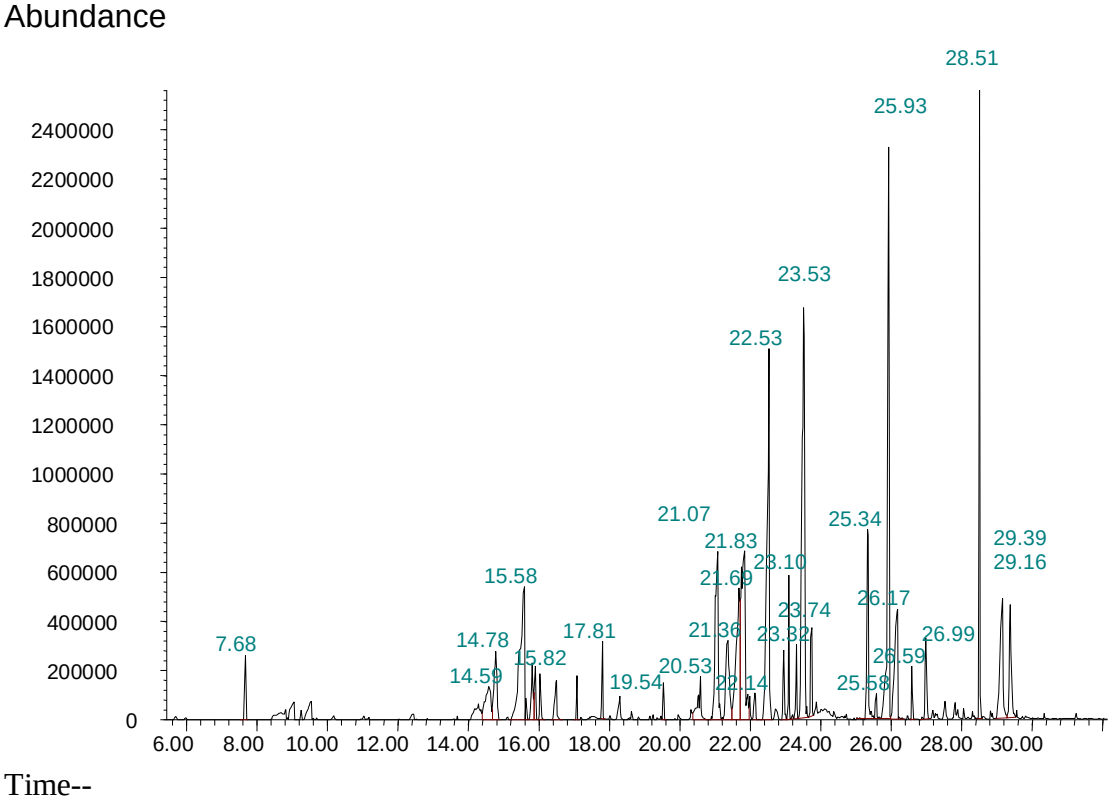

Figure 13c: GC-MS chromatogram for isolate Chy 2–3.

Secondary metabolites from Chy 15–5 analyzed had the following chemical compounds, Amide: Acetamide, 2-chloro-N-(2-ethyl-6-methylphenyl)-N-(2-methoxy-1-methylethyl)- (23.715 min). Acids: Hexanoic acid (10.814 min), Heptanoic acid (12.426 min), Cyclohexanecarboxylic acid (13.165 min), Benzenecarboxylic acid (14.263 min), Nonanoic acid (15.428 min), n-Decanoic acid (16.659 min), Dodecanoic acid (19.123 min), Tetradecanoic acid (21.363 min), Pentadecanoic acid (22.393 min), n-Hexadecanoic acid (23.558 min), 9-Octadecenoic acid, (E)- (25.081 min), Octadecanoic acid (25.372 min), 1-Phenanthrenecarboxylic acid, 1,2,3,4,4a,9,10,10a-octahydro-1,4a-dimethyl-7-(1-methylethyl)-,[1R-(1.alpha.,4a.beta.,10a.alpha.)]- (27.791 min), Hexadecanoic acid, 4-

nitrophenyl ester (36.817 min) and Octadecanoic acid, ethenyl ester (41.744 min). Ketone: 2-Pentadecanone, 6, 10, 14-trimethyl- (22.236 min). Hydrocarbons: Tetradecane (17.085 min), Pentadecane (18.339 min), Hexadecane (19.526 min), Pentadecane, 2,6,10,14-tetramethyl- (20.736 min), Hexadecane, 2,6,10,14-tetramethyl- (21.856 min), Nonadecane (22.774 min), Heneicosane (24.700 min), Docosane (25.596 min), Octadecane (26.447 min), Eicosane (28.821 min), Tricosane (29.583 min), Squalene (30.658 min), Nonacosane (31.262 min), Tetracosane (32.315 min) and Octacosane (33.592 min) **(Figure 13d)**.

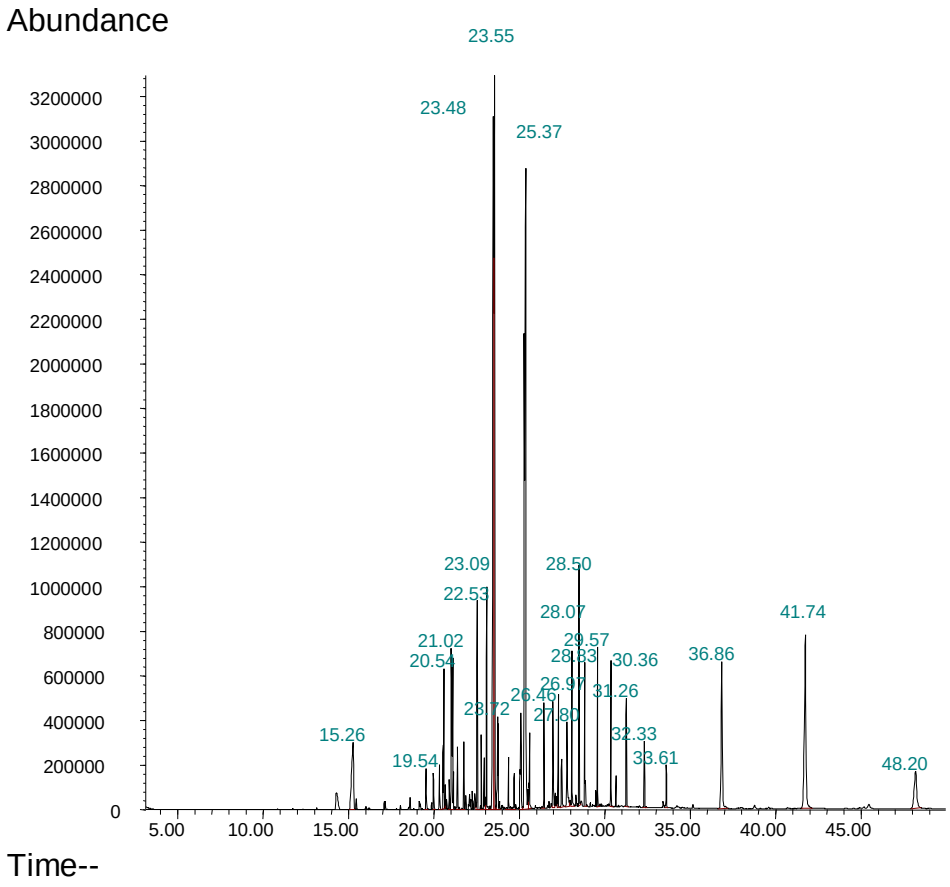

Figure 13d: GC - MS chromatogram for isolate Chy 15-5.

Isolate CHY 15-10 produced secondary metabolites with the following chemical compounds, Amides: L-Prolinamide (20.982 min) and N-(4-Methoxyphenyl)-2-hydroxyimino-acetamide (23.289 min). Amines: Pyrimidine-2(1H)-thione, 4,4,6-trimethyl-1-(1-phenylethyl)- (29.336 min). Acids: Butanoic acid, 3-methyl- (8.395 min), Butanoic acid, 2-methyl- (8.619 min), Benzenecarboxylic acid (14.420 min), Benzeneacetic acid (15.338 min), Benzenepropanoic acid (16.413 min), Dodecanoic acid (19.146 min), n-

Hexadecanoic acid (23.535 min), Heptadecanoic acid 924.364 min), 9-Octadecenoic acid, (E)- (25.081 min), Octadecanoic acid (25.350 min), 1,2- Benzenedicarboxylic acid, mono(2-ethylhexyl) ester (28.463 min) and Hexadecanedioic acid (29.717 min). Hydrocarbons: Tetradecane (17.085), Hexadecane (19.526 min), Nonadecane (22.774 min), Tridecane (23.759 min), Heneicosane (26.447 min), Tetracosane (27.276 min), Pentacosane (28.060 min), Octacosane (29.538 min), Triacontane (30.344 min), Squalene (30.658 min), Nonacosane (31.240 min), Heptadecane, 9-octyl- (32.024 min) and Heptadecane (32.315 min). Alcohols: 1,4-Dioxane-2,6-dimethanol (15.719 min) and Phenol, 3,5-dimethoxy- (22.460 min). Pyrrolizidines: Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro- (21.744 min) **(Figure 13e)**.

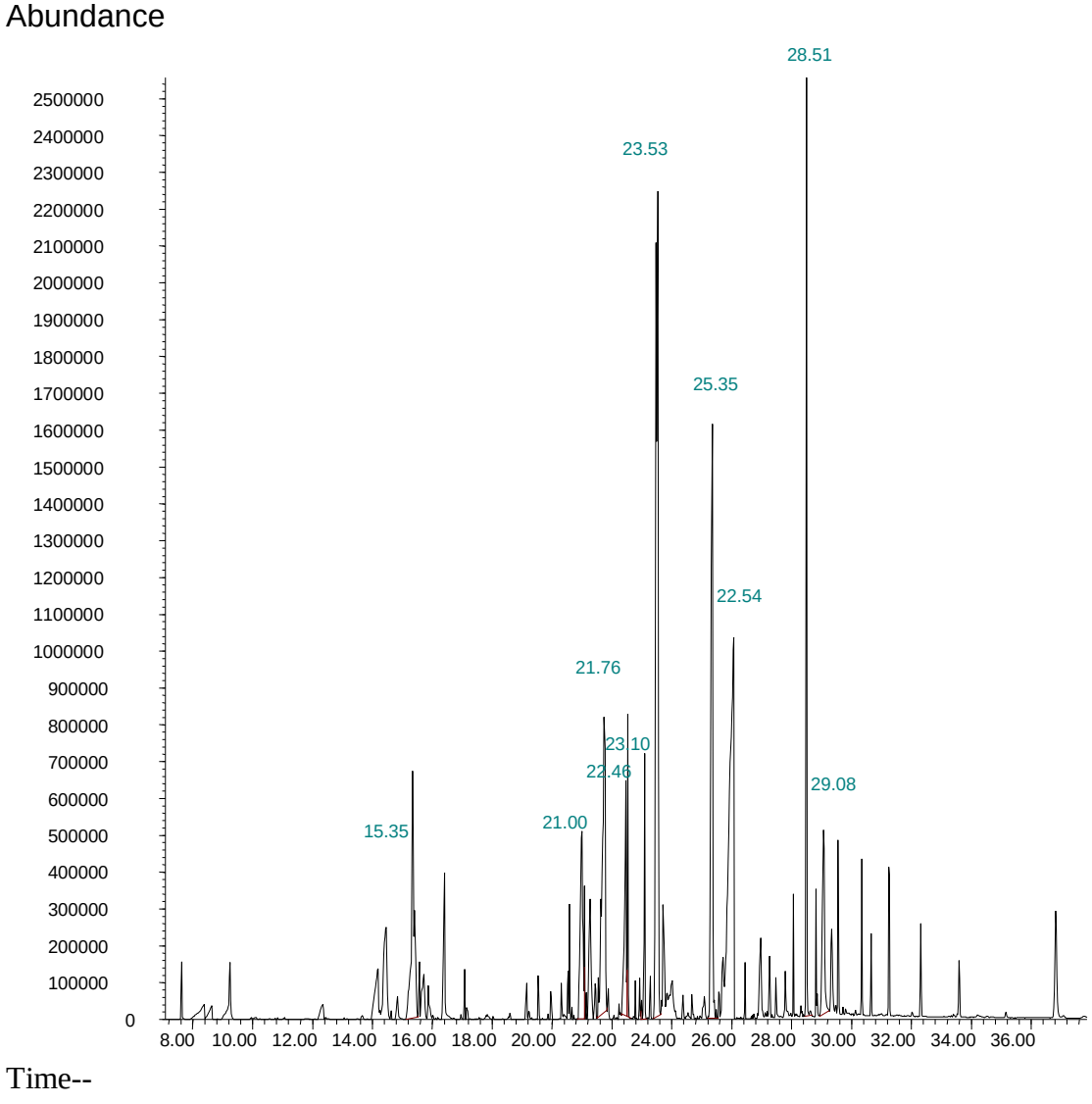

Figure 13e: GC - MS chromatogram for isolate Chy 15-10.

5.0 CHAPTER FIVE: DISCUSSION

The aim of this study was to determine the physiochemical characteristics of *Streptomyces* isolates and screening for production of enzymes and secondary metabolites that have got industrial applications.

The isolates had been isolated from two Kenyan National Parks namely Chyulu and Ruma National parks. Differential media was used to revive the isolates. The medium had fermentable carbohydrate, nitrogen, organic and inorganic compounds. Morphological studies were carried out and characteristics of the isolates compared with the standard characteristics described in Bergy's manual. All the isolates were Gram positive as confirmed in a previous study carried out by Nonoh *et al*. (2010). They formed colored, tough and leathery colonies that were hard to pick from the culture media. Microscopic studies also showed that the isolates cells formed long branched network of mycelia a characteristic of *Streptomyces sp* as previously described by Kieser *et al.* (2000).

Physiochemical studies were carried out since most enzymes applied for industrial purposes have limitations, in exhibiting low activity and low stability at wide range of pH and temperature and secondly 30 - 40 % of production cost of industrial enzymes is estimated to be accounting for the cost of growth medium (Giarrhizzo *et al*., 2007). The study was therefore to optimize cultural conditions in order to achieve higher enzyme activities. Physiochemical characterization of the isolates Chy 4-10, Chy 15-10, Chy 15-5 and Chy 2-3 on pH showed optimal growth of the isolates at pH range of 6 - 9. These results were in accordance to Gava (1998) who reported that majority of Actinomycetes

isolated from rhizosphere and non-rhizosphere soil grows at a pH range varying from 6.5 to 8.0. In addition, the wide pH range is an advantage when it comes to production of enzymes adapted to alkaline conditions in order to have good enzymatic stability. Isolate Ruj 7-1 yielded good growth, characterized by abundant mycelium, in culture media with pH 6.0 suggesting its tolerance to acidic condition. This isolate would therefore be useful in production of acid tolerance enzymes. The optimum pH level allows for optimal metabolic reactions characterized by enzymes hence the increase in growth of microorganisms (Moreira & Siqueira, 2002). Isolates Chy 4-10, Chy 15-10, Chy 15-5, Chy 2-3 and Ruj 7-1 showed optimum growth at 30 ℃, 32.5 ℃, 30 ℃, 32.5 ℃ and 27.5 ℃ respectively. Minimal growth was recorded below 27.5 ℃ and above 32.5 ℃. Isolates Chy 15-10 and Chy 2-3 had a wider temperature range (15 ℃ – 32.5 ℃) hence they would produce enzymes that are more stable when temperatures exceed 27.5 ℃. These results also confirmed that isolates Chy 4-10, Chy 15-10, Chy 15-5 and Chy 2-3 originated from a relatively warmer ecosystem than isolate Ruj 7-1 (27.5 ℃). Therefore, for isolation of enzymes from these isolates, temperature during fermentation would be different. According to Goodfellow *et al.* (1990), bacterial growth rates increase with temperature up to the optimum temperature, at which the growth rate is maximal. Enzymatic processes are thought to limit further increases in growth rates at temperatures above the optimum temperature (Goodfellow *et al.*, 1990).

Growth of the isolates in culture medium with varying NaCl levels (0 g/l to 32.5 g/l) confirmed tolerance to saline conditions. All the isolates recorded growth in absence of

sodium chloride but isolate Chy 4-10, Chy 15-10 and Chy 2-3 indicated an increase in growth at 5 g/l sodium chloride concentration which was similar to that of *Nocardiopsis kunsanensis* sp. nov., a moderately halophilic actinomycete (Chun *et al.,* 2000). Isolate Chy 15-5 and Ruj 7-1 grew optimally at higher NaCl concentrations (10 g/l and 17.5 g/l) respectively meaning they were more tolerant and also required higher NaCl concentrations for them to grow. In terms of enzyme stability, isolate Ruj 7-1 would likely produce enzymes that are more stable to a wide range of NaCl concentrations compared to the other isolates.

Most microorganisms obtain their energy through a series of orderly and integrated enzymatic reactions leading to the biooxidation of a substrate that is frequently a carbohydrate. The organisms use the carbohydrate differently depending on their enzyme complement. Some organisms ferment sugars such as glucose anaerobically, while others use the aerobic pathway (Williams *et al*., 1989). Glucose was the best carbon source for growth of isolates Chy 4-10 (0.601 nm), Chy 15-5 (0.719 nm) and Chy 2-3 (0.475 nm). This meant that glucose would give optimum growth during fermentation processes than the other sugars. Sucrose also indicated a considerable growth of the isolate Chy 4-10 (0.473 nm) meaning it could act as an alternative source of carbon for this isolate. Mannose would also be an alternative source of carbon to isolate Chy 15-5 (0.250 nm) besides glucose. Xylose was the preferred carbon source for isolates Chy 15-10 (0.899 nm) and Ruj 7-1 (0.900 nm) hence it would be the ideal carbon source during fermentation processes of isolates. All the isolates hydrolyzed starch a characteristic that confirm their

role in the decomposition of organic matter in the habitats (Crawford, 1988; Kieser *et al*., 2000). Degradation of hydrogen peroxide by producing an enzyme catalase was positive in all isolates. Methyl red test was negative for Chy 15-10 but positive for the other isolates indicating ability of the isolates to oxidize glucose with production and stabilization of high concentrations of acid end products (Harold's, 2002). There was no production of tryptophanase enzyme and hence the negative indole production results in all the isolates. However, the ability to produce hydrogen sulfide from substrates such as sulfur containing amino acids and organic sulfur was confirmed positively in isolates Chy 15-10, Chy 4-10, Chy 15-5 and Chy 2-3. Reduction of nitrates to nitrites was only positive in isolates Chy 2-3 and Ruj 7-1. Degradation of urea through enzyme urease was confirmed positive in isolates Chy 4-10, Chy 15-5 and Ruj 7-1.

Streptomyces are an important source of enzymes and bioactive products (Bull *et al*., 1992). Most produce secondary metabolites that have antibacterial, anti-fungal, anti-tumor or antiprotozoal activities making them a target for isolation in large-scale screening programs in industries.

Growth of the isolates on solid media containing the various substrates as the only carbon sources demonstrated that these isolates secreted enzymes. All the studied isolates hydrolyzed lipids, skim milk and starch. Tween 80 was hydrolyzed by all the other isolates apart from isolate Chy 4-10. Isolate Chy 4-10 recorded highest degradation of starch (27.33 ± 0.33^{a}) and skim milk (21.33 ± 2.4^{a}) whereas Ruj 7-1 had the highest degradation of Tween 20 (23.00 ± 0.58^{a}) and Tween 80 (17.33 ± 0.88^{a}). Hydrolysis of the various

substrates was an indication of the ability in the various isolates to produce lipases, proteases, amylases and esterases that are industrially important enzymes. These enzymes also play an important role in promotion of plant growth and biological control of plant diseases (Moreira & Siqueira, 2002). Starch is the most important organic reserve compound of plants and among the good starch decomposers are the Actinomycetes which produce organic acids, CO_2, and dextrin during the decomposition process (Moreira & Siqueira, 2002).

Enzymatic index (EI) was determined by directly correlating the diameters of the halo of degradation and that of colony (Lin *et al.*, 1991). The EI is a practical tool that facilitates and speeds the selection and the comparison of the enzymatic production of different isolates. Fungaro and Maccheroni (2002) suggested that EI larger than 1.0 were indicative of excretion of enzymes. It was observed that all the isolates possessed at least one enzymatic activity tested **(Table 7)**.

The amylolytic activity was observed in all isolates with Chy 4-10 showing the highest EI of (5.2 ± 1.000). Isolate Chy 4-10 had a higher EI when compared with *Actinomyces pyogenes* that had an EI of 1.2 cm in a previous study carried out by Aysha *et al.* (2006).

Among the producers of esterases, four isolates showed positive results apart from isolate Chy 4-10. Isolate Ruj 7-1 had the highest EI of (5.3 ± 0.500). This was an indication of the potential this isolate had as producer of these enzymes. In this experiment, all the isolates produced extracellular lipases although EI differed among the isolates due to the differences in levels of lipase production. Isolate Ruj 7-1 gave the highest EI in lipase

activity (4.2 ± 1.500) followed by isolate Chy 15-5 (4.1 ± 1.500). This is a positive indication of the potential these isolates have in terms of lipase production.

All the isolates produced proteases though the best proteolytic production was observed in isolate Chy 4-10 (7.3 ± 0.000). These results form a platform for further studies and also revealed alternative sources of amylase, lipase, esterase and protease enzymes with applicable biotechnological potential in different areas such as in the nutrition, detergent, paper, pharmaceutical, textile and leather industries.

Actinomycetes are a prolific source of secondary metabolites. Around 23,000 bioactive secondary metabolites produced by microorganisms have been reported and over 10,000 of these compounds are produced by Actinomycetes, representing 45 % of all bioactive microbial metabolites discovered (Berdy, 2005). Among Actinomycetes, around 7,600 compounds are produced by *Streptomyces* species (Berdy, 2005). Streptomyces species are distributed widely in aquatic and terrestrial habitats Pathom-aree *et al.* (2006) and are of commercial interest due to their unique capacity to produce novel bioactive compounds. The main source for the bioactive secondary metabolites is soil streptomycetes, but a wide variety of structurally unique and biologically active secondary metabolites have recently been isolated from marine Actinomycetes, including those from the genus *Streptomyces* (Cho *et al.*, 2001; Sanchez-Lopez *et al.*, 2003; Lee *et al.*, 2005; Jensen *et al.*, 2005).

Production of secondary metabolites commonly precedes the development of aerial hyphae, when the growth rate of bacterial filaments has decreased and sporulation starts (Bibb, 2005). Much of the published data indicate that the most important environmental

signal triggering secondary metabolism is nutrient starvation, particularly that of phosphate (Sola-Landa *et al.*, 2003). The signaling networks behind the regulation of secondary metabolism in streptomycetes have recently been reviewed (Bibb, 2005).

Many of these secondary metabolites are potent antibiotics, which has made streptomycetes the primary antibiotic-producing organisms exploited by the pharmaceutical industry (Berdy, 2005).

Besides antibiotics, which present the largest group of bioactive secondary metabolites, these antimicrobial compounds show several other biological activities i.e. antagonistic agents**,** including antibacterials, antifungals, antiprotozoans as well as antivirals, pharmacological agents, including antitumorals, immunomodulators, neurological agents and enzyme inhibitors, agrobiologicals, including insecticides, pesticides and herbicides, and compounds with regulatory activities, such as growth factors, siderophores or morphogenic agents. To detect simultaneous bioactivities for a given compound, pharmacological and agricultural screens are increasingly being used in combination with antimicrobial tests. This has revealed several novel therapeutic and agrobiological agents and previously unknown biological activities for antibiotics (Berdy, 1995; Sanglier *et al.*, 1996; Berdy, 2005).

Progress has been made recently on drug discovery from Actinomycetes by using high-throughput screening and fermentation, mining genomes for cryptic pathways, and combinatorial biosynthesis to generate new secondary metabolites related to existing pharmacophores (Baltz, 2008).

In this research, five Actinobacteria isolates were studied for the production of secondary metabolites. Yields of the secondary metabolites produced were determined and isolate Chy 15-10 produced the highest yield (0.62 g/l) whereas Ruj 7-1 produced the least (0.14 g/l). The five isolates were also tested for their *in vitro* activity on type culture collection of gram-positive (*S. aureus* NCTC 10788) and gram-negative bacteria (*E. coli* NCTC 10418). All the isolates had inhibitory effects on *S. aureus* but with different levels of inhibition. Isolate Chy 4-10 showed stronger inhibition against *S. aureus* NCTC 10788 with an inhibition diameter of 24 mm followed by isolate Chy 2-3 (20 mm) whereas isolate Chy 15-10 recorded the weakest inhibition of 13 mm. The results were an indication that although all the isolates showed activity on the test microorganism, the degree of activity was varying due to the different or varying concentrations of the active ingredient. On the Gram negative *E. coli* NCTC 10418, isolates Chy 15-10 and Chy 2-3 showed no antagonistic effect on the test organism. However, isolate Chy 15-5 and Ruj 7-1 showed inhibitory effects on the test organism with zones of inhibition measuring 18 mm and 15 mm indicating higher chances of producing secondary metabolites that would counteract growth of Gram negative bacteria.

GC - MS analysis of the secondary metabolites was carried out and profiles of the fractions indicated the presence of different number of chemical compounds with different retention times and abundance. Among the compounds identified comprised of amides, amines, acids, pyrrolizidines, ketones, quinones, alcohols and hydrocarbons. Some of these compounds have been detected from Actinobacteria and documented. Quinone related

compounds with antitumor activity have been isolated from different marine Actinomycetes. *Streptomyces chinaensis* AUBN1/7 isolated from marine sediment samples of Bay of Bengal, India, is the producer of 1-hydroxy-1-norresistomycin and resistoflavin (Gorajana *et al.,* 2005). These compounds together with resistomycin and tetracenomycin D have also been produced by *Streptomyces* sp. B8005 isolated from sediments of the Laguna de Términos at the Gulf of México (Kock *et al.,* 2005). Resistomycin was isolated, in addition, from *Streptomyces* sp. B4842 from mud sediment of a coastal site of Mauritius, Indian Ocean (Kock *et al.,* 2005). 1-hydroxy-1-norresistomycin (Gorajana *et al.,* 2005) and resistoflavin (Gorajana *et al.,* 2007) showed cytotoxic activity against human gastric adenocarcinoma HMO2 and hepatic carcinoma HePG2 cell lines. Apart from the common or documented quinones obtained from *Streptomyces*, a unique group of quinone compound was detected from isolate Chy 4-10 in this study. p-Benzoquinone2-hydroxy-5-(methylthio)- from isolate Chy 4-10 was detected at 25.932 min retention time with an abundance of 9.493 %. The potential of this compound as an antitumor is still not known.

Two new cytotoxic quinones of the angucycline class, marmycins A and B have been isolated from the culture broth of *Streptomyces* strain CNH990 isolated from a sediment sample collected at a depth of 20 m at the entrance to the Sea of Cortez, 5 km east of Cabo San Lucas, México (Martin *et al.,* 2007). In cytotoxic assays using the human cell line of colon adenocarcinoma HCT-116, marmycin A showed an IC_{50} of 60.5 nM, almost 18 times more potent than marmycin B, which showed an IC_{50} of 1.09 μM. Marmycin A was further

evaluated for its *in vitro* cytotoxicity showing a mean IC_{50} value of 0.022 μM against 12 human tumor cell lines (breast, prostate, colon, lung, leukemia). In the same assays marmycin B was significantly less potent with a mean IC_{50} value of 3.5 μM (Martin *et al.*, 2007).

Streptomyces luteoverticillatum 11014 Li *et al.* (2006) isolated from underwater sediment at 20 m depth collected off the coast of Taipingjiao, Qingdao, China, is the producer of four known butenolides: (4*S*)-4,10- dihydroxy-10-methyl-undec-2-en-1,4-olide Cho *et al.* (2001), (4*S*)-4,10-dihydroxy-10-methyl-dodec-2-en- 1,4-olide (Mukku *et al.,* 2000; Cho *et al.,* 2001) and two diastereomeric (4*S*)-4,11-dihydroxy-10-methyl-dodec-2-en-1,4- olides. The four butenolides showed cytotoxic activity against human leukemia K562 with IC_{50} values of 8.73, 6.29, and 1.05 μmol/ml and murine lymphoma P388 cell lines with IC_{50} values of 0.34, 0.19, and 0.18 μmol/ml, respectively.

In this study, a butenolide was detected in isolate Ruj 7-1. 4, 8, 12, 16-Tetramethylheptadecan-4-olide was detected at 26.940 min retention time and its activity is still unknown. This butenolide could be having novel antitumoral suppression activity in its application.

A new class of 2-alkylidene-4-oxazolidinone, exhibiting an unprecedented antibiotic pharmacophore, was isolated from a marine actinomycete (NPS8920) (Macherla *et al.*, 2007; Michelle *et al.*, 2008). A series of three compounds in this class, lipoxazolidinones A to C, have been isolated, with the most potent activity against Anti-methicillin-resistant Staphylococcus aureus (MRSA) and Anti-vancomycin-resistant Enterococci (VRE).

In this study, two chemical compounds relating with lipoxazolidinones were detected; 2-Piperidinone from isolate Chy 4-10 at 14.151 min retention time and 2-Pyrrolidinone from isolate Chy 2-3 at 12.449 min retention time. The activity of these two chemical compounds is unknown and further purification and testing would provide more information on their activities.

Today, the urgent need for new antimalarials requires the discovery of small and inexpensive molecules. Using an SYBR Green bioassay on the parasite's erythrocytic stages Jacques *et al.* (2008) carried out research on secondary metabolite produced by a marine actinomycete *Salinispora tropica* that had significant antimalarial activity. A pure amide chemical compound, salinosporamide A, was tested for its inhibitory activity against parasite development *in vitro* (*P. falciparum*) and in vivo (*P. yoelii*). The finding demonstrated that natural products remain one of the most important sources of medicines against the parasite. In this research, all the studied isolates produced amides that were detected at different retention times; N-(4-Methoxyphenyl)-2-hydroxyimino-acetamide at 23.289 min, L-Prolinamide at 20.982 min, Acetamide,2-chloro-N-(2-ethyl-6-methylphenyl)-N-(2-methoxy-1-methylethyl)- at 23.737 min, Formamide,N-(2,4-diamino-1,6-dihydro-6-oxo-5-pyrimidinyl)- at 29.157 min, N-Methoxymethyl-N-methylformamide at 11.015 min, Propanamide, 2-methyl- at 9.694 min and Propanamide, N,N-dimethyl- at 17.667 min. Activities of these amide chemical compounds need to be researched upon for they could be holding novel properties that would help in tackling the challenges of malaria at hand.

Other groups of chemical compounds were also detected such as alcohols and hydrocarbons. Vanillin which is a chemical intermediate in the production of pharmaceuticals Hocking and Martin (1997) was also detected in isolates Chy 15-10 and Chy 15-5.

5.1 CONCLUSION

Morphological studies showed that the isolates were Gram positive and they formed long branched network of mycelia. The isolates formed colored, tough, leathery and filamentous colonies that were hard to pick from the culture media.

Physiochemical characterization of the isolates showed that optimal growth of the isolates was observed at pH values 6 and above. Optimum growth temperatures were observed between 27.5 ℃ to 32.5 ℃. Growth of the isolates in culture medium at different NaCl levels (varying between 5 and 32.5 g/l) indicated tolerance to salinity and an adaptability of these isolates to varying NaCl concentrations.

All the isolates hydrolyzed starch a characteristic that confirms their role in the decomposition of organic matter in the natural habitats as well as production of amylase-like enzyme. In addition, they all hydrolyzed Tween 20, Tween 80 and skim milk; indicating that they are an important source of lipase, esterase and protease enzymes. Glucose and xylose were the most utilized sugars for growth of the isolates whereas arabinose, inositol and mannitol were the least preferred sugars.

The isolates showed antimicrobial activity and also produced a wide range of chemical compounds indicating they could be an important source of different antimicrobial compounds. *Streptomyces* have been well known during the last seventy years as prolific producers of new bioactive compounds, antitumor drugs included (Berdy, 2005). With the increasing development of oceanographic studies leading to the

isolation of new Actinomycetes from marine sources, new prolific genera in the production of useful compounds have been found, such as *Salinispora*. Moreover, protected terrestrial ecosystems may harbor a myriad of new Actinomycetes providing novel structural diversity to be discovered as evidenced by this study. Besides, the continuous effort to unravel the biosynthesis of the already known compounds and the isolation and characterization of their biosynthesis gene clusters may lead to the development of new antitumor compounds, hopefully with improved therapeutic properties, by using combinatorial biosynthesis approaches. And lastly, adoption of innovative techniques such as coculturing, cross species induction and biofilm development, may further facilitate the discovery of new and useful antimicrobial compounds.

5.2 RECOMMENDATIONS

1. Further purification procedures of the proteins secreted by these isolates through various chromatographic techniques should be done. This will help to determine their molecular weights and also aid in further characterization of the proteins.
2. More enzymatic research should be carried out on the isolates to fully optimize their potential.
3. More research should be carried out to exploit these untapped secondary metabolites released by these microorganisms since they could be of great importance in drug products discovery processes. This could aid in the discovery of broad spectrum therapeutic agents, against pathogens which are becoming increasingly resistant to existing antimicrobials.
4. Tracking the compounds respective activity should be done, in order to find out which chemical compound is responsible for the bioactivity and whether they have a novel mode of action, as this could lead to the development of new therapeutic agents against harmful pathogens.
5. Different culture media and different cultural conditions (e.g. Temperature, pH etc) should be used to optimize the production of compounds and/or metabolites with potential antimicrobial activity.

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APPENDICES

1. Differential broth (g/l)

12.5g Glycerol

1.0g Arginine

1.0g Sodium Chloride

1.0g Di-potassium hydrogen phosphate

0.5g Magnesium sulphate

0.01g Ferrous sulphate heptahydrate

0.001g Copper sulphate pentahydrate

0.001g Zinc sulphate sextahydrate

0.001g Manganese sulphate sextahydrate

1000 ml of distilled water

2. Basal broth media (g/l)

1 % sugar

1.0g Sodium Chloride

1.0g Di-potassium hydrogen phosphate

0.5g Magnesium sulphate

0.01g Ferrous sulphate heptahydrate

0.001g Copper sulphate pentahydrate

0.001g Zinc sulphate sextahydrate

0.001g Manganese sulphate sextahydrate

1000 ml of distilled water

3. International Streptomyces Project broth medium II (ISP_2) – (g/l)

10g Yeast extract

4g Malt extract

4g Glucose

1000 ml of distilled water

4. NaCl broth (g/l)

5g Peptone

3g Yeast extracts

5g Glucose

NaCl

1000 ml of distilled water

pH 7-7.2.

5. Utilization of sugars by the isolates

	Sugars										
Isolate	**Arab**	**Fruc**	**Gala**	**Gluc**	**Inos**	**Lact**	**Mann**	**Mano**	**Meli**	**Sucr**	**Xylo**
Chy4-10	0.038	0.273	0.182	0.601	0.004	0.194	0.068	0.149	0.078	0.473	0.383
	±0.002	±0.020	±0.003	±0.021	±0.001	±0.006	±0.001	±0.001	±0.005	±0.016	±0.017
Chy15-10	0.037	0.079	0.14	0.509	0.017	0.019	0.01	0.499	0.199	0.204	0.899
	±0.003	±0.002	±0.002	±0.009	±0.003	±0.002	±0.002	±0.004	±0.003	±0.005	±0.015
Chy15-5	0.029	0.118	0.036	0.719	0.039	0.241	0.029	0.25	0.174	0.159	0.13
	±0.002	±0.004	±0.004	±0.014	±0.001	±0.004	±0.002	±0.002	±0.003	±0.001	±0.002
Chy 2-3	0.059	0.119	0.051	0.475	0.085	0.198	0.021	0.241	0.186	0.191	0.099
	±0.003	±0.003	±0.001	±0.023	±0.011	±0.001	±0.002	±0.051	±0.004	±0.006	±0.002
Ruj 7-1	0.021	0.097	0.029	0.325	0.019	0.119	0.049	0.181	0.129	0.127	0.9
	±0.002	±0.004	±0.001	±0.013	±0.001	±0.006	±0.001	±0.003	±0.002	±0.002	±0.012

6. Effect of pH on growth of isolates

Isolate	**pH Levels**			
	pH3	**pH6**	**pH7**	**pH9**
Chy 4-10	0.173±0.008	0.536±0.003	0.604±0.004	0.529±0.002
Chy 15-10	0.178±0.004	0.518±0.002	0.569±0.001	0.610±0.005
Chy 15-5	0.205±0.004	0.513±0.007	0.549±0.004	0.591±0.006
Chy 2-3	0.193±0.005	0.408±0.004	0.443±0.002	0.489±0.002
Ruj 7-1	0.215±0.007	0.619±0.002	0.548±0.002	0.357±0.003

7. Effect of temperature on growth of isolates

Isolate	Temperature								
	15°C	**20°C**	**25°C**	**27.5°C**	**30°C**	**32.5°C**	**36°C**	**37°C**	**38°C**
Chy 4-10	0.155±0.003	0.215±0.006	0.324±0.006	0.472±0.002	0.61±0.005	0.578±0.007	0.494±0.004	0.409±0.002	0.339±0.003
Chy 15-10	0.158±0.003	0.227±0.002	0.433±0.002	0.508±0.003	0.586±0.006	0.602±0.001	0.508±0.004	0.43±0.003	0.372±0.002
Chy 15-5	0.173±0.002	0.237±0.002	0.367±0.002	0.417±0.002	0.562±0.004	0.514±0.001	0.428±0.004	0.382±0.002	0.338±0.001
Chy 2-3	0.166±0.003	0.254±0.003	0.305±0.004	0.368±0.002	0.429±0.003	0.518±0.003	0.476±0.003	0.378±0.001	0.288±0.001
Ruj 7-1	0.253±0.004	0.443±0.002	0.523±0.003	0.653±0.007	0.558±0.004	0.415±0.003	0.362±0.004	0.28±0.003	0.148±0.003

8. Effect of sodium chloride on growth of isolates

	Sodium Chloride concentration (g/l)					
Isolate	**0**	**5**	**10**	**17.5**	**25**	**32.5**
Chy 4-10	0.391±0.006	0.411±0.008	0.265±0.004	0.180±0.004	0.071±0.130	0.026±0.003
Chy 15-10	0.126±0.003	0.423±0.013	0.140±0.001	0.039±0.003	0.019±0.002	0.010±0.001
Chy 15-5	0.142±0.001	0.147±0.001	0.169±0.001	0.090±0.003	0.041±0.002	0.023±0.003
Chy 2-3	0.131±0.002	0.388±0.021	0.156±0.003	0.057±0.002	0.031±0.002	0.009±0.002
Ruj 7-1	0.219±0.009	0.269±0.004	0.374±0.006	0.584±0.008	0.131±0.002	0.035±0.003

9. Compounds identified by GC - MS data analyses.

9.1: Compounds identified from isolate Chy 4–10.

Retention Time (min)	**Compound Name**	**% Total**	**% Quality**
6.648	2-Pentanone, 4-hydroxy-	0.275	83
10.187	Ethanone, 1-(2-methyl-1-cyclopenten-1-yl)-	0.717	72
12.001	2-Pentene, 4,4-dimethyl-, (Z)-	1.618	79
14.151	2-Piperidinone	1.003	72
15.248	Caprolactam	1.610	74
15.808	5-Methoxy-2,4-dimethyl-furan-3-one	12.237	70
16.391	Benzenepropanoic acid	0.763	90
17.063	Sulfurous acid, 2-ethylhexyl hexyl ester	0.334	72
17.175	Propenylguaethol	0.230	75
17.555	Phenol, 3-methoxy-	0.690	70
17.667	Propanamide, N,N-dimethyl-	0.362	73

18.698	Pyrimidine, 2-methoxy-5-methyl-	0.855	70
19.213	4-Methylformanilide	0.325	77
19.526	Borane, diethyl(decyloxy)-	0.230	72
19.952	Benzene, 3,5-dimethyl-1-(phenylmethyl)-	0.658	91
20.512	2,6-Diisopropylnaphthalene	0.290	91
20.669	Tridecane, 1-iodo-	0.188	70
20.736	Tridecane, 5-propyl-	0.309	77
20.937	(Z)-2-Methylimino-4,5-tetramethylenetetrahydro-1,3-oxazine	0.320	75
21.206	7-Acetyl-1,7-diazabicyclo[2.2.0]heptane	0.560	72
21.408	2-Hexenoic acid, 5-hydroxy-3,4,4-trimethyl-, (E)-	0.589	75
21.587	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-	1.761	86
21.744	Heneicosane	1.230	86

22.057	Pyridine, 2-methoxy-5-nitro-	0.236	72
22.416	Phenol, 3,5-dimethoxy-	0.560	83
22.528	Phthalic acid, isobutyl nonyl ester	0.616	83
22.774	Nonadecane	0.400	90
23.065	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	0.890	99
23.222	1,4-Dioxaspiro[4.5]decane, 6-methylene-	1.002	72
23.468	1,2-Benzenedicarboxylic acid, butyl 2-methylpropyl ester	1.560	78
23.513	n-Hexadecanoic acid	0.560	94
23.715	Acetamide, 2-chloro-N-(2-ethyl-6-methylphenyl)-N-(2-methoxy-1-methylethyl)-	2.260	83
24.028	Phosphonic acid, bis(1-methylethyl) ester	0.966	83

24.364	1,4-Anthracenedione, 5,6,7,8-tetrahydro-2-methoxy-5,5-dimethyl-	0.484	89
24.678	Heptadecane	0.656	83
25.327	Octadecanoic acid	3.307	99
25.932	p-Benzoquinone, 2-hydroxy-5-(methylthio)-	9.493	78
26.940	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)-	1.992	90
27.477	1-Phenanthrenecarboxylic acid, 7-ethenyl-1,2,3,4,4a,4b,5,6,7,8,10,10a-dodeca hydro-1,4a,7-trimethyl-, methyl ester, [1R-(1.alpha.,4a.	0.651	73
27.813	1-Phenanthrenecarboxylic acid, 1,2,3,4,4a,9,10,10a-octahydro-1,4a-dimethyl-7 -(1-methylethyl)-, [1R-(1.alpha.,4a.beta.,10a.alpha.)]-	0.923	94
28.485	Phthalic acid, 6-ethyloct-3-yl 2-ethylhexyl ester		83
28.821	Hexacosane	0.368	90

29.000	Formamide, N-(2,4-diamino-1,6-dihydro-6-oxo-5-pyrimidinyl)-	1.960	72
29.023	Pyrimidine-2(1H)-thione, 4,4,6-trimethyl-1-(1-phenylethyl)-	1.230	82
29.291	l-Phenylalanine, N-(2,6-difluorobenzoyl)-, methyl ester	1.600	78
29.560	Triacontane	0.676	91
30.344	Heptadecane	0.604	91
30.658	Squalene	0.369	93
31.240	Nonacosane	0.599	96
32.315	Triacontane	0.314	94
33.592	Tetracosane	0.246	83
36.817	Heptamethyl-3-phenyl-1,4-cyclohexadiene	0.796	77

9.2: Compounds identified from isolate Ruj7-1

Retention Time (min)	Compound Name	% Total	% Quality
9.156	2-Butenal, 3-methyl-	0.360	73
15.584	1,4-Dioxane-2,6-dimethanol	18.055	78
18.071	1,1'-Bicyclohexyl, 2-methyl-, trans-	0.630	71
19.101	Dodecanoic acid	1.077	95
19.302	Naphthalene, 1,6-dimethyl-	0.890	58
19.526	Hexadecane	0.456	97
21.340	Tetradecanoic acid	2.988	95
22.236	Diethyldithiophosphinic acid	0.690	78
22.505	Phthalic acid, isobutyl octyl ester	1.181	90
22.707	1-Tridecanol	0.919	91
23.065	2,5-Cyclohexadien-1-one, 2,6-bis(1,1-dimethylethyl)-4-ethylidene-	6.090	70
23.446	Dibutyl phthalate	27.546	94
23.715	Acetamide, 2-chloro-N-(2-ethyl-6-methylphenyl)-N-(2-methoxy-1-methylethyl)-	2.175	98

25.260	Octadecanoic acid	3.804	99
26.940	4,8,12,16-Tetramethylheptadecan-4-olide	0.804	95
27.253	Heptadecane	1.389	97
28.060	Octadecane	1.580	97
28.485	1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester	2.297	91
28.821	Hexacosane	1.615	98
29.359	Phenol, 4,4'-(1-methylethylidene)bis[2,6-dichloro-	0.990	93
29.560	Heptacosane	1.954	98
29.919	Oxalic acid, monoamide, monohydrazide, N-(2,5-dimethylphenyl)-N2-(4-methylbe nzylideno)-	0.980	93
30.031	Octadecane, 1-iodo-	1.009	93
30.658	Squalene	2.283	97
30.882	Octacosane	1.008	90

31.240	Tetracosane	1.023	97
31.890	Nonacosane		96
32.315	Triacontane	1.003	99

9.3: Compounds identified from isolate Chy 2-3

Retention Time (min)	**Compound Name**	**% Total**	**% Quality**
5.416	Acetic acid, 2-methylpropyl ester	0.073	83
7.678	1,2-Ethanediol, mono-acetate	0.397	78
8.709	2-Propenoic acid, 2-methyl-	1.742	70
9.044	Hexanoic acid, 2-methyl-	0.960	72
9.515	Pentanoic acid	0.662	83
9.694	Propanamide, 2-methyl-	0.630	80
11.015	N-Methoxymethyl-N-methylformamide	0.313	78
12.449	2-Pyrrolidinone	0.520	89

12.516	Furan, tetrahydro-2,5-dipropyl-	0.072	75
12.830	2-Ethylpiperidine	0.230	86
13.681	Cyclohexane	0.500	72
14.778	Thiophene, 2,3-dihydro-	2.319	86
15.450	Benzeneacetic acid	1.319	76
15.808	Caprolactam	1.419	83
16.032	p-Dioxane-2,5-dimethanol	0.566	76
16.480	Benzenepropanoic acid	0.0103	97
17.063	Tetradecane	0.332	98
17.802	5-Oxohexanethioic acid, S-t-butyl ester	0.822	75
18.272	Cyclohexanol, 4-methoxy-	0.890	75
18.630	4-Ethyl hydrogen itaconate	0.385	72
19.146	Dodecanoic acid	0.900	99
19.526	Hexadecane	0.860	96

20.579	2,6-Diisopropylnaphthalene	0.369	93
21.072	L-Prolinamide	0.290	77
21.363	3-Pyrrolidin-2-yl-propionic acid	2.133	86
21.766	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-	2.710	83
22.528	Phenol, 3,5-dimethoxy-	2.890	83
22.953	Bicyclo[3.1.0]hex-2-ene, 5,6-diphenyl-	1.900	72
23.088	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	1.256	99
23.311	5,10-Diethoxy-2,3,7,8-tetrahydro-1H,6H-dipyrrolo[1,2-a;1',2'-d]pyrazine	1.230	79
23.535	n-Hexadecanoic acid	1.036	98
23.737	Acetamide, 2-chloro-N-(2-ethyl-6-methylphenyl)-N-(2-methoxy-1-methylethyl)-	1.089	78
25.327	Octadecanoic acid	2.364	99
25.574	p-Fluorophenoxyacetic acid	1.357	82
25.910	2,5-Piperazinedione, 3,6-bis(2-methylpropyl)-	13.999	72

26.581	Benzene, 1-ethyl-4-(1-methylethyl)-	1.230	75
26.985	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)-	1.520	93
28.508	1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester	0.980	91
28.821	Hexacosane	0.900	95
29.157	Formamide, N-(2,4-diamino-1,6-dihydro-6-oxo-5-pyrimidinyl)-	2.530	78
29.381	Pyrimidine-2(1H)-thione, 4,4,6-trimethyl-1-(1-phenylethyl)-	1.710	77
30.344	Tetracosane	0.254	97
36.795	16-Hentriacontanone	0.301	78
41.700	Octadecanoic acid, ethenyl ester	0.532	88

9.4: Compounds identified from isolate Chy 15-5.

Retention Time (min)	**Compound Name**	**% Total**	**% Quality**

10.814	Hexanoic acid	0.590	83
12.426	Heptanoic acid	0.003	79
13.165	Cyclohexanecarboxylic acid	0.360	97
14.263	Benzenecarboxylic acid	0.895	93
15.248	Caprolactam	2.010	91
15.428	Nonanoic acid	0.532	93
16.010	Phthalic anhydride	0.870	81
16.659	n-Decanoic acid	0.191	98
17.085	Tetradecane	0.348	97
17.152	Vanillin	0.850	97
18.048	.alpha.-Cedrene oxide	0.197	76
18.339	Pentadecane	0.600	97
18.586	Tributyl phosphate	0.185	73
19.123	Dodecanoic acid	0.680	99
19.526	Hexadecane	0.603	98

19.862	Dodecanoic acid, 1-methylethyl ester	0.166	90
20.310	1,1'-Biphenyl, 4-(1-methylethyl)-	0.585	96
20.512	2,6-Diisopropylnaphthalene	1.370	95
20.736	Pentadecane, 2,6,10,14-tetramethyl-	0.330	94
20.893	2-(p-Tolylmethyl)-p-xylene	0.590	78
21.363	Tetradecanoic acid	1.867	99
21.856	Hexadecane, 2,6,10,14-tetramethyl-	0.629	95
22.236	2-Pentadecanone, 6,10,14-trimethyl-	0.780	89
22.393	Pentadecanoic acid	0.623	99
22.528	1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester	1.530	73
22.774	Nonadecane	1.411	80
22.931	Benzene, (1-methyldodecyl)-	1.980	76
23.087	2,5-Cyclohexadien-1-one, 2,6-bis(1,1-dimethylethyl)-4-ethylidene-	1.862	86

23.558	n-Hexadecanoic acid	6.182	98
23.715	Acetamide, 2-chloro-N-(2-ethyl-6-methylphenyl)-N-(2-methoxy-1-methylethyl)-	3.010	73
24.364	Heptadecanoic acid	2.270	91
24.700	Heneicosane	1.312	98
25.081	9-Octadecenoic acid, (E)-	3.361	99
25.372	Octadecanoic acid	11.471	99
25.596	Docosane	3.260	99
26.447	Octadecane	1.911	97
27.477	Androst-5-en-17-ol, 4,4-dimethyl-	1.903	75
27.791	1-Phenanthrenecarboxylic acid, 1,2,3,4,4a,9,10,10a-octahydro-1,4a-dimethyl-7 -(1-methylethyl)-, [1R-(1.alpha.,4a.beta.,10a.alpha.)]-	2.650	99
28.485	Phthalic acid, decyl 2-ethylhexyl ester	2.224	72
28.821	Eicosane	1.570	98
29.583	Tricosane	1.346	98

30.658	Squalene	1.416	98
31.262	Nonacosane	1.723	99
32.315	Tetracosane	0.887	97
33.592	Octacosane	0.804	98
36.817	Hexadecanoic acid, 4-nitrophenyl ester	0.367	78
37.959	Stigmastanol	0.467	78
41.744	Octadecanoic acid, ethenyl ester	4.108	83

9.5: Compounds identified from isolate Chy 15–10.

Retention Time (min)	**Compound Name**	**% Total**	**% Quality**
7.633	1,2-Ethanediol, monoacetate	0.230	74
8.395	Butanoic acid, 3-methyl-	0.560	83
8.619	Butanoic acid, 2-methyl-	0.123	74
9.224	Dimethyl sulfone	0.269	85
14.196	2-Piperidinone	1.481	86

14.420	Benzenecarboxylic acid	0.560	95
14.845	1H-Pyrrole-2-carboxylic acid	0.234	91
15.338	Benzeneacetic acid	3.036	64
15.540	Caprolactam	0.589	76
15.719	1,4-Dioxane-2,6-dimethanol	1.002	89
16.413	Benzenepropanoic acid	1.117	97
17.085	Tetradecane	1.230	97
17.17	Vanillin	0.840	93
19.146	Dodecanoic acid	0.980	99
19.526	Hexadecane	0.589	97
20.579	2,6-Diisopropylnaphthalene	0.258	95
20.982	L-Prolinamide	0.789	77
21.273	3,7-Dimethyloctyl acetate	3.088	78
21.744	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-	12.539	96

22.460	Phenol, 3,5-dimethoxy-	3.409	77
22.528	1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester	0.968	94
22.774	Nonadecane	0.879	98
23.087	2,5-Cyclohexadien-1-one, 2,6-bis(1,1-dimethylethyl)-4-ethylidene-	1.125	79
23.289	N-(4-Methoxyphenyl)-2-hydroxyimino-acetamide	0.456	91
23.535	n-Hexadecanoic acid	11.074	98
23.759	Tridecane	2.067	94
24.364	Heptadecanoic acid	1.456	95
25.081	9-Octadecenoic acid, (E)-	1.790	97
25.350	Octadecanoic acid	5.746	99
26.044	cis-2-Thioxo-5,6-trimethylene-2,3,5,6-tetrahydropyrimidin-4(1H)-one	26.891	77
26.447	Heneicosane	2.369	98
26.962	1-Phenanthrenecarboxylic acid, 1,2,3,4,4a,9,10,10a-octahydro-1,4a-dimethyl-7	1.386	75

	-(1-methylethyl)-, methyl ester, [1R-(1.alpha.,4a.beta.,		
27.276	Tetracosane	0.646	99
27.477	1-Phenanthrenecarboxylic acid, 7-ethenyl-1,2,3,4,4a,4b,5,6,7,8,10,10a-dodeca hydro-1,4a,7-trimethyl-, methyl ester, [1R-(1.alpha.,4a.	0.890	86
27.791	1-Phenanthrenecarboxylic acid, 1,2,3,4,4a,9,10,10a-octahydro-1,4a-dimethyl-7 -(1-methylethyl)-, [1R-(1.alpha.,4a.beta.,10a.alpha.)]-	0.789	99
28.060	Pentacosane	0.855	98
28.463	1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester	3.480	87
29.336	Pyrimidine-2(1H)-thione, 4,4,6-trimethyl-1-(1-phenylethyl)-	1.470	78
29.538	Octacosane	0.929	96
29.717	Hexadecanedioic acid	0.456	74
30.344	Triacontane	0.976	98
30.658	Squalene	1.009	91

31.240	Nonacosane	1.467	98
32.024	Heptadecane, 9-octyl-	0.870	93
32.315	Heptadecane	0.892	96