CHARACTERIZATION OF MYXOBACTERIA BY USING MOLECULAR TOOLS AND IDENTIFICATION OF GENE RESPONSIBLE FOR ANTI-MICROBIAL ACTIVITY

M.Sc. Thesis

2019

Submitted to:

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Tribhuvan University

Kirtipur, Kathmandu, Nepal

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Dedicated to

my

Grandparents

ACKNOWLEDGEMENT

After an intensive period of nine months, today is the day: writing this note of thanks is the finishing touch on my thesis. It has been a period of intense learning for me, not only in the scientific arena, but also on a personal level. Writing this thesis has had a big impact on me. I would like to reflect on the people who have supported and helped me so much directly and indirectly throughout this period.

Foremost, I would like to express my sincere gratitude to my supervisors Prof. Dr. Rajani Malla, Former Head, Central Department of Biotechnology and Ms. Pragati Pradhan, Lecturer, Central Department of Biotechnology their continuous support, patience, motivation, enthusiasm, and immense knowledge.

I want to thank Mr. Rajindra Napit and Ms. Apshara Parajuli for their guidance that helped me in all the time of research and writing of this thesis.

I am very grateful to Prof. Dr. Krishna Das Manandhar, Head, Central Department of Biotechnology for his encouragement support and permission to use lab for work. .I acknowledge with thanks the support rendered by all the faculties viz., Prof. Dr. Ganga Prasad Kharel, Prof. Dr. Tribikram Bhattarai, Prof. Dr. Tilak Ram Shrestha, Prof. Dr. Mohan Kharel, Dr. Gauri Shankar Manandhar, Dr. Suresh Subedi, Dr. Pramod Aryal, Mrs. Jarina Joshi, Dr. Smita Shrestha, Mrs. Alina Shree Sapkota, Mrs. Priti Regmi and Ph.D. scholars Mr. Guna Raj Dhungana, Mrs. Prasamsha Panta and Mr. Bhuwan Saud for their encouragement, knowledge and constructive criticism during the work.

Special thanks to Anupam Poudel and Bikram Prajapati who helped me during collection of samples.It will be inadequate to express my sincere thanks to Himani Upreti, Indu Gyanwali, Pooja Pathak, Prashant Paudel, Sawan Kumar Chaudhary, Sishir Gautam, Yujeen Chapagain and all friends for their continuous support and co-operation along with Samiram Subedi, Devraj Maidali, Siddharth Gautam and all them for their support. I would also like to thank all the non-teaching staffs of Central Department of Biotechnology for their co-operation and help.

I would like to thank University Grant Commission for providing Masters Research Support Award MRS-76/77-S&T-5 which helped me to overcome the difficulties and encouraged me during the work.

Last but not the least, I would like to thank my respected parents: Mr. Dhruba Raj Subedi and Mrs. Kalpana Subedi along with my wife Mrs. Bindu Sigdel Subedi and the younger

brother Mr. Sudhan Kumar Subedi for their spiritual and unconditional support, understanding, encouragement and continuous inspiration throughout my life.

GLOSSARY ACRONYMS

AT	Acyl Transferase
ATCC	American Type Culture Collection
CMC	Carboxy Methyl Cellulose
C.V	Crystal Violet
DNA	De-oxyribo Nucleic Acid
LB	Luria-Burtani
NA	Nutrient Agar
NCBI	National Center for Biotechnology Information
NRP	Non-Ribosomal Protein
PKS	Poly Ketide Synthase
WCX	Water Cycloheximide Agar

SYMBOLS

µl	Microlitre
gm	Gram
mg	Milligrams
mg/L	Milligrams per litre
ml	Milliliter

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ABSTRACT

The emergence of antibiotic-resistant pathogenic microorganisms and rapid development of resistance to chemotherapeutic drug have necessitated the discovery of structurally diverse and mechanistically distinct antimicrobial compounds. Myxobacteria have great potential to contribute to the pool of natural products for drug discovery such as antibacterial, antiviral, antitumor, antifungal, cytotoxic, immunosuppressive, antimalarial, and anti-oxidative drugs. Myxobacteria are Gram-negative unicellular rod shaped bacteria that occur everywhere in soils. They are characterized by an unusual way of life, as they move by gliding or creeping on surfaces. Using exoenzymes they lyse different biological macromolecules as well as whole microorganisms such as bacteria and yeasts. Upon exhaust of nutrients they form myxospores and fruiting bodies. Within the prokaryotes myxobacteria show a unique cooperative social behavior, based on a communication system of cell-to-cell interaction. Myxobacteria form fruiting bodies that display bright colors and therefore are often identified with the help of stereomicroscope. The aim of this study is to explore the novel myxobacteria that can produce potential anti-microbial metabolites. In this study, thirteen isolates were isolated and the isolates with different morphology was further processed. 16 S' rDNA sequences of 13 different isolates were sequenced of which 8 isolates after phylogenetic analysis were further processed for amplification of the ketosynthase gene and acyltransferase gene which reveal the absence of both genes. The crude extracts inhibited the *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* which may be due to the other metabolites rather than the ketides and acyl-transferases. Myxobacteria have a very large genome size compared to other bacteria, and produce a number of structurally unique biologically active secondary metabolites. Many compounds isolated from myxobacteria act on unique cellular targets, which, at the time of their discovery, were not targeted by other secondary metabolites. Most of the isolates also showed the cellulolytic activity. There is a persistent requirement for new bioactive compounds so that the demand for new natural products in therapy can be met. The aim of this study was to explore novel myxobacteria that can produce potential anti-microbial metabolites and enzyme which can be further used for the medicinal and industrial purpose.

Keywords: Myxobacteria: Phylogenetic analysis, anti-microbial activity

CHAPTER 1
INTRODUCTION

1.1 Background

Antibiotic resistance is increasing day by day creating global threat. As a result simple infections become untreatable and Life-saving interventions like organ transplantation has become more difficult, resulting in death. Now, the solution to this antibiotic crisis is very urgent as either new and novel compound has to be discovered which has not been used before and it needs to happen as quickly as resistance develops in the microbial population or completely new way to deal with those diseases has to be developed keeping side effect at minimal. There are numerous approaches to either reduce the selection for antibiotic resistance or to replace the antibiotic altogether modification of existing drugs and discovery of new compounds. They are herbal remedies, bacteriocins, and predatory bacteria-Bdellovibrio bacteriovirus (BALOs) MYXO, Bacteriophages and each approach has its own benefits and costs. Myxobacteria are group of microorganisms that is known for having anti-microbial and anti-cancerous activity. Myxobacteria in terms of antimicrobial activity differs from other microorganism in a way that it produces cocktails of bioactive compounds along with traditional type of antibiotics. In either case, the need of vigorous research is eminent and should happen at lightning speed given the status of antibiotics resistance right now all around the world.

Myxobacteria were found nearly everywhere from Antarctica to the tropics, and from water bodies to terrestrial. The genuine habitat of myxobacteria is the soil, as long as the pH is slightly acid to slightly alkaline, i.e. between 5 and 8. Frequently myxobacteria are found on the dung of herbivorous animals, on decaying plant material and on the bark of trees; occasionally they have also been found on the surface of plant leaves. Myxobacteria are prolific producers of a variety of bioactive secondary metabolites including antibacterial, antifungal, antiviral and antitumor compounds. Myxobacteria have been regarded as ‘‘microbe factories’’ for active secondary metabolites, because they have great potential as a prolific source of new drugs for drug discovery programs. Myxobacteria are widely distributed in natural ecosystem. However, currently less than 10% of all the natural species have been isolated. Therefore, it would be of great potential economic value to improve our capacity to isolate new myxobacteria, in order to provide more strains for drug screening and development.

1.2 Current studies

There are many researches for the development of the new drugs that can cure the global threat of the antibiotics resistance. Recently, many studies related to the capability of myxobacteria to synthesize the mixture of secondary metabolites including the ribosomal proteins and the non-ribosomal proteins are under progress. The studies suggest that the myxobacterial metabolites are not only capable for the treatment of the multi-drug resistant bacterial infection but is also capable of treating the cancer. Isolation and purification of the myxobateria is tedious, so many researchers are in search of new methods of isolation of myxobacteria.

1.3 Hypothesis

H_0: Myxobacteria cannot produce secondary metabolites which showed potent anti-microbial property.

H_1: Myxobacteria can produce secondary metabolites which showed potent anti-microbial property.

1.4 Objectives

1.4.1 General Objective

- To explore the novel myxobacteria and their secondary metabolites that have potential anti-microbial activities.

1.4.2 Specific Objectives

- To isolate novel Myxobacteria from soil, bark and dung samples collected within Kathmandu
- To identify isolates genetically
- To extract the crude secondary metabolites from the culture
- To test antimicrobial activity of myxobacterial extract
- To identify the gene responsible for the antibacterial activity
- To identify the cellulase producing isolates

1.5 Rationale

The global issue of increase in antibiotic resistance has also been a serious threat to Nepal. Previous study also showed that the prevalence rate of antibiotic resistance in Nepal is very high. The problem of antibiotics resistance is even more serious in the country like Nepal as rule and regulation that is needed to halt or prevent the advent of antibiotics resistance is minimal. Preventing antimicrobial resistance is one of the

priority area of government of Nepal as it is one of the goal of sustainable development goals which is committed by government. This emergence of antibiotic resistance demands newer antibiotics to be developed. Myxobacteria in terms of antimicrobial activity differs from other microorganism in a way that it produces cocktails of bioactive compounds along with traditional type of antibiotics, which could be a probable and potential solution to the antibiotics resistance as many bioactive compounds simply means, resistance will be less common than that of in traditional antibiotics.

CHAPTER 2
LITERATURE REVIEW

Myxobacteria are Gram-negative bacteria belonging to the delta subgroup of proteobacteria, which can be isolated from different habitats like soil, herbivore dung, decaying plant mass and tree bark, showing that they are able to adapt to diverse environments. Compared to other bacteria, myxobacteria have some noticeable features: The capability to glide on solid surfaces, building swarms, degrading macromolecules, and preying on living microorganisms are their main characteristics. Myxobacteria possess a sort of "social" behavior as they swarm and collectively hunt for other microorganisms such as bacteria and fungi. For that reason, myxobacteria can be called "micropredators". They are characterized by an unusual way of life, as they move by gliding or creeping on surfaces. Using exo-enzymes they lyse different biological macromolecules as well as whole microorganisms such as bacteria and yeasts. Upon exhaust of nutrients they form myxospores and fruiting bodies. Within the prokaryotes myxobacteria show a unique cooperative social behavior, based on a communication system of cell-to-cell interaction. Myxobacteria synthesize a large number of biologically active secondary metabolites.

2.1 Biology of Myxobacteria

2.1.1 Habit and Habitat

Myxobacteria were found nearly everywhere from Antarctica to the tropics, and from water bodies to terrestrial (Dawid, 2000). The genuine habitat of myxobacteria is the soil, as long as the pH is slightly acid to slightly alkaline, i.e. between5 and 8. Frequently myxobacteria are found on the dung of herbivorous animals, on decaying plant material and on the bark of trees (Reichenbach and Dworkin, 1992); occasionally they have also been found on the surface of plant leaves (Rückert, 1981). Isolation technologies for myxobacteria usually involve the incubation of natural samples on a selective solid medium, to allow for the formation of fruiting body (Rosenberg et al., 1984; Dworkin, 1996). However, many myxobacteria species could not formed its fruiting body under current culture conditions and resulted in missing numerous strains. Until now, the recognized myxobacteria are only classified into three suborders, six families, 23 genera and approximately 50 species (Shimkets, 2006). The works about the myxobacteria were very limited mainly due to insufficiency incubation strategy and limited strain information. Myxobacteria are very common and ubiquitous organisms in soil (Li, 2014). In soils according to their frequency of occurrence, the following species are most typical: *Na. exedens, Cc. coralloides, So. cellulosum*, different *Polyangium* species, *Mx.*

fulvus, different *Cystobacter* species, and *Mx. Stipitatus* . The typical species living on the bark of trees and on rotting wood are *Sg. aurantiaca*, *Cm. apiculatus*, *Cc. coralloides*, *Mx. fulvus*, *Cm. pediculatus*, and different *Haploangium* species. Dung pellets of herbivores are preferentially colonized by *Mx. fulvus, Cc. coralloides, Cb. fuscus, Cb. ferrugineus, Ar. serpens*, *Sg. erecta*, *Mx. virescens*, *Mx. xanthus*, and *Cb. velatus* (Reichenbach and Dworkin, 1992).

Myxobacteria are capable of multicellular, social behavior (Reichenbach, 1993). The distinctive feature of myxobacteria is that the vegetative cells aggregate into large mounds and then a matured fruiting body formed, by means of a peculiar gliding motility under starvation conditions (Rosenberg et al., 1984; Dworkin, 1996). Myxobacteria play important roles in establishing ecological function, as result of gliding motility, complex life cycle, fruiting bodies formation, degrade insoluble macromolecules and the production of bioactive compounds (Dawid, 2000).

The myxobacteria feed by preying on other living microbial species, and also consume insoluble macromolecular debris such as cell walls, proteins, polysaccharides and nucleic acids. To feed on such materials, myxobacteria first must attain a high cell density, enabling its excreted lytic enzymes to reach adequate levels. Thus, their life-sustaining behavior is social, focused on maintaining a high high-density population or, in the case of fruiting bodies, a potential for reaching high densities that are key for how such cells gather nutrients (Dworkin, 2007).

2.1.2 Morphogenesis of Myxobacteria

Myxobacteria shows different variation in its morphological structure in different stages of life. The formation of fruiting bodies is induced by nutritional deficiency and is controlled by nutrient concentrations, pH, cations and temperature (Dworkin, 1996). It is a cooperative morphogenesis by the vegetative swarming cells. The process is a sequence of the following steps:(1) a large number(10^5-10^7) of swarming cells loose their physical individuality during morphogenesis; (2) Vegetative growth of the rods ceases; (3) In certain positions of the swarm the cells assemble to form aggregates; (4) Molecules formed on the cell surface cause that cells stick together; (5) The consequence is an unstructured agglutination of masses of cells that autolyse to about 65-90%; (6) The formation of special structural elements (stem, base plate, sporangiole wall) starts; (7) The characteristic (specific) shape of the fruiting body is formed; (8) During the maturation phase the vegetative rod cells are transformed into myxospores by cellular morphogenesis (Dawid, 2000).

The formation of myxospores occurs inside the maturing sporangioles and fruiting bodies. The vegetative rod cells undergo a cellular morphogenesis by becoming shorter and rounder. The whole vegetative cell turns into a myxospore. The change in shape is conspicuous: Myxospores are much shorter and thicker than vegetative cells, they appear rounded, strongly light-refringent and surrounded by a thin capsule. As survival cells myxospores differ from eubacterial endospores (e.g. in the genus *Bacillus*) by structure, physiological properties and way of formation (Reichenbach and Dworkin, 1992). Myxospores are dehydrated, resistant against dryness, heat, ultra-sonication and ultraviolet radiation. Due to their dry resistance myxospores, harvested from agar plate cultures on sterile filter paper strips, and dried in a desiccator, will survive for 10-25 years. Inside the cells myxospores contain granules of polyphosphates and polysaccharides, surrounded by an envelope of ribosome-like particles, and lipid inclusions. The contents in myxospores of other biomolecules shows strong differences to that in vegetative cells (Fink and Zissler, 1989).

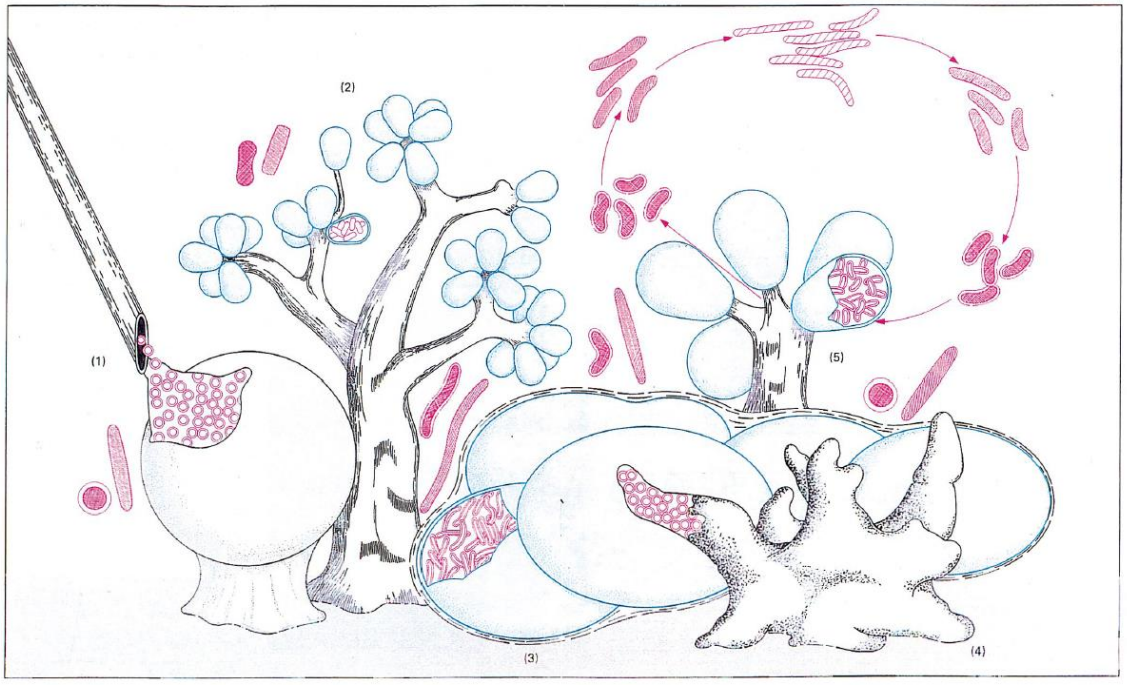

Figure 1: Fruiting bodies of myxobacteria, partly broken open to show the myxospores inside (Dawid, 2000).

[Sporangioles with tough shells are given in blue, slimy material in black. On the immediate left of each fruiting body the vegetative cell is shown, further left a myxospore typical of that genus. (1) *Myxococcus*: soft and slimy, so that material may be removed easily with the needle of a syringe. (2) *Cm. crocatus*. (3) *Cystobacter*. (4) *Corallococcus*: hard, cartilaginous slime. (5) *Sg. aurantiaca*: shown above the fruiting body in the cycle of cellular morphogenesis from myxospore to the vegetative cell and back to the myxospore. The figures are not drawn to scale.

2.1.3 Motility of Myxobacteria

Myxobacteria moves by gliding, a property it shares with many other bacteria, including some of the cyanobacteria, *Cytophaga*, *Sporocytophaga, Beggiatoa, Vitreoscilla* and *Flexibacter*. Although these organisms cannot swim in aqueous media and do not possess flagella. They can move by gliding over solid surfaces. Myxobacteria does not have visible organelles of locomotion. This movement is accompanied by bending of cells and by secretion of slime. Extracellular motility organelles such as flagella are lacking (Burchard, 1984). Several models have been proposed to explain the gliding mechanism. Burchard proposed that it is based upon bundles of filaments and tubules that are arranged longitudinally as well as diagonally beneath the cell membrane (Burchard, et al., 1977). Waves of contraction are supposed to propel the cells (Macrae and Mccurdy, 1976). Lunsdorf and Reichenbach (1989) detected a strictly ordered chain-like structure beneath the outer cell membrane. This structure consisting of rings and elongate components by contraction causes propagating waves that seem to act on the cell surface, thus propelling the gliding cell. The slime excreted during movement is visible as a slime track and has been described as a protein-polysaccharide-lipid complex (Gnosspelius, 1978). It is involved in the proteolytic activities of myxobacteria by denaturing native proteins of lysed prey cells and thus supplying the proteolytic exoenzymes of myxobacteria with appropriate substrates. However, the spatial and temporal variability at a community level is less well known.

In spite of their cellulolytic and bacteriolytic activities myxobacteria are susceptible to antibiotics in being sensitive to erythromycin, neomycin, kanamycin, streptomycin, and tetracycline (Reichenbach and Dworkin, 1992; McCurdy, 1969). Rather unusual for Gram-negative bacteria, almost all myxobacteria are sensitive against actinomycin. This is due to the strongly lipophilic cell surface (Dworkin, 1969).

2.1.4 Taxonomy of Myxobacteria

Currently the monophyletic order Myxococcales comprises 3 suborders, 10 families, 29 genera, and 58 species.

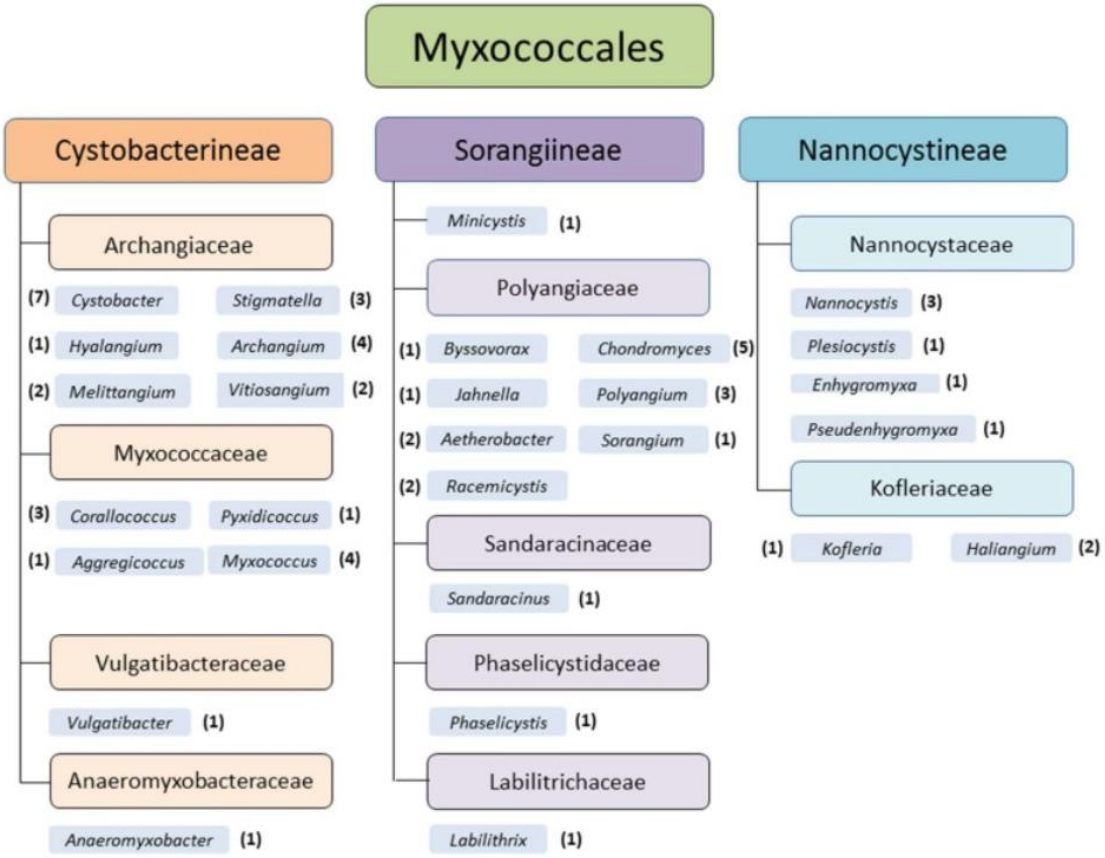

Figure 2: Monophyletic order Myxococcales (delta-proteobacteria), suborders, families, and genera of myxobacteria (status May 2018). The number of species within the genera is mentioned in brackets (Mohr, 2018)

2.2 Culture methods of myxobacteria

Myxobacteria can be isolated by different methods, some of which are described below.

2.2.1 Rabbit dung method

Rabbit dung pellets are natural medium for myxobacteria growth. Fruiting bodies can be induced in a shorter time (within 2–3 days) by sterilized rabbit dung pellets, and most of fruiting bodies are Myxococci that are easy to be purified. However, natural rabbit dung pellets are rich in organic matter, and meanwhile are easily contaminated by molds during the induction process, covering the fruiting bodies of myxobacteria (Zhang et al., 2003).

2.2.2 Placing soils on bacterial smears

In this method, small quantities of soils or plant materials were placed on wet bacterial cells streaked on plain agar, and formation of fruiting bodies are investigated. Mostly the cells of *Brevibacterium lactofermentum* ATCC 13869 cultivated in nutrient broth were used, but these were replaceable with other bacterial cells (Yamanaka et al., 1987).

2.2.3 Filter paper method

For the isolation of cellulose-degrading myxobacteria, slices of filter paper were placed on top of the ISCX agar (KNO_3 0.1%, $FeCl_3 \cdot 6\ H_2O$ 0.02%, K_2HPO_4 0.1%, $MnSO4 \cdot 7\ H_2O$ 0.01%, $CaCl_2 \cdot 2\ H_2O$ 0.1%, $MgSO4 \cdot 7\ H_2O$ 0.1%, yeast extract 0.002%, agar 1.5%, pH 7.2). They were then inoculated in the center of the filter paper with a pea-sized aliquot of the soil samples. While they grew slowly in the inorganic salt filter paper; they took 7–14 days or longer time to grow up (Zhang et al., 2013).

2.2.4 Agar medium

Myxobacteria are grown in the agar medium consisting $CaCl_2$ and cycloheximide. Cycloheximide is used as a fungicide. Myxobacteria grew rapidly in the WCX medium, and they could be observed under optical microscope after culture for 3 days (Zhang et al., 2013).

2.3 Myxobacteria as multi-producers of secondary metabolites

Myxobacteria has very large genomes of up to 13 million nucleotides. Although Myxobacteria was discovered by Ronald Thaxter in 1892 but their ability to produce useful biochemicals such as antibacterial, antiviral, antitumor, antifungal, cytotoxic, immunosuppressive, antimalarial, and antioxidative properties was far later extensively studied. It is because they are slow growers having doubling time i.e., 4-14hr in laboratory condition and also to get homogenous single cell suspension needs repeated months of sub culturing and for extraction of secondary metabolites needs scaling fermentation. Myxobacterial strains have antibacterial (29%) or anti-fungal (54%) properties and till date at least 100 natural product core structures and around 500 derivatives have been found from 7500 different strains like volatile substances from different compound classes, including ketones, esters, lactones, sulfur and nitrogen-containing molecules, and additional terpenes, linear or cyclic polyketides (PKs) and non-ribosomal polypeptides (NRPs) which possess high pharmacological importance. Unlike metabolites from other microorganisms, myxobacterial metabolites are not glycosylated (Bode and Müller, 2006; Kaur et al., 2006). Most of the compounds are novel and some similar to that found in other bacteria but their mode of action being different hence

giving a good alternative for drug resistance like hybrid PK/NRP metabolites (>50%) found in Myxobacteria in contrast to purely PK or NRP natural products synthesized by the actinomycetes and Bacilli.

Table 1: Potential Use of Myxobacteria (Kumar et al., 2017)

Myxobacteria	**Secondary metabolites**	**Activity against**
Sorangium cellulosum	Thuggacins	*M. tuberculosis*
Chondromyces crocatus	Crocacin	Gram positive bacteria
Sandaracinus amylolyticus	Indiacens	Both Gram(+) and (-)
Pyxidicoccus fallax	Disciformycins	MRSA, VRSA
Corallococcus coralloides	Coralmycins	Gram negative bacteria
Chondromyces pediculatus	Pedein A and B ,Miuraenamides	Fungi
Stigmatella aurantiaca	Aurafurons A and B	
Archangium gephyra	Aurafurons, cyrmenins	
Cystobacter armeniaca	Cyrmenins	Fungi

In addition to the discovery of new structures, their corresponding biosynthetic pathways are attaining more and more interest (Bode and Muller, 2006).Knowledge of the biosynthetic pathway offers the possibility to manipulate it genetically and therewith to increase yields or obtain new derivatives (Bode and Muller, 2005). This information can also be used to express biosynthetic gene clusters in heterologous hosts (Wenzel and Muller, 2005).Within the last decade, a large number of biosynthetic gene clusters have been identified. This genetic information enables the prediction of unknown pathways and subsequently new structures (Menzella and Reeves, 2007).

The origin of two large groups of secondary metabolites, the polyketides and non-ribosomal peptides, are products of multi-step biosynthetic pathways. The underlying biosyntheses is directed by complex biosynthetic machineries, such biosynthetic machineries are polyketide synthases (PKS) and non-ribosomal peptide synthetases (NPRS). Aurafuron (Henkel et al., 1999) and soraphen (Young and Taylor, 2008) for instance are myxobacterial metabolites generated by pure PKSs (Kunze et al., 2005). While myxochelin (Harada, 2004) is a myxobacterial representative resulting of a NRPS pathway.

2.3.1 Non-Ribosomal Peptide Synthase

Non-ribosomal peptide synthetases, which, unlike the ribosomes, are independent of messenger RNA, synthesizes non-ribosomal peptides. Only one type of peptide can be synthesized by each non-ribosomal peptide synthetase. Non-ribosomal peptides often have cyclic and/or branched structures, can contain non-proteinogenic amino acids including D-amino acids, carry modifications like *N*-methyl and *N*-formyl groups, or are glycosylated, acylated, halogenated, or hydroxylated. Cyclization of amino acids against the peptide "backbone" is often performed, resulting in oxazolines and thiazolines; these can be further oxidized or reduced. On occasion, dehydration is performed on serines, resulting in dehydroalanine. This is just a sampling of the various manipulations and variations that non-ribosomal peptides can perform. Non-ribosomal peptides are often dimers or trimers of identical sequences chained together or cyclized, or even branched. Non-ribosomal peptides are a very diverse family of natural products with an extremely broad range of biological activities and pharmacological properties. They are often toxins, siderophores, or pigments. Non-ribosomal peptide antibiotics, cytostatics, and immuno-suppressants are in commercial use (Schwarzer et al., 2003).

Nonribosomal peptides are synthesized by one or more specialized nonribosomal peptide-synthetase (NRPS) enzymes. The NRPS genes for a certain peptide are usually organized in one operon in bacteria and in gene clusters in eukaryotes. However the first fungal NRP to be found was ciclosporin. It is synthesized by a single 1.6MDa NRPS (Turgayet al., 1992). The enzymes are organized in modules that are responsible for the introduction of one additional amino acid. Each module consists of several domains with defined functions, separated by short spacer regions of about 15 amino acids (Fischbach and Walsh, 2006).

The biosynthesis of nonribosomal peptides shares characteristics with the polyketide and fatty acid biosynthesis. Due to these structural and mechanistic similarities, some nonribosomal peptide synthetases contain polyketide synthase modules for the insertion of acetate or propionate-derived subunits into the peptide chain (Wang et al., 2014).

2.3.2 Polyketide synthase

Polyketide synthases (PKSs) are a family of multi-domain enzymes or enzyme complexes that produce polyketides, a large class of secondary metabolites, in bacteria, fungi, plants, and a few animal lineages. The biosyntheses of polyketides

share striking similarities with fatty acid biosynthesis (Khosla et al., 1999; Jenke-Kodama et.al, 2005).The compound backbone of a polyketide (PK) is constructed by acyl-CoAs. The biosynthetic principle of a polyketide synthase (PKS) is similar to fatty acid biosynthesis and comparable to NRPSs. There are three essential domains in a minimal module, as well as some additional ones. The three core domains are the acyltransferase (AT), the ketosynthase (KS) and the acyl carrier protein (ACP). Accordingly, PKSs can be classified into three groups with the following subdivisions: Type I polyketide synthases are large, highly modular proteins; Iterative Type I PKSs reuse domains in a cyclic fashion; NR-PKSs — non-reducing PKSs, the products of which are true polyketides; PR-PKSs — partially reducing PKSs; FR-PKSs — fully reducing PKSs, the products of which are fatty acid derivatives; Modular Type I PKSs contain a sequence of separate modules and do not repeat domains (with the exception of trans-AT domains); Type II polyketide synthases are aggregates of monofunctional proteins; Type III polyketide synthases do not use ACP domains(Castoe, et al., 2007).

Polyketide synthases are an important source of naturally occurring small molecules used for chemotherapy (Koehn and Carter, 2005). For example, many of the commonly used antibiotics, such as tetracycline and macrolides, are produced by polyketide synthases. Other industrially important polyketides are sirolomus (immunosuppressant), erythromycin (antibiotic), lovastatin (anticholesterol drug), and epothilone B (anticancer drug) (Wawrir, et al., 2005).

2.4 Other Applications of Myxobacteria

Due to their nutritional behavior and based on their specialization in degradation of biomacromolecules, members of the order Myxococcales can be divided into two groups: predators (the majority), which are able to lyse whole living cells of other microorganisms by exhausting lytic enzymes, and cellulose-decomposers, the latter are represented by the genera *Sorangium* and *Byssovorax (*Reichenbach, 1999*)*. But, as mentioned for the (facultative) anaerobic myxobacteria, it is also highly likely that further cellulose-degrading genera exist, which successfully resisted standard cultivation attempts. Due to the predatory behavior of the myxobacteria, it may be also used in agriculture as the biopesticides.

CHAPTER 3
MATERIALS AND METHODS

3.1 Materials

A complete list of materials, equipment, media, chemicals, and reagents used in this study are listed in Appendix B.

3.2 Methodology

3.2.1 Study design and duration

This was explorative study conducted from March 2019 to October 2019.

3.2.2 Study Site

This research work was conducted at Central Department of Biotechnology, Tribhuvan University, Kirtipur, Kathmandu.

3.2.3 Sample collection and storage

30 different samples (soil, dung, bark) were collected from the three different areas of Kathmandu valley, viz., Bhaktapur, Kirtipur and Shivapuri National Park. Samples were stored at 4°C for further use. Samples were collected from the region which is rich in organic wastes.

3.3 Isolation and Purification of Myxobacteria

Myxobacteria were isolated by *Escherichia coli* baiting technique from the samples. The samples were inoculated into the WCX media ($CaCl_2.2H_2O$: 0.5%, Agar: 1%) containing 100µg/ml cycloheximide where *E. coli* culture was inoculated as the nutrients for the myxobacteria. The plates were incubated at 30°C for 2-3 weeks.

Colony morphology and fruiting bodies were observed using the stereomicroscope to identify myxobacteria. The fruiting bodies and the cells were picked up and streaked on WCX media with *E.coli* and VY/2 medium (Barkers yeast 0.5%, $CaCl_2.2\ H_2O$ 0.1%, agar 1.5%, pH 7.2) and incubated at 30°C. This step was repeated till the isolates were obtained in pure culture.

3.4 Identification of Myxobacteria

3.4.1 Morphological Identification

3.4.1.1 Grams' Staining

A loop of the bacterial culture was taken and smear was prepared in the clean grease free slide and heat-fixed. Then the smear was stained with the crystal violet (C.V.) for 1 minute. Then iodine was flooded over the C.V. stained slide for 1 minute. Then the slide was washed quickly with the decolorizing solution and washed with distilled water. Then the smear was stained with safranin for a minute. The slide was washed and observed under oil-immersion microscope.

Grams' Staining of the bacterial culture were performed in two different stages of their growth; one at its vegetative growth in NA and next in the starvation condition in WCX Agar to observe the different morphological variation according to the nutrition medium.

3.4.2 Molecular identification (16S rRNA Sequencing)

3.4.2.1 Extraction of Genomic DNA

Genomic DNA of the bacterial samples confirmed morphologically were isolated by using the Zymo Research Soil Microbe DNA Miniprep*TM*, Catalog No. D6001. The agarose gel (1%) electrophoresis of the extracted was performed.

3.4.2.2 16S' rRNA Sequencing

The 16S' rRNA segment of the myxobacterial genomic DNA was amplified by using thermal cycler- Polymerase Chain Reaction under following conditions using the enlisted ingredients.

Table 2: Primers used in 16S' rRNA Sequencing

Primers:	**Forward Primer:** 5'- AACGCGAAGAACCTTAC -3'
	Reverse Primer: 5'- CGGTGTGTACAAGGCCCGGGAACG -3'

Table 3: Ingredients required and reaction conditions for 16S' rRNA sequencing

Particulars:	**Volume per reaction mixture**
Zymo *Taq*^{TM} PCR Premix(2X)	12.5 µl
Forward primer(10 pmol)	1.0 µl
Reverse primer(10 pmol)	1.0 µl

Template gDNA	1.0 µl		
Nuclease Free Water(NFW)	9.5 µl		
Total	25.0 µl		
Reaction Condition:			
Reaction	**Temperature**	**Time**	**Cycles**
Enzyme activation	95°C	10 minutes	
Step I:			
Denaturation	95°C	30 seconds	15 cycles
Annealing	56.7°C decreasing 0.5°C per cycle	30 seconds	
Extension	72°C	40 seconds	
Step II:			
Denaturation	95°C	30 seconds	20 cycles
Annealing	49.7°C	30 seconds	
Extension	72°C	40 seconds	

The products obtained after polymerase chain reaction was observed by agarose gel (1%) electrophoresis and the size was compared with DNA marker of 100 base pairs.

The amplified 16S' rRNA amplicons were sent to Xcelris Labs Ltd, Old Premchandnagar Road, Opp Satyagrah Chhavani, Sattelite, Ahmedabad-380015, Gujarat, India for sequencing the amplified segments. The sequences obtained from the Xcelris Labs Ltd, were analyzed by using Chromas.lnk software.

3.4.2.3 NCBI Blast and Phylogenetic tree Analysis

The FASTA sequences obtained are processed for NCBI nBLAST and compared with the other myxobacterial strains for the molecular identification of the samples by the help of percentage identity. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version X (Kumar et al., 2018) by using neighbor-joining method with bootstrapping of 1000.

3.5 Extraction of crude metabolites

Isolated myxobacetria were sub-cultured in 100ml LB broth with 50µg/ml cyclohexamide at 30˚C on shaking incubator for 2 days. *E.coli* (200µl, 0.5 O.D at 600nm) was also sub-cultured on 50ml LB broth at 37˚C in shaking incubator 24 hrs. Both the broth were centrifuged at 4000rpm for 10mins. Both the Cell pellets were transferred to 100ml fresh WCX broth and incubated at 30˚C in shaking incubator for 2-3 days. After 2 days broth was observed under microscope so that only myxobacteria remains then only further procedure could be done.100ml myxobacterial broth plus 100ml ethyl acetate were taken and shaken for 60mins at room temperature. Then the mixture was centrifuged at 4000rpm for 20mins. Organic phase (top) was filtered and stored at -20˚C (supernatant). Rota-evaporator was used for concentration at 37°C.

3.6 Determination of Anti-Microbial Activity

The Myxobacterial extracts were diluted in ethyl acetate and 20 µl of the extract dilution plus 80 µl LB broth and 100 µl of a 10^7 CFU/mL of bacterial solutions (active log phase) were added to each well of a sterile 96-well plate and the final volume in each well was 200 µl. Each concentration was tested. Only LB broth with no extracts was kept as negative control. The stock solution of extract was used and was serially diluted to various range of concentration with LB broth and then was incubated at 37℃ for 3 to 4 hrs .It was carried out in microtitre plate in which 0.02% resazurin was added after incubation to detect the anti-microbial activity. Here purple color indicate death or inhibition while pink referred to the viable state of bacteria. Resazurin (7-Hydroxy-3H-phenoxazin-3-one 10-oxide) is a blue non-fluorescent dye that becomes pink and fluorescent when reduced to resorufin by oxidoreductases within viable cells. Resorufin is further reduced to hydroresorufin (uncoloured and nonfluorescent). It is used as an oxidation-reduction indicator in cell viability assays for both aerobic and anaerobic respiration. Thus, Resazurin antimicrobial assay was used as a pre-screening tool for the extracts with antimicrobial potential.

3.7 Identification of Anti-microbial gene

Presence of the polyketide synthesis (PKS) gene responsible for the antimicrobial activity were detected by the amplification of the ketosynthetase(KT) gene and the acyltransferase(AT) genes by using thermal cycler- Polymerase Chain Reaction under following conditions(Komaki et al., 2008)using the enlisted ingredients.

Table 4: Ingredients and Reaction condition for detecting the genes

Particulars:	**Volume per reaction mixture**
Zymo *Taq*^{TM} PCR Premix(2X)	12.5 µl
Forward primer(10 pmol)	1.0 µl
Reverse primer(10 pmol)	1.0 µl
Template gDNA	1.0 µl
Nuclease Free Water(NFW)	9.5 µl
Total	25.0 µl
For Ketosynthetase (KT) gene:	**Forward Primer:** 5'- GCSATGGAYCCSCARCARCGSVT -3'
	Reverse Primer: 5'- GTSCCSGTSCCRTGSSCYTCSAC -3'

Reaction Condition:			
Reaction	**Temperature**	**Time**	**Cycles**
Enzyme activation	95°C	10 minutes	
Denaturation	94°C	30 seconds	25 cycles
Annealing	66°C	30 seconds	
Extension	72°C	1 minute	
Final Extension	72°C	5 minutes	
Hold	4°C	∞	

For Acyltransferase (AT) gene:	**Forward Primer:** 5'- TTCSTSTTYMCSGGVCAGG -3'
	Reverse Primer: 5'- GSGGGCYSABYTCSABGAA -3'

Reaction Condition:			
Reaction	Temperature	Time	Cycles
Enzyme activation	95°C	10 minutes	
Denaturation	94°C	30 seconds	35 cycles
Annealing	64°C	30 seconds	
Extension	72°C	1 minute	

Final Extension	72°C	5 minutes
Hold	4°C	∞

The products obtained after polymerase chain reaction was observed by agarose gel (1%) electrophoresis and the size was compared with DNA marker of 100 base pairs.

3.8 Cellulase Assay

The ability of myxobacteria to produce cellulose enzyme was detected by growing the isolated bacterial culture on the Carboxy-methyl-cellulose (CMC) agar. CMC can bind with the Congo red while the degraded molecule can't bind giving the halozone which confirms the presence of cellulose due to growth of bacteria.

Bacterial isolates were spreaded over the CMC agar plate and incubated at 30°C for 48 hrs. The production of cellulose was screened by flooding the plate with 1% Congo red for 15 minutes. The unbinded Congo red was discarded and then 1M NaCl solution was added over the plate and left for 15 minutes. The excess of NaCl solution discarded and the halozone was observed.

CHAPTER 4
RESULTS

4.1 Isolation and Purification of Myxobacteria

Isolation was performed by the *E. coli* baiting method on the WCX agar plate, where the growth of fruiting bodies were observed with the help of stereo-microscope as in figures in photographs at the end of this report. The following table shows the location of the sample and source of the isolates and their morphology.

Table 5: Sources and morphology of the isolates

S.N.	Location	Source	Isolates	Morphology
1	Bhaktapur	Soil	M2	Colonies: mucilaginous; fruiting bodies: not observed, rounded cell aggregates were observed
2			M4	Colonies: swarm; fruiting bodies: pin-head type observed
3			M9	Colonies: pink swarm; fruiting bodies : reddish-brown Granules
4	Shivapuri	Bark	M5	Colonies: mucilaginous; fruiting bodies: not observed, rounded cell aggregates were observed
5		Soil	M8	Colonies: mucilaginous; fruiting bodies: not observed, rounded cell aggregates were observed
6	Kirtipur	Cow Dung	M6	Ovoid and brown fruiting bodies; radial pattern within swarm area
7			M10	Colonies: mucilaginous; fruiting bodies: not observed, rounded cell aggregates were observed
8			M14	Colonies: mucilaginous; fruiting bodies: not observed, rounded cell aggregates were observed

4.1 Identification of Myxobacteria

4.1.1 Gram Staining

4.1.1.1 Gram staining of vegetative cells

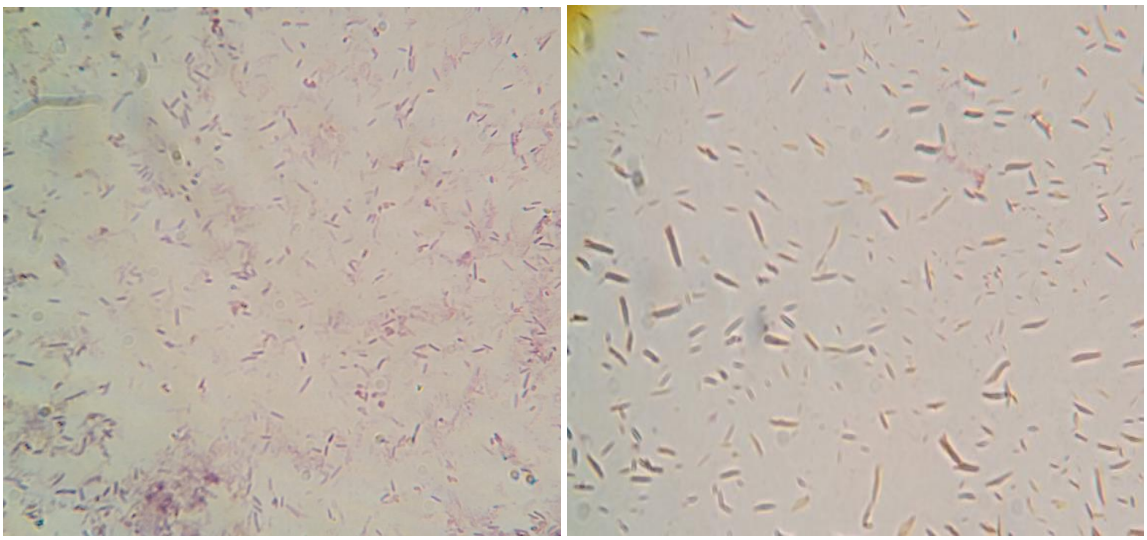

Figure 3: Gram Staining of Myxobacterial cultures in vegetative stage (Make the pictures separate)

Myxobacteria are slender rod shaped Gram negative bacteria in vegetative stage.

4.1.1.2 Gram Staining of cells on starvation

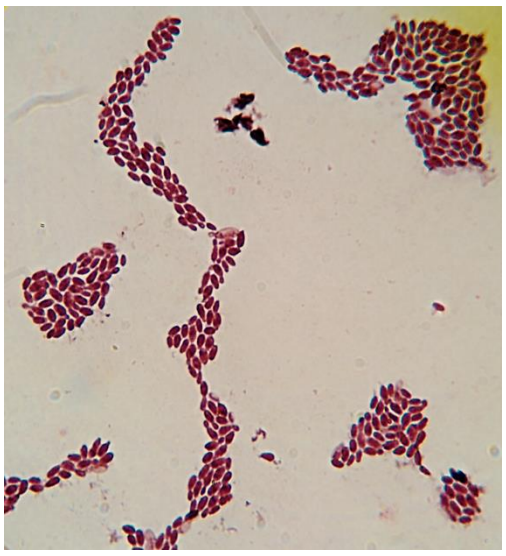

Figure 4: Gram staining of Myxobacteria in Starvation condition

This results shows that the myxobacteria changes its slender rod structure into the spherical shape and colonises at the starvation condition.

4.1.2 Molecular Identification of Myxobacteria

4.1.2.1 Genomic DNA

The genomic DNA of isolates were extracted and the agarose (0.8%) gel electrophoresis was performed as in photograph.

4.1.2.2 16S' rRNA Sequencing By using 16 S rRNA primers, the bands of amplified product was observed at size of 400 base pairs in 16 putative isolates of myxobacteria.

Table 6: Table showing the similarity and percentage identity of isolates after NCBI blast

S.N.	Isolates	Similarity with	Percentage identity
1	M2	*Myxobacterium* NU-2	85.46%
2	M4	*Byssovorax cruenta*	83.82%
3	M5	*Polyangium sp.*	94.90%
4	M6	*Archangium minus*	82.96%
5	M8	*Byssovorax cruenta*	84.21%
6	M9	*Corallococcus coralloides*	86.57%
7	M10	*Vitiosangium cumulatum*	82.73%
8	M14	*Vitiosangium cumulatum*	95.51%

The chromatogram of 16S' rRNA sequencesof the myxobacterial samples are in appendix and the phylogenetic tree of each samples are illustrated below:

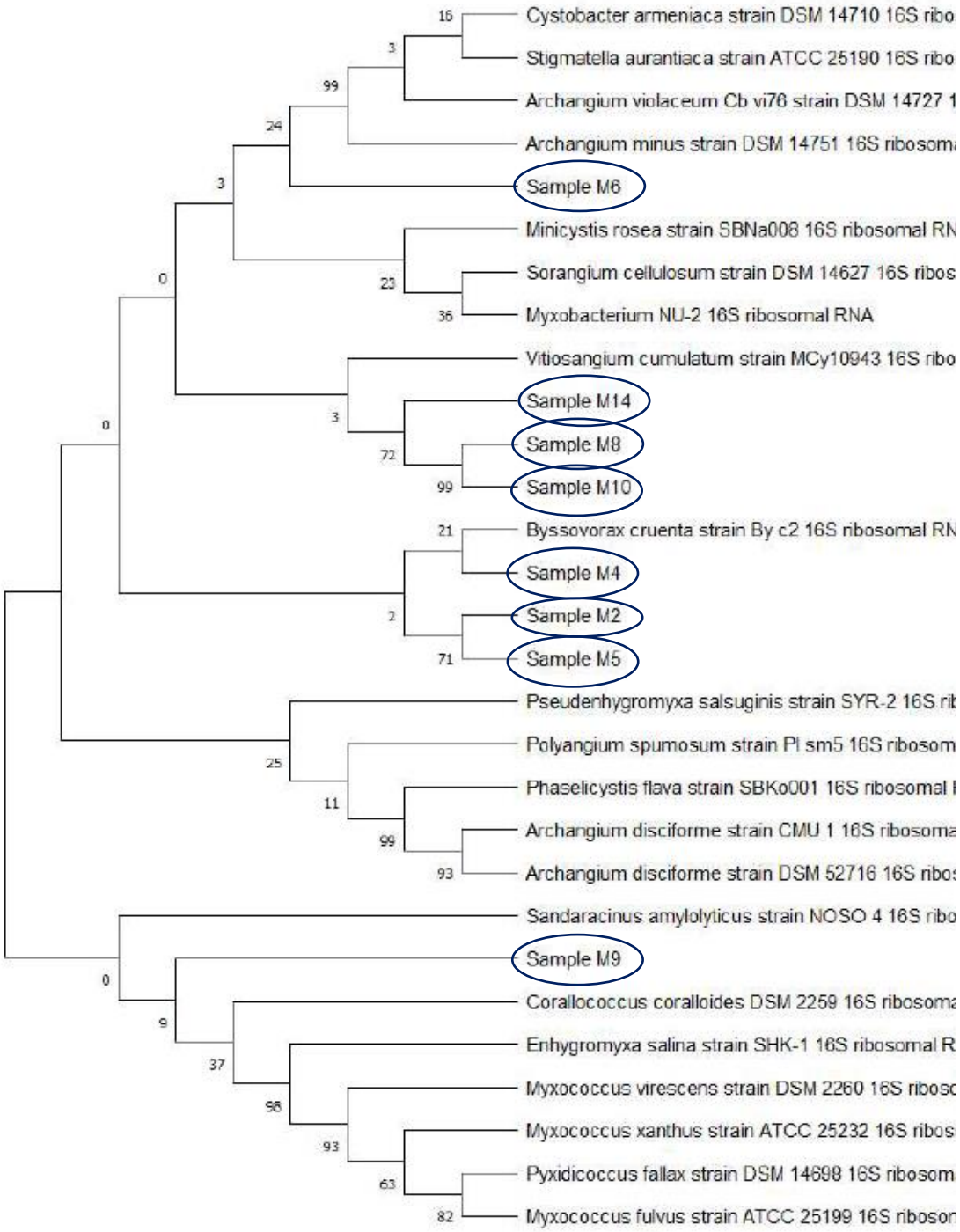

Figure 5: Phylogenetic tree of the isolated myxobacteria constructed using the neighborjoining method based on 16S rRNA gene sequences aligned to their closest type strains. Phylogeny was tested with 1000 bootstrap replications.

4.2 Determination of minimum Inhibitory concentration

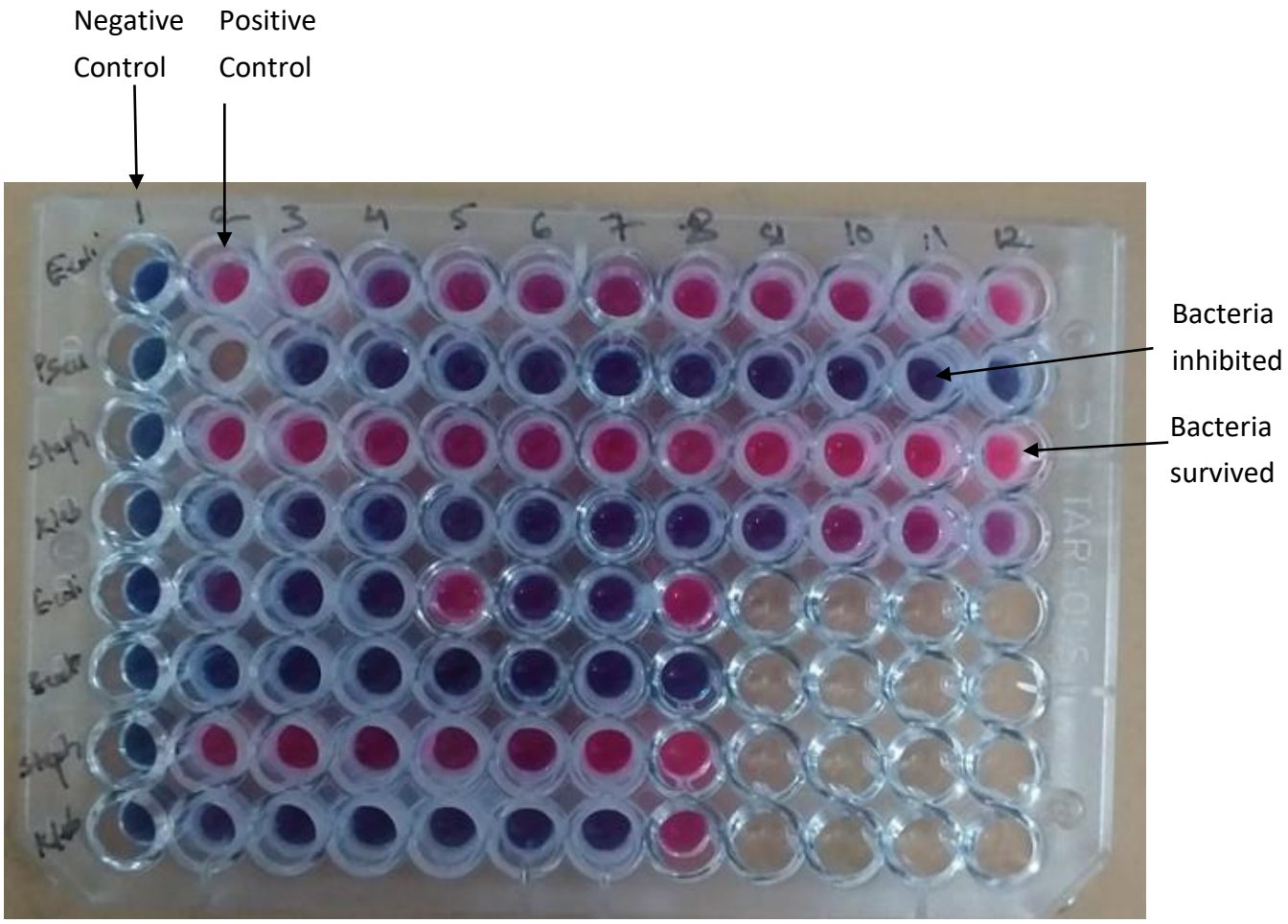

Figure 6: Resazurin Assay for anti-microbial activity

(Purple color indicate death or inhibition while pink referred to the viable state of bacteria. The figure indicates that *Pseudomonas aeruginosa* and *Klebsiella pneumonia* were inhibited by the most of the extracts. Resazurin antimicrobial assay was used as a pre-screening tool for the extracts with antimicrobial potential.)

4.3 Amplification of keto- synthase gene and Acyl-transferase gene

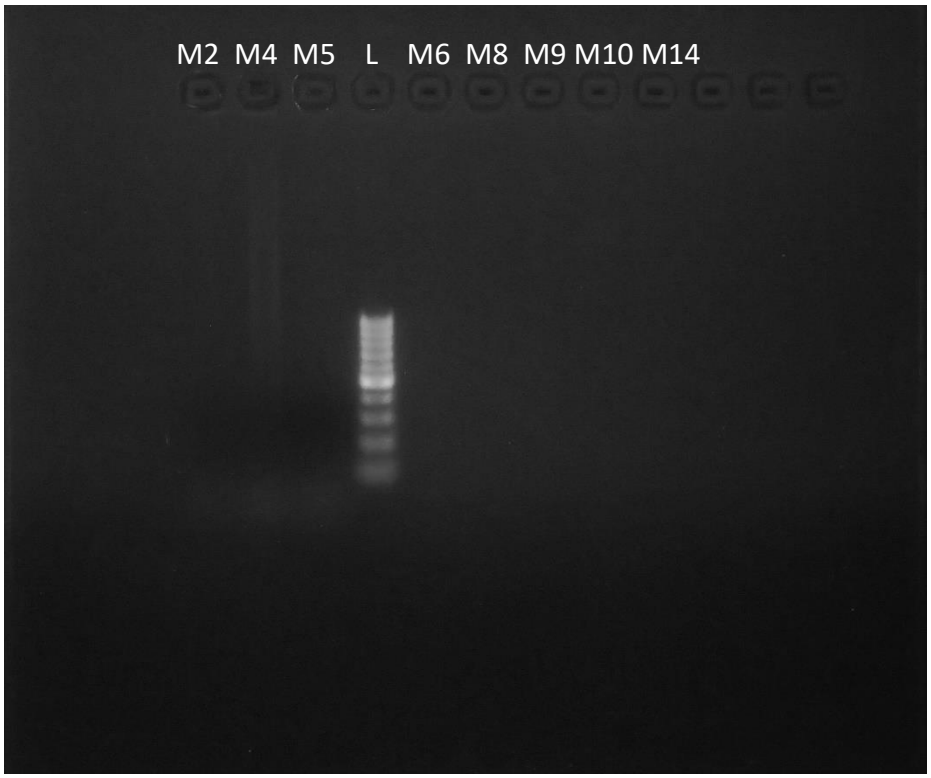

Figure 7: Agarose gel electrophoresis of the amplified product of the ketosynthase gene

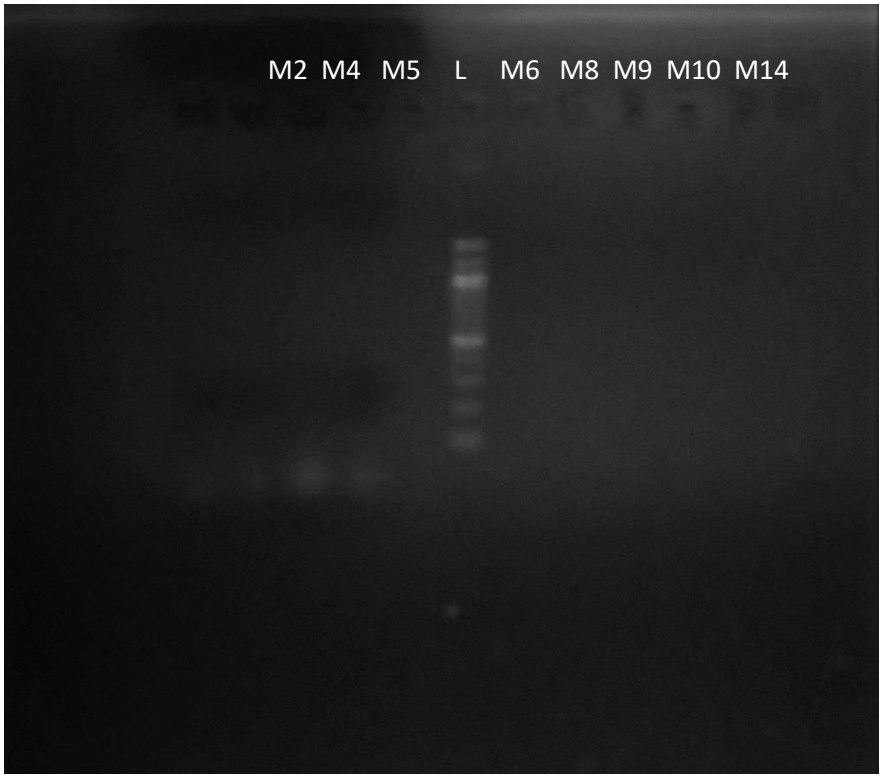

Figure 8: Agarose gel (1%) electrophoresis of the amplified product of the acyl-transferase gene

4.4 Cellulase Assay

Myxobacteria feed on the cellulosic masses present on the forest, soil and dung, so some of them are capable of secreting cellulose enzyme, which is confirmed by cellulose assay on CMC agar plate. Most of the isolates showed the cellulolytic activity which are tabulated below.

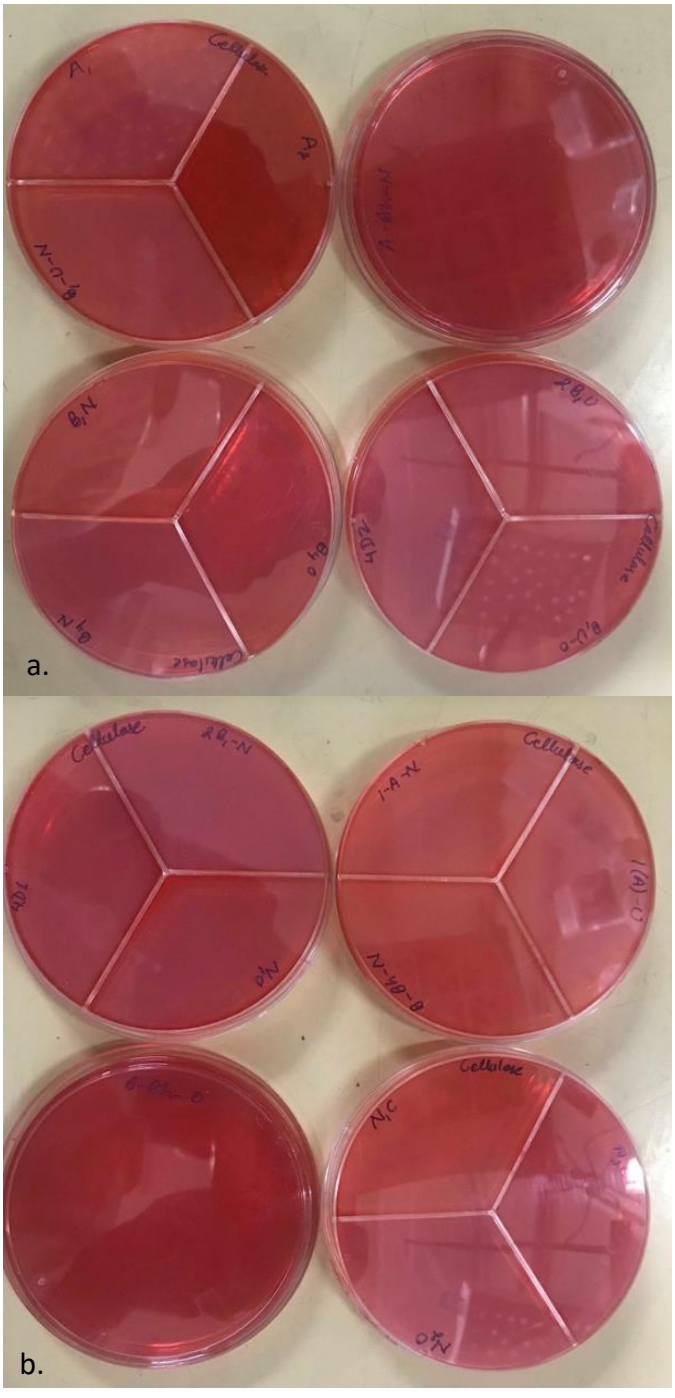

Figure 9:(a), (b): Cellulase assay of myxobacterial isolates.

(The dark red indicates the binding of Congo red with the CMC agar while the halozone represents the degradation of cellulose due to the production of the cellulose enzyme)

Table 7: Cellulolytic activities of isolates

S.N.	**Isolates**	**Halozone on cellulose assay**	**Inferences**
1	M2	Halozone absent	Presence of cellulose on media, non-cellulolytic bacteria
2	M4	Halozone present	Absence of cellulose in media, cellulolytic bacteria
3	M5	Halozone present	
4	M6	Halozone absent	Presence of cellulose on media, non-cellulolytic bacteria
5	M8	Halozone present	Absence of cellulose in media, cellulolytic bacteria
6	M9	Halozone present	
7	M10	Halozone present	
8	M14	Halozone present	

CHAPTER 5
DISCUSSION

The emergence of antimicrobial resistance has threatened patients' lives worldwide and imposed an economic burden on the health care systems and patients. Myxobacteria have recently received a remarkable attention because of their significant potential for the production of bioactive compounds. Myxobacteria are Gram-negative, usually soil-dwelling bacteria that feed on other microorganisms or macromolecular organic matter (Reichenbach and Dworkin, 1992; Reichenbach, 1993). They are unique among prokaryotes for their complicated multicellular behavior, especially the morphogenesis of fruiting bodies, and therefore are considered as social bacteria (Shimkets, 1990; Dworkin, 1996).The unique morphogenetic traits of myxobacteria are the primary bases not only of their taxonomy, but also of the isolation techniques (Reichenbach and Dworkin, 1992). Using morphogenesis-dependent enriching and isolation techniques, myxobacteria are found nearly everywhere (Reichenbach, 1993; Dawid, 2000). The myxobacteria can be divided into two suborders, four families, 12 genera and approximately 40 species according to their morphological characters (McCurdy, 1969; Reichenbach and Dworkin, 1992). According to the homologies of 16S rRNA sequences, the myxobacteria are located in delta-division of Proteobacteria (Oyaizu and Woese, 1985) and form three sublines, i.e. *Myxococcus*, *Chondromyces* and *Nannocystis* (Ludwig *et al*., 1983; Shimkets and Woese, 1992).

Myxobacteria were isolated from the soil, dung and the bark samples from the different places of the Kathmandu valley which are grown on the WCX media by *E. coli* baiting method, where the myxobacteria feeds upon the bacteria for their growth.(How many isolates from how many sample). This method of isolation also make the culture prone to contamination by other bacteria during the purification process. They show different morphological characters based on the supplement of the nutrients. They are slender rods in the nutrient rich condition while they become spherical in shape in starvation condition and colonises to form the fruiting bodies. In general, the isolation of myxobacteria of all types can be a time-consuming and often tedious process. They are usually obtained from herbivore dung, decaying plant material and bark of living and dead trees by placing samples of these materials in a moist chamber (Reichenbach and Dworkin, 1991) and observing them frequently for the development of characteristic fruiting bodies. They can also be recovered from soil by baiting with herbivore dung and by placing soil on streaks of bacterial or yeast cells on agar plates (Singh, 1947). Under starvation conditions, myxobacteria start a multicellular development cycle. Cells

aggregate, building characteristic multi-cellular fruiting bodies containing myxospores which are resistant against extreme environmental conditions like temperature changes, desiccation, and UV radiation. As soon as environmental conditions become more favorable, the myxospores are retransformed into normal vegetative cells. The fruiting bodies that form are picked and streaked in the hope of obtaining a pure culture. Often, however, the fruiting bodies are contaminated with other bacteria, and an involved purification process is usually necessary (Reichenbach and Dworkin, 1991).

Myxobacteria have the potentiality to carry out cooperative social behavior in their life cycle, especially the fruiting body morphogenesis (Shimkets, 1990; Dworkin, 1996). The social characters of myxobacteria unfortunately limit the isolation techniques, i.e. individual myxobacterial cells are not readily growing into colonies and consequently this determines that the isolation of myxobacteria has normally to be achieved by scattering soil samples on baiting agar and picking up the fruiting bodies raised (Reichenbach and Dworkin, 1992).

The use of 16S rRNA gene sequences to study bacterial phylogeny and taxonomy has been by far the most common housekeeping genetic marker used for a number of reasons. These reasons include its presence in almost all bacteria, often existing as a multigene family, or operons; the function of the 16S rRNA gene over time has not changed, suggesting that random sequence changes are a more accurate measure of time (evolution); and the 16S rRNA gene (1,500 bp) is large enough for informatics. The analysis of 16 S' rRNA sequence reveals that the isolates Isolate M2 has highest similarity with Myxobacterium NU-2. The isolates M4 and M8 has high similarity with the *Byssovorax cruenta* while M6 has high percentage identity with *Archangium minus* and M5 has similarity with *Polyangium sp.* M9 shows the similarity with both *Corallococcus coralloides* while M14 has high percentage identity with *Vitiosangium cumulatum* followed by isolates M10. A divergence of 4 % in the 16SrRNA gene sequence from its closest relatives has been used for the proposal of a novel genus in myxobacteria (Garcia et al., 2010).

In case of antibacterial activity of the myxobacteria resazurin assay was used .Resazurin (7-Hydroxy-3H-phenoxazin-3-one 10-oxide) is a blue non-fluorescent dye that becomes pink and fluorescent when reduced to resorufin by oxidoreductases within viable cells. Resorufin is further reduced to hydroresorufin (uncoloured and nonfluorescent). It is used as an oxidation-reduction indicator in cell viability assays for both aerobic and anaerobic respiration. From the experiment, it is found that ATCC culture of *Pseudomonas aeruginosa* and *Klebsiella pneumonia* are susceptible to most of the extracts. Other bacterial samples used like *Staphylococcus aureus* and *E. coli* are found

to be resistant to the extracts. Although these extracts inhibits the two bacteria but it was found that the myxobacterial isolates lacks the polyketide synthase (PKS) gene and the acyl-transferase (AT) gene. This result suggest that the antimicrobial activity is due to metabolites other than the polyketides and the acyltransferase. This inhibition may be due to the other genes including non-ribosomal proteins. Myxobacteria are prolific producers of a variety of bioactive secondary metabolites including antibacterial, antifungal, antiviral and antitumor compounds. Remarkably, Reichenbach found that 60-80% of the myxobacteria tested had antibacterial or antifungal activity. About 50 basic structures and 300 structural variants have been described from these cultures, and most appear to be unique to myxobacteria. Even though these cultures are attractive sources of new compounds, they have not received wide attention in pharmaceutical bioactive metabolite screening programs primarily because they are difficult to isolate (Karwowski et al., 1996).

Screening for extracellular cellulase production by bacteria and fungi is often done on agar plates containing CMC as substrate (Dashtban et al., 2009). The detection of the cellulolytic activity in these cases is achieved by staining or precipitation of undigested CMC in plate regions which were not exposed to cellulolytic activity, while areas exposed to cellulase give clear halos surrounding the source of the enzyme. This method is popular because large numbers of samples can be monitored and compared simultaneously and quickly. Over the decades, a variety of dyes have been introduced for this differential staining, Congo red and the Gram's iodine are the most commonly used ones. Despite its wide use, the plate clearing assay based on CMC–agar is notorious for its low specificity, producing halos around other polymer-degrading enzymes like amylase and agarase, independent of the presence or absence of a cellulase substrate (e.g., CMC). The artifacts occur likewise with other gelling agents like agarose and Gelrite and with other staining methods like Congo red. Among these problems, particularly the substrate-independent formation of halos seriously impacts one of the most important criteria of enzyme assays, their specificity and precludes any chances to quantify cellulase activities (Johnsen and Krause, 2014).

CHAPTER 6
SUMMARY

Myxobacteria are unique among bacteria due to their complicated multicellular morphogenesis and behavior. They have been isolated from a variety of environment and substrates particularly from soil of tropical to temperate region. The genuine habitat of myxobacteria is the soil, as long as the pH is slightly acid to slightly alkaline, i.e. between5 and 8. Frequently myxobacteria are found on the dung of herbivorous animals, on decaying plant material and on the bark of trees Myxobacteria are micropredators of various microorganisms including Gram-positive and Gram-negative bacteria. Antibiotics and enzymes produced by myxobacteria kill microorganisms and lyse cells from which biomacromolecules are destroyed. Antibiotics and enzymes degrade structural cell macromolecules such as polysaccharides, proteases and nucleases.

In this study, twenty one isolates were isolated and the isolates with different morphology was further processed. Isolation of the myxobacteria is a tedious process as the myxobacteria are the slow growers and it may get contaminated with other bacteria due to its predatory action. The transfer of the fruiting bodies from one medium to the next is also the challenging factor as the fruiting bodies may burst due to the mechanical stress during transfer. 16 S' rDNA sequences of 13 different isolates were sequenced. After the phylogenetic analysis, 8 samples showing the higher percentage identity with myxobacterial sequence in the NCBI gene library were further processed for amplification of the ketosynthase gene and acyltransferase gene which reveal the absence of both genes. The crude extracts inhibited the *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* which may be due to the other metabolites rather than the ketides and acyl-transferases. The inhibition of the bacterial samples by the extract may be due to the other metabolites such as other ribosomal proteins, non-ribosomal proteins, hybrids of ketides and non-ribosomal proteins. Most of the isolates were also found to have the cellulolytic activity. Myxobacteria are also found in the bark, dead and decaying materials in the environment and they prey on other bacteria for their survival, they are capable of secreting many enzymes for degradation of polysaccharides, proteins, lipids and nucleic acids.

Exploration of myxobacteria will be the promising source of antibiotics due to its ability to synthesize cocktails of the diverse metabolites. The enzymes production may also help in the industries and degrading the complex molecules. The predatory behavior makes its possibility for the use in agricultural fields as the biopesticedes.

CHAPTER 7
CONCLUSION

Myxobacteria, the group of slender rod shaped bacteria with varying morphology in different stages of life cycle shows the social behavior and colonizes to form the visible fruiting bodies. Myxobacteria can be isolated from the soil, bark and dung of the herbivorous animals. They secretes the enzymes as well as the other metabolites which makes their survival easier. With the help of the secretions, they prey on other bacteria and get nutrition. Some of the myxobacteria were also capable to degrade the cellulose. The metabolites produced by the myxobacteria may have the high potential in the treatment of diseases.

CHAPTER 8
RECOMMENDATIONS

Though the myxobacteria produces variety of secondary metabolites, they are not explored properly. Certain factors like the tedious method of culture, purification and the long incubation time periods may be the causes that hampers the exploration of myxobacteria and its metabolites. So, determination of the appropriate method of isolation and purification in low time will be helpful for further research works. Furthermore, identification of the potent drug for the treatment of microbial diseases and cancer may help for the maintenance of the healthy future generation. Further studies should be carried out like phytopharmacology, isolation and identification of active biocompounds which may be followed by development of lead molecules. Hence, Myxobacteria can be explored for the identification of the new drugs as well as for the industrial purpose due to its ability to degrade the complex organic macromolecules.

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APPENDICES

Appendix A

FASTA sequences of the myxobacterial isolates obtained from 16 S rRNA sequencing

>Sample_M2

AACAGTAGCATCTGATCTACGATTACTAGCGATTCCGACTTCATGGAGTCGAGTTGCAGACTCCA
ATCCGGACTACGACATACTTTATGAGGTCCGCTTGCTCTCGCGAGGTCGCTTCTCTTTGTATATG
CCATTGTAGCACGTGTGTAGCCCTACTCGTAAGGGCCATGATGACTTGACGTCATCCCCACCTTC
CTCCAGTTTATCACTGGCAGTCTCCTTTGAGTTCCCGGCCGAACCGCTGGCAACAAAGGATAAG
GGTTGCGCTCGTTGCGGGACTTAACCCAACATTTCACAACACGAGCTGACGACAGCCATGCAGC
ACCTGTCTCAGAGTTCCCGAAGGCACCAATCCATCTCTGGAAAGTTCTCTGGATGTCAAGAGTA
GGTAAGGTCCTTTTCCGCGTAAAAA

>Sample_M4

GGCAGAAAATTAACGAGGAAGTGCATCTGATCACGATTACTAGCGATTCCGACTTCATGGAGTC
GAGTTGCAGACTCCAATCCGGACTACGACGCACTTTATGAGGTCCGCTTGCTCTCGCGAGGTCG
CTTCTCTTTGTATGCGCCATTGTAGCACGTGTGTAGCCCTACTCGTAAGGGCCATGATGACTTGA
CGTCATCCCCACCTTCCTCCAGTTTATCACTGGCAGTCTCCTTTGAGTTCCCGGCCGGACCGCTGG
CAACAAAGGATAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATTTCACAACACGAGCTGAC
GACAGCCATGCAGCACCTGTCTCAGAGTTCCCGAAGGCACCAATCCATCTCTGGAAAGTTCTCTG
GATGTCAAGAGTAGGTAAGGTTCCTTCGCGTTAAA

>Sample_M5

ATCAGCGAATGCTGATCCGCGATTACTAGCGATTCCGACTTCACGCAGTCGAGTTGCAGACTGC
GATCCGGACTACGATCGGGTTTCTGGGATTGGCTCCCCCTCGCGGGTTGGCGACCCTCTGTCCC
GACCATTGTATGACGTGTGAAGCCCTACCCATAAGGGCCATGAGGACTTGACGTCATCCCCACC
TTCCTCCGGTTTGTCACCGGCAGTCTCATTAGAGTGCCCTTTCGTAGCACTAATGACAAGGGTTG
CGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAGCCATGCAGCACCTG
TGTTCCGGTTCTCTTGCGAGCACTTCCAAATCTCTTCGGAATTCCAGACATGTCAAGGGTAGGTA
AGGTTCTTTCGCGTTAAAATTA

>Sample_M6

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TGCGCCATTGTAGCACGTGTGTAGCCCTACTCGTAAGGGCCATGATGACTTGACGTCATCCCCAC
CTTCCTCCAGTTTATCACTGGCAGTCTCCTTTGAGTTCCCGGCCGGACCGCTGGCAACAAAGGAT
AAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATTTCACAACACGAGCTGACGACAGCCATGC

AGCACCTGTCTCAGAGTTCCCGAAGGCACCAATCCATCTCTGGAAAGTTCTCTGGATGTCAAGA
GTAGGTAAGGTTCTTCGCGTTAA

>Sample_M8

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CCATTGTAGCACGTGTGTAGCCCTACTCGTAAGGGCCATGATGACTTGACGTCATCCCCACCTTC
CTCCAGTTTATCACTGGCAGTCTCCTTTGAGTTCCCGGCCGGACCGCTGGCAACAAAGGATAAG
GGTTGCGCTCGTTGCGGGACTTAACCCAACATTTCACAACACGAGCTGACGACAGCCATGCAGC
ACCTGTCTCAGAGTTCCCGAAGGCACCAATCCATCTCTGGAAAGTTCTCTGGATGTCAAGAGTA
GGTAAGGTCCCTTCGCGTTAAA

>Sample_M9

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ATGCGCCATTGTAGCACGTGTGTAGCCCTACTCGTAAGGGCCATGATGACTTGACGTCATCCCC
ACCTTCCTCCAGTTTATCACTGGCAGTCTCCTTTGAGTTCCCGGCCGAACCGCTGGCAACAAAGG
ATAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATTTCACAACACGAGCTGACGACAGCCAT
GCAGCACCTGTCTCAGAGTTCCCGAAGGCACCAATCCATCTCTGGAAAGTTCTCTGGATGTCAA
GAGTAGGTAAGGTTCTTCGCGTTAAA

>Sample_M10

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CTCCAGTTTATCACTGGCAGTCTCCTTTGAGTTCCCGGCCGGACCGCTGGCAACAAAGGATAAG
GGTTGCGCTCGTTGCGGGACTTAACCCAACATTTCACAACACGAGCTGACGACAGCCATGCAGC
ACCTGTCTCAGAGTTCCCGAAGGCACCAATCCATCTCTGGAAAGTTCTCTGGATGTCAAGAGTA
GGTAAGGCCTCTTTCCGCGTTCAA

>Sample_M14

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CCTCCGGTTTGTCACCGGCAGTCTCATTAGAGTGCCCTTTCGTAGCAACTAATGACAAGGGTTGC
GCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACGGCCATGCAGCACCTGT
GTTCCGGTTCTCTTGCGAGCACTTCCAAATCTCTTCGGAATTCCAGACATGTCAAGGGTAGGTAA
GTTCCTTTCCCCGGTTAATAGTC

>SAMPLE_M15

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CTCCAGTTTATCACTGGCAGTCTCCTTTGAGTTCCCGGCCGGACCGCTGGCAACAAAGGATAAG
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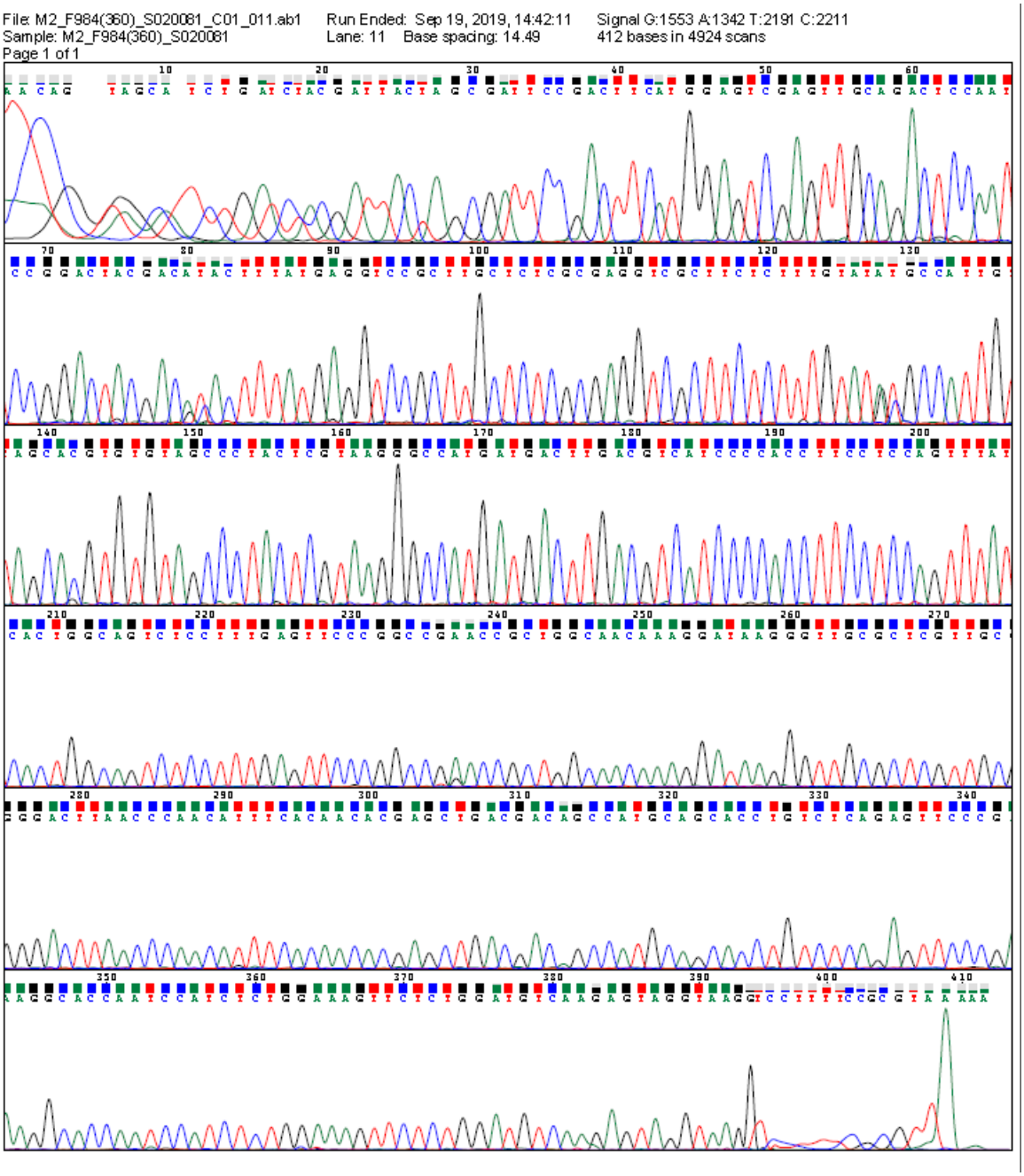


Figure 10: Chromatogram of the sequences of sample M2 generated by Chromas software.

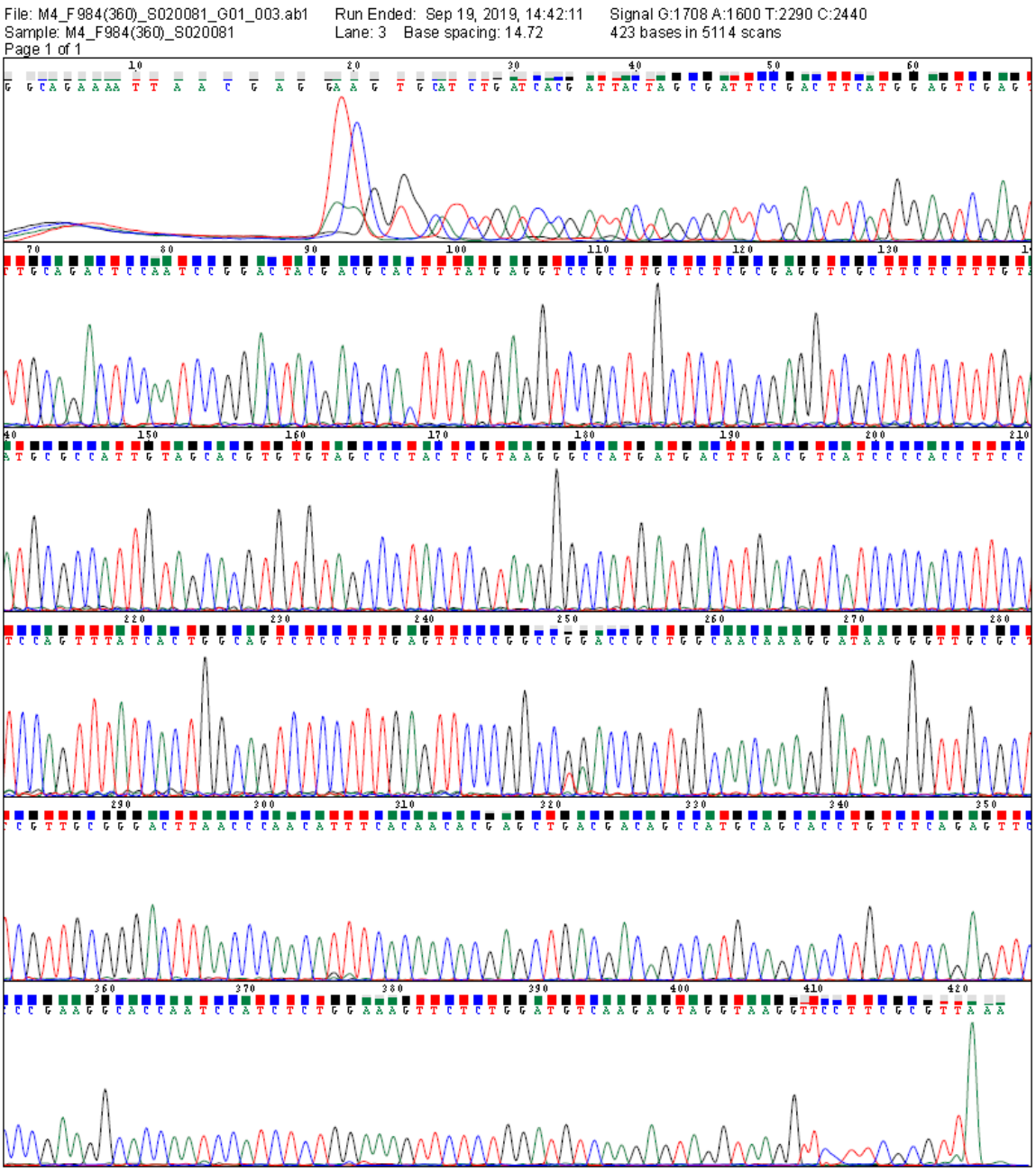


Figure 11: Chromatogram of the sequences of sample M4 generated by Chromas software

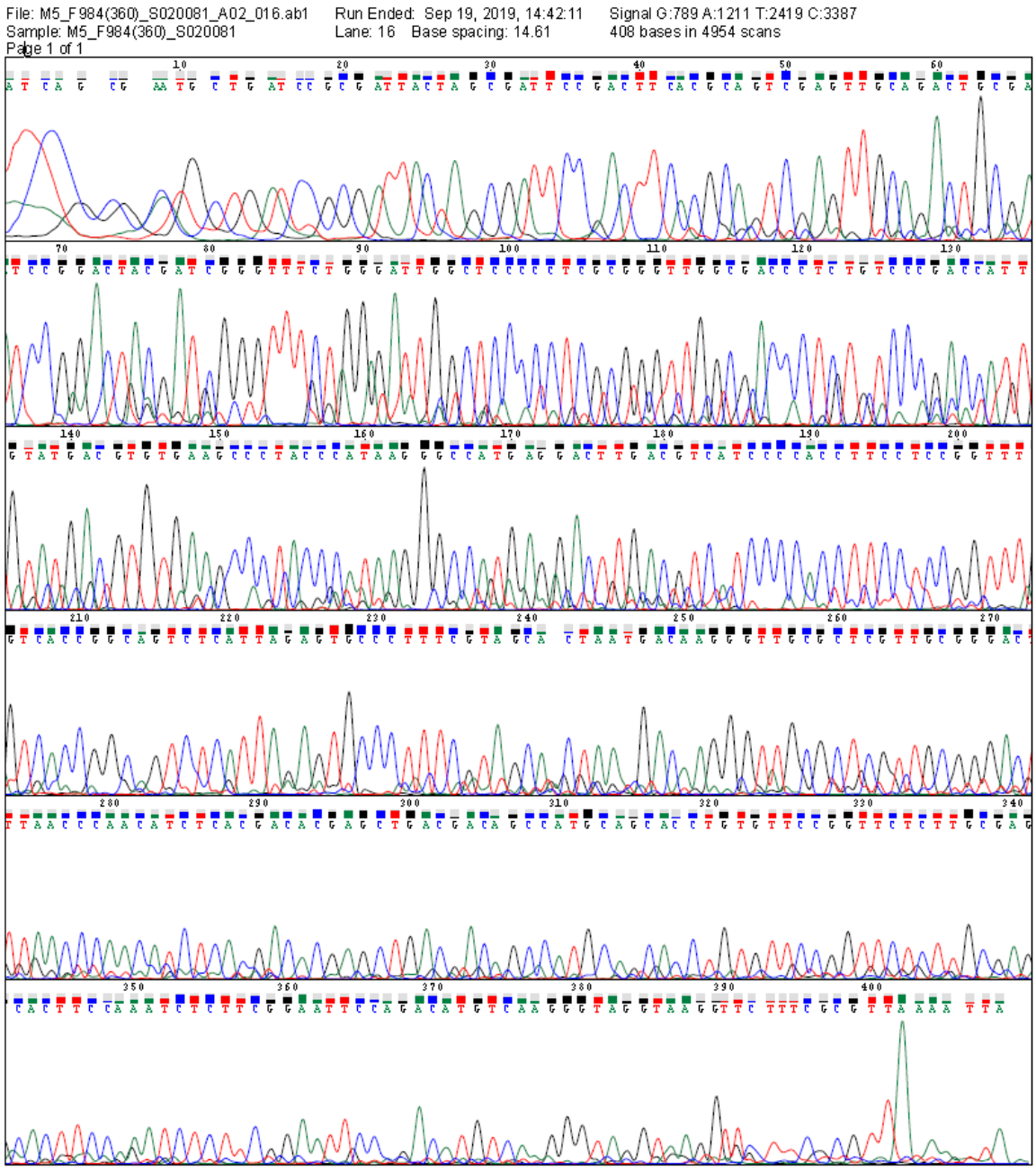


Figure 12: Chromatogram of the sequences of sample M5 generated by Chromas software

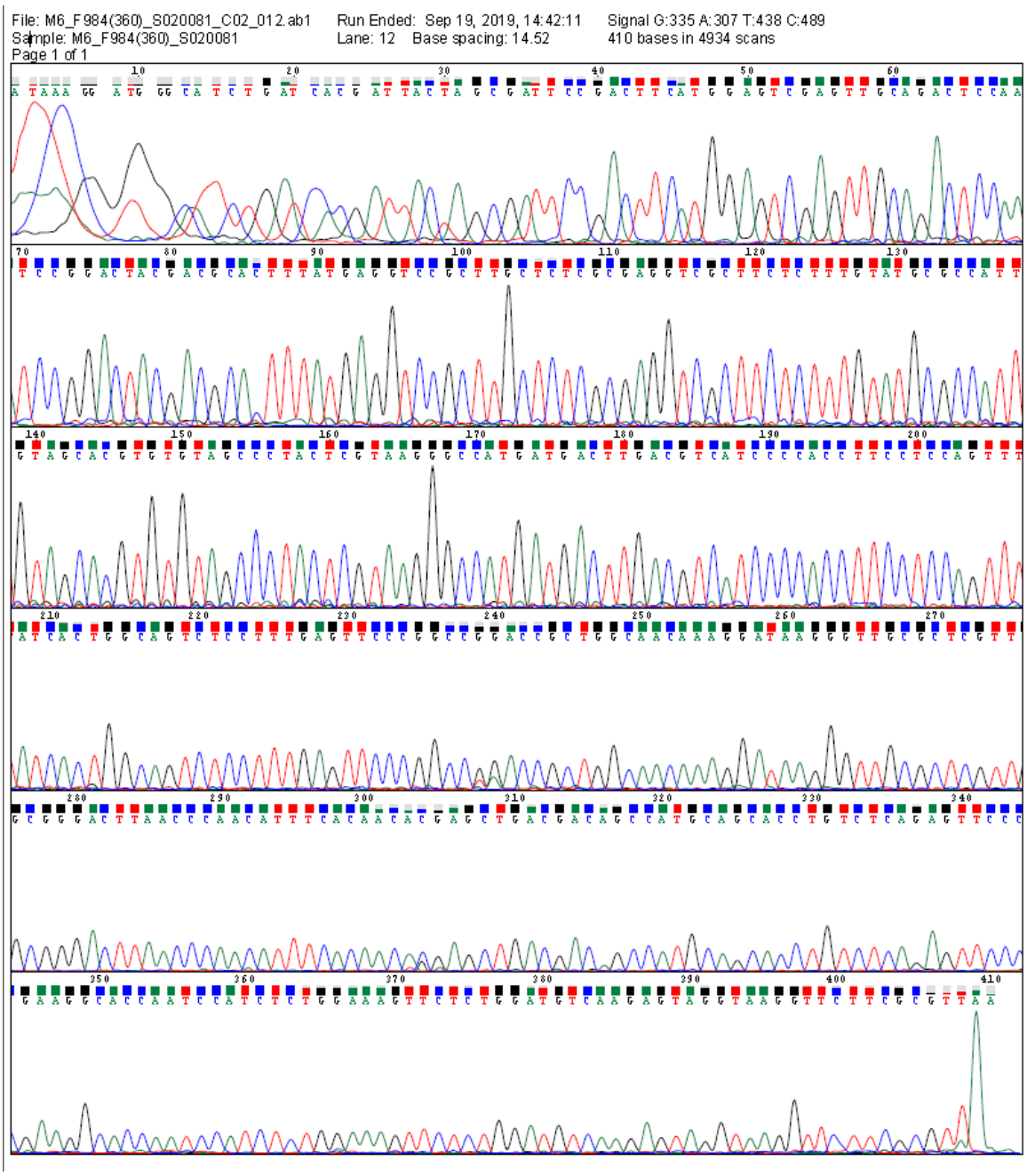


Figure 13: Chromatogram of the sequences of sample M6 generated by Chromas software

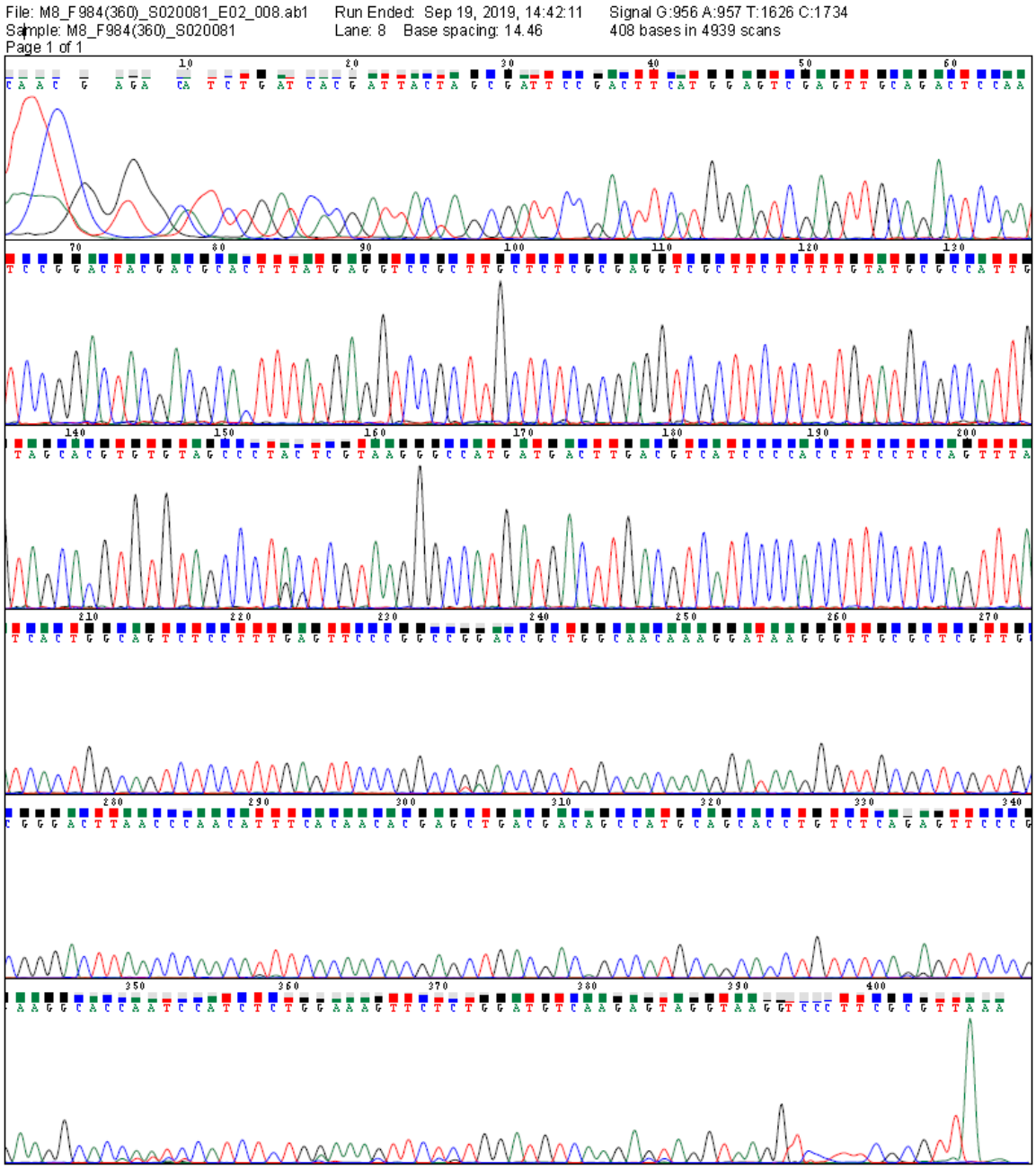


Figure 14: Chromatogram of the sequences of sample M8 generated by Chromas software

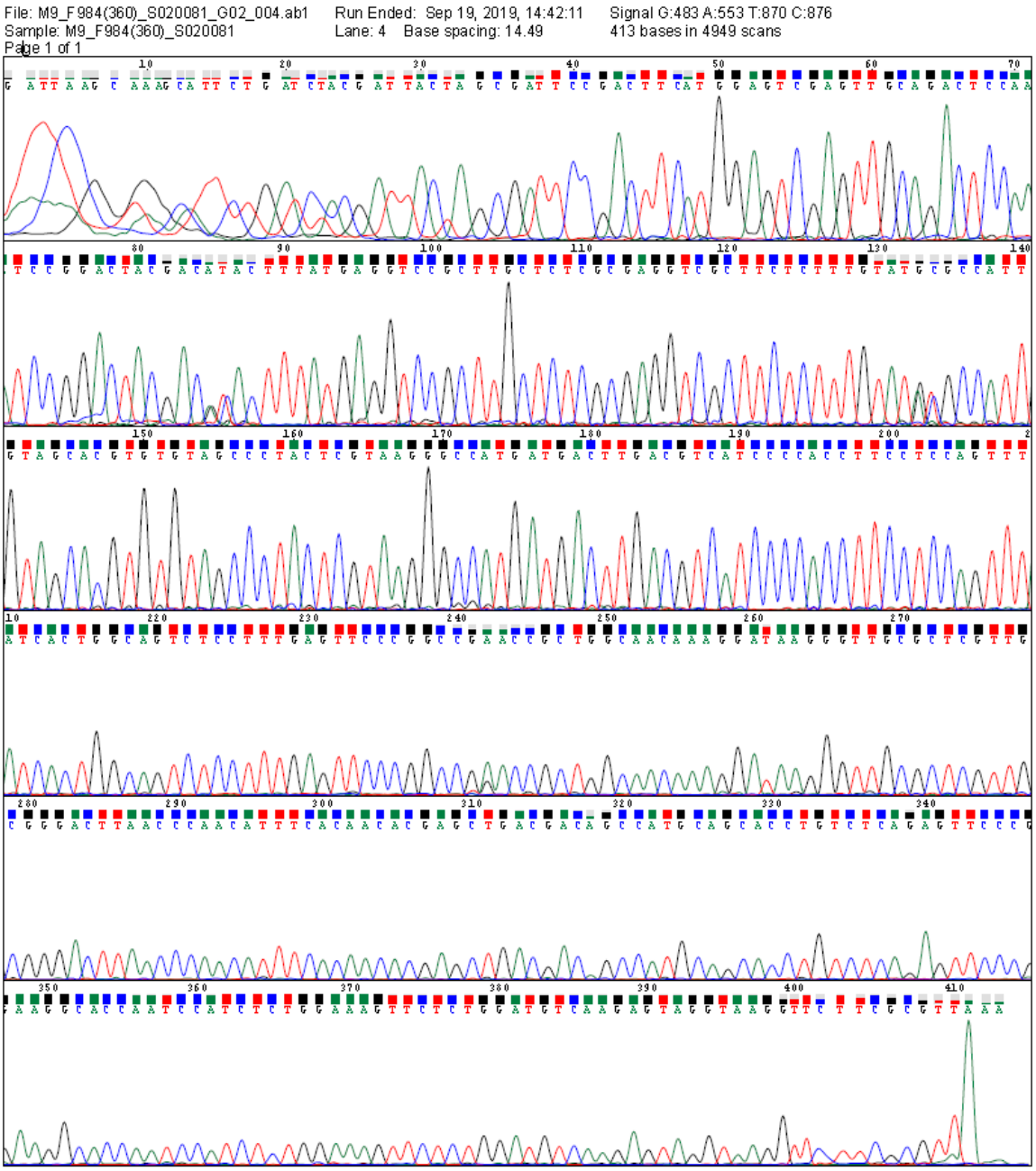


Figure 15: Chromatogram of the sequences of sample M9 generated by Chromas software

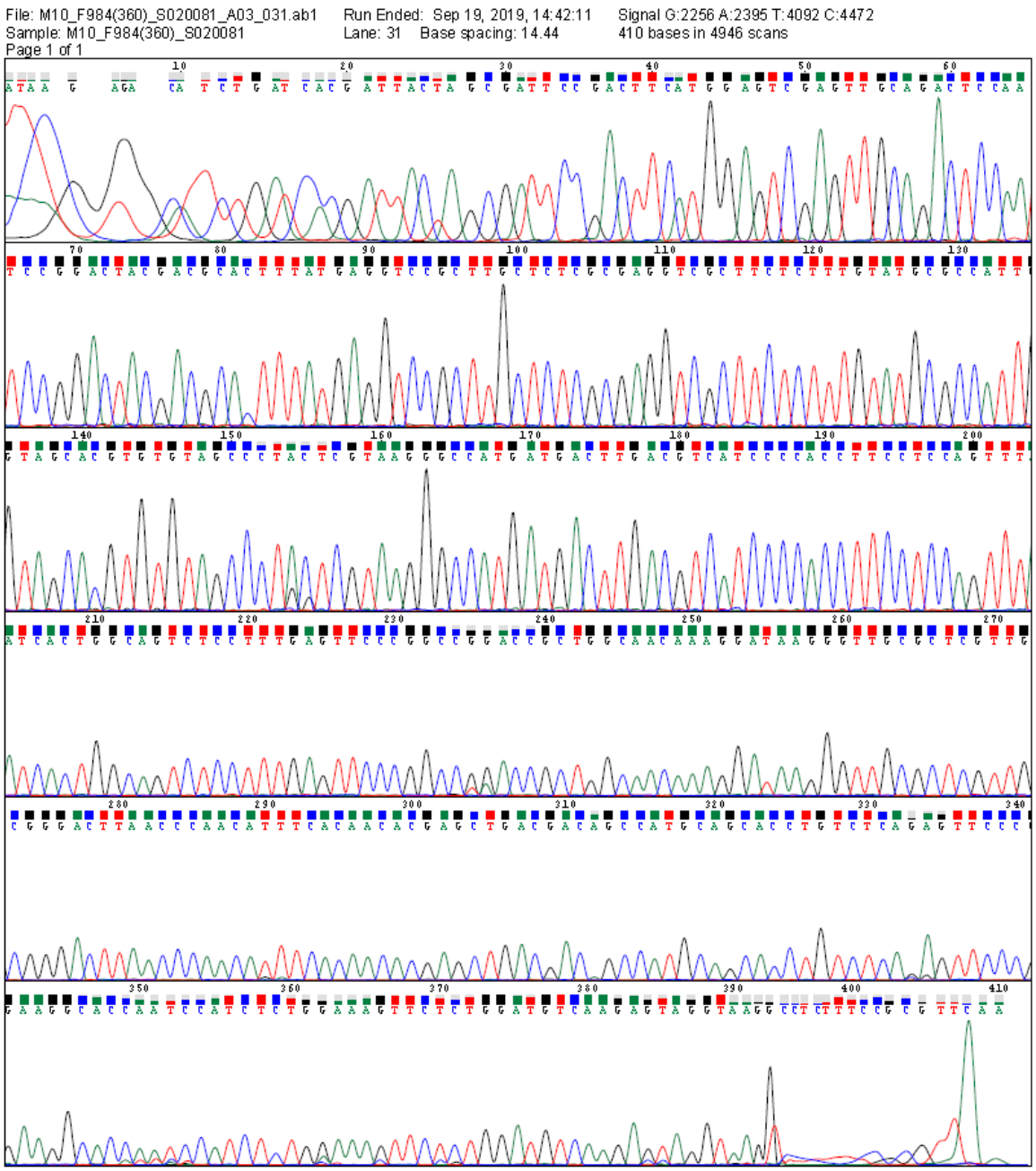


Figure 16: Chromatogram of the sequences of sample M10 generated by Chromas software

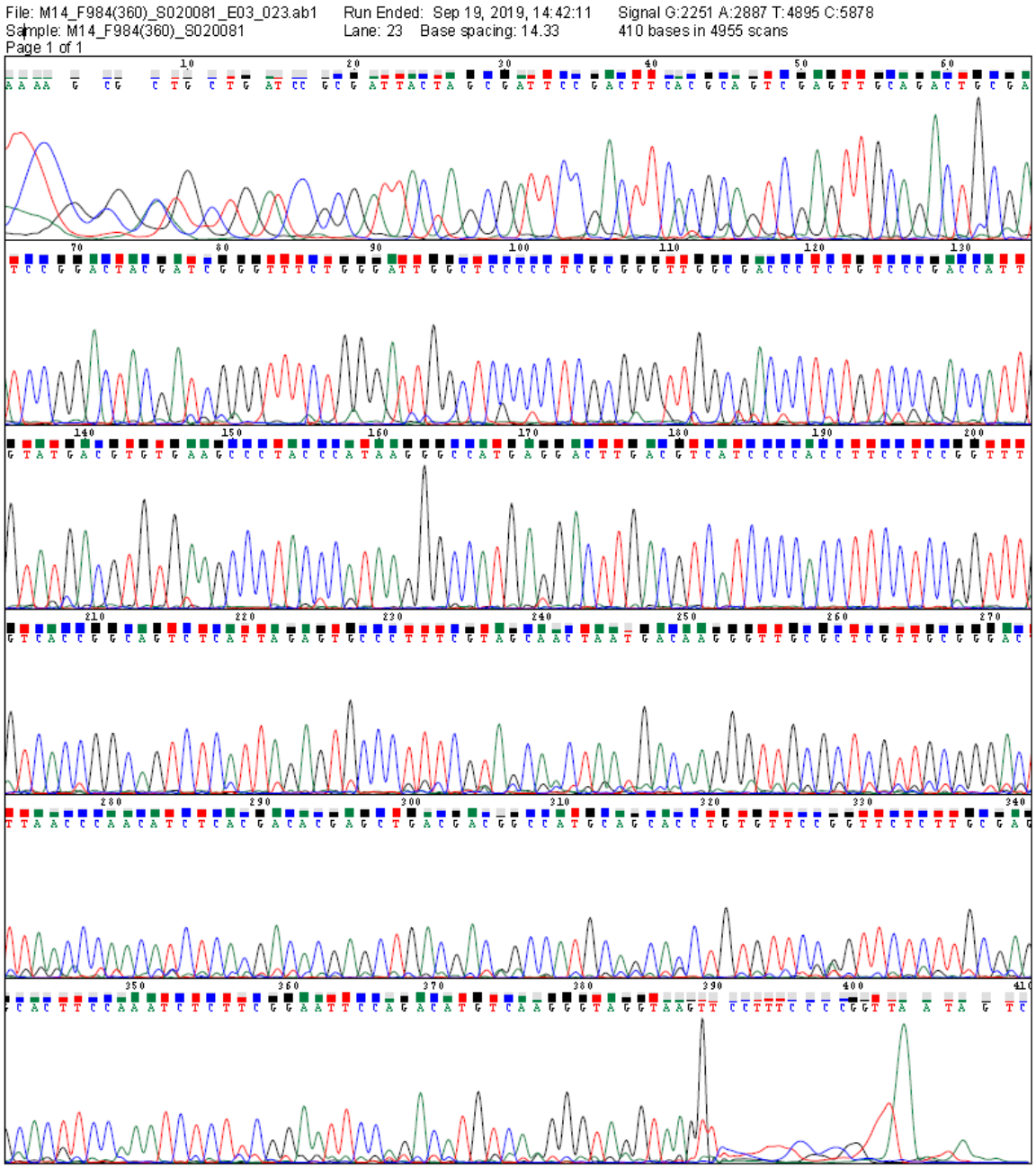


Figure 17: Chromatogram of the sequences of sample M14 generated by Chromas software

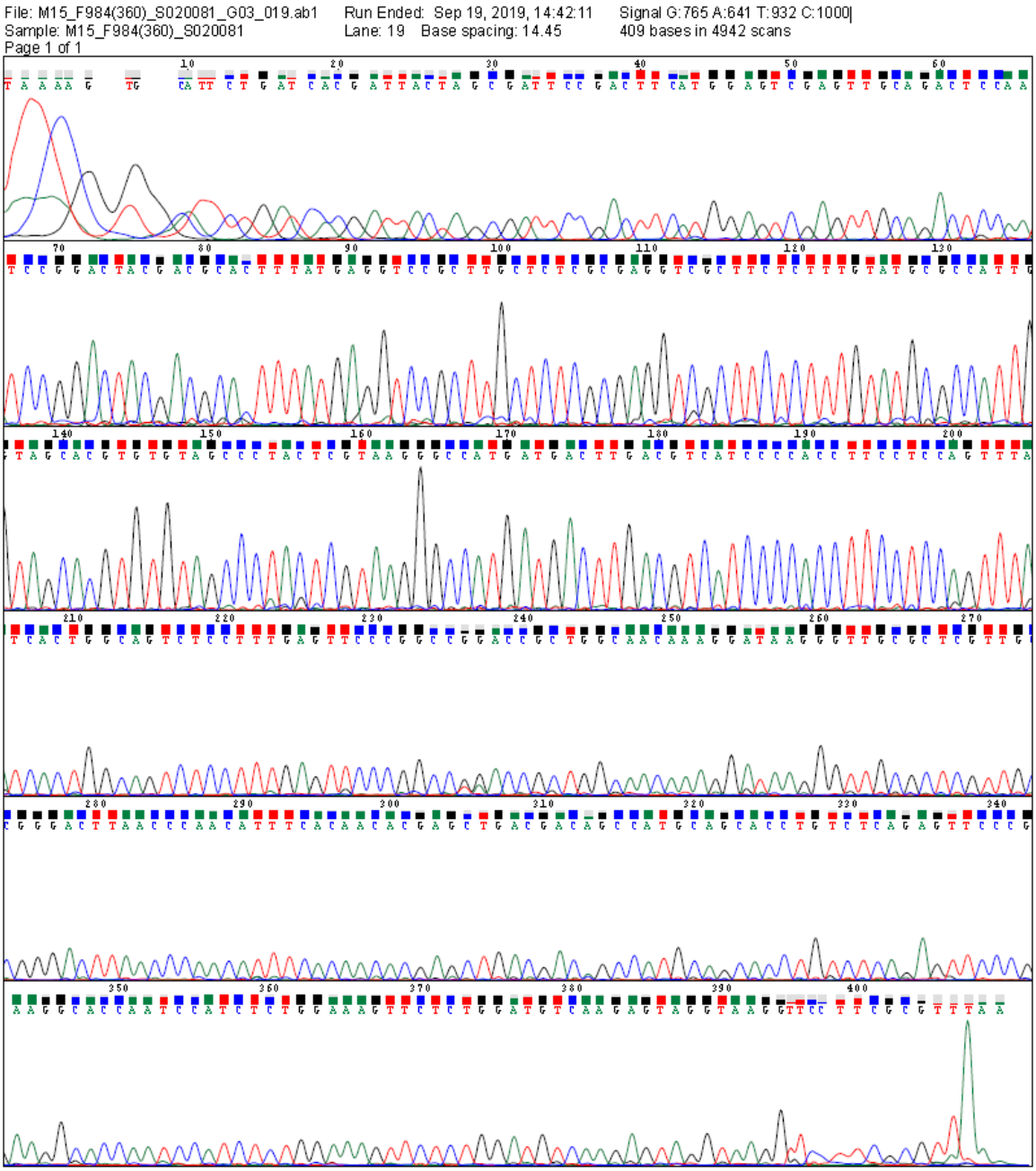


Figure 18: Chromatogram of the sequences of sample M15 generated by Chromas software

Appendix B

Equipments and materials used:

a) Autoclave

b) Incubator

c) Microscope

d) Centrifuge

e) Hot air oven

f) Refrigerator

g) Weighing machine

h) Pipette

i) Glass wares

j) Magnetic Stirrer

k) Rota-evaporator

l) Stereo-microscope

m) Electrophoresis tank

n) Gel-Doc

o) Thermal Cycler (PCR)

p) Laminar Air Flow

Media Used (Hi-media manufacturer):

a) Nutrient Agar

b) Nutrient broth

c) WCX broth

d) WCX agar

e) Luria-Brutani broth

f) CMC agar

g) Muller Hinton Broth

Chemicals and Reagents Used:

a) Congo red

b) Ethidium bromide

c) Agarose

d) Calcium chloride

e) Cycloheximide

f) Ethyl acetate

g) Primers

h) Zymogen Premix (2X)

Staining and Reagents

1. Gram Staining Reagent: Crystal violet solution(20 g crystal violet, 9 g ammonium oxalate and 95 g ethanol in 1000ml of distilled water), Gram's Iodine, Acetone-alcohol Decolorizer and safranin.

Appendix C

Composition and Preparation of different Microbiological Culture Media and Reagents.

A. Culture Media

1. Nutrient Agar (NA)

Peptic digest of animal tissue	5.0 gm
Beef extract	1.5 gm
Yeast extract	1.5 gm
Sodium chloride	5.0 gm
Agar	15 gm
Distilled water	1000 ml

2. Luria Burtani broth (LB- broth)

Yeast extract	5.0 gm
Sodium chloride	10.0 gm
Enzyme casein hydrolase	10.0 gm
Distilled water	1000 ml

3. Nutrient Broth (NB)

Peptic digest of animal tissue	5.0 gm
Sodium chloride	5.0 gm
Beef extract	1.5 gm
Yeast extract	1.5 gm
Final pH at 25˚C	7.4±0.2

4. **WCX agar:**

$CaCl_2.2H_2O$	0.1%
Agar	1.5%
pH	7.2

5. CMC agar:

Carboxymethylcellulose	1%
K_2HPO_4	0.2%
Agar	1%
MgSO4	0.03%
$(NH_4)_2SO_4$	0.25%
Gelatin	0.2%
pH	7±0.2

PHOTOGRAPHS

Figure 19: Sample collection at Budhanilkantha and Kirtipur

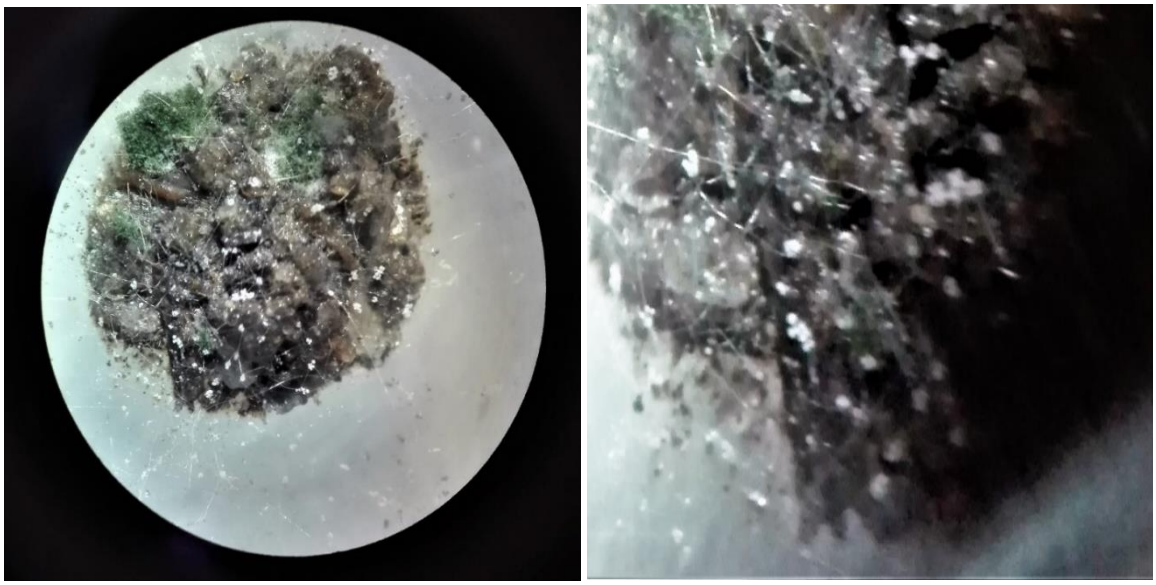


Figure 20: Fruiting bodies induction from soil samples in WCX agar

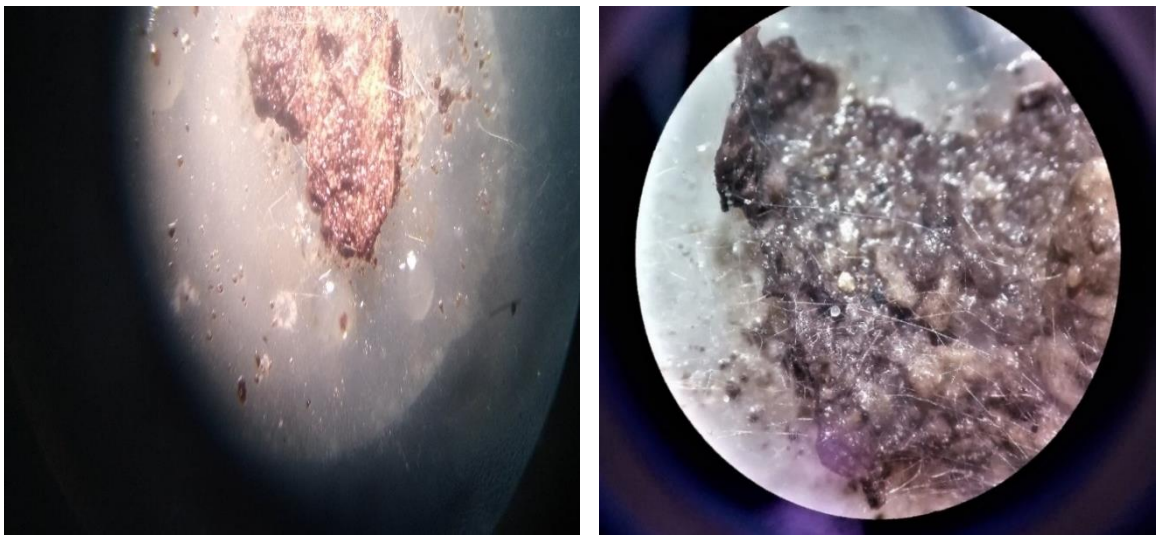


Figure 21: Fruiting bodies observed from bark and soil sample

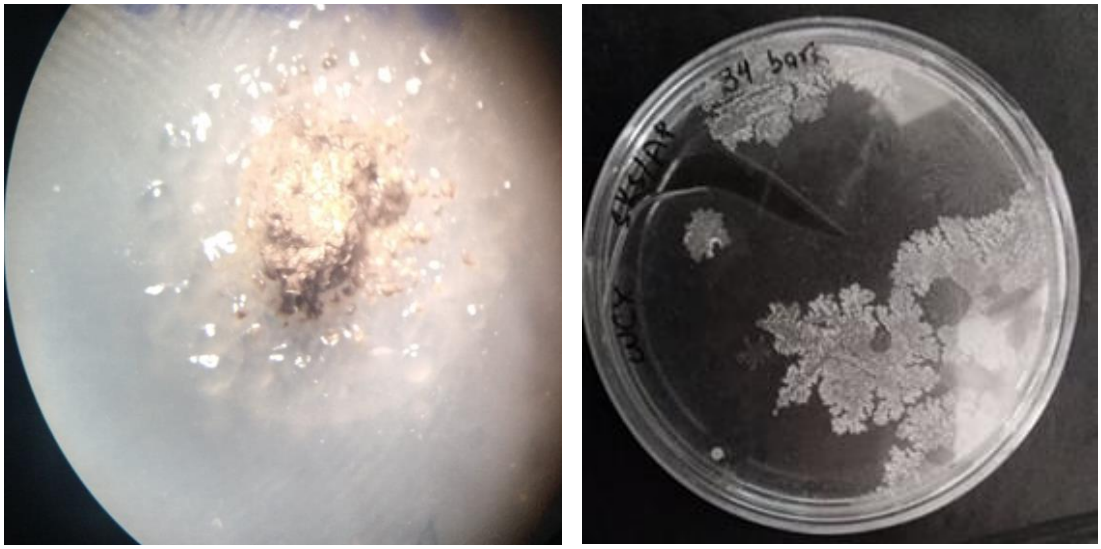


Figure 22: Observation of Myxobacterial growth during subculture

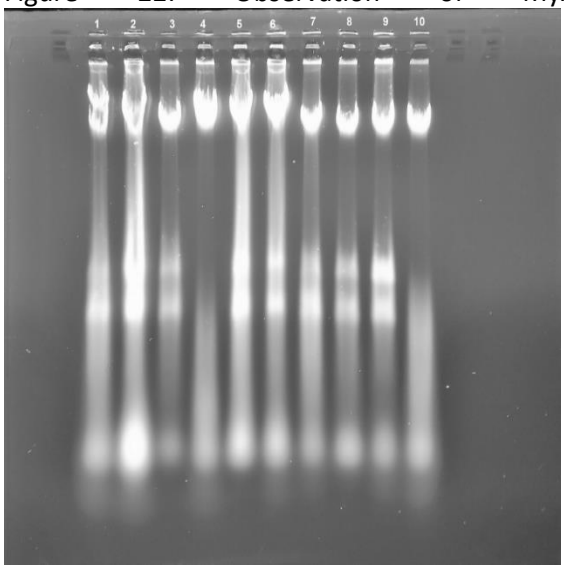


Figure 23: Agarose gel (0.8%) electrophoresis of genomic DNA of Myxobacterial isolates

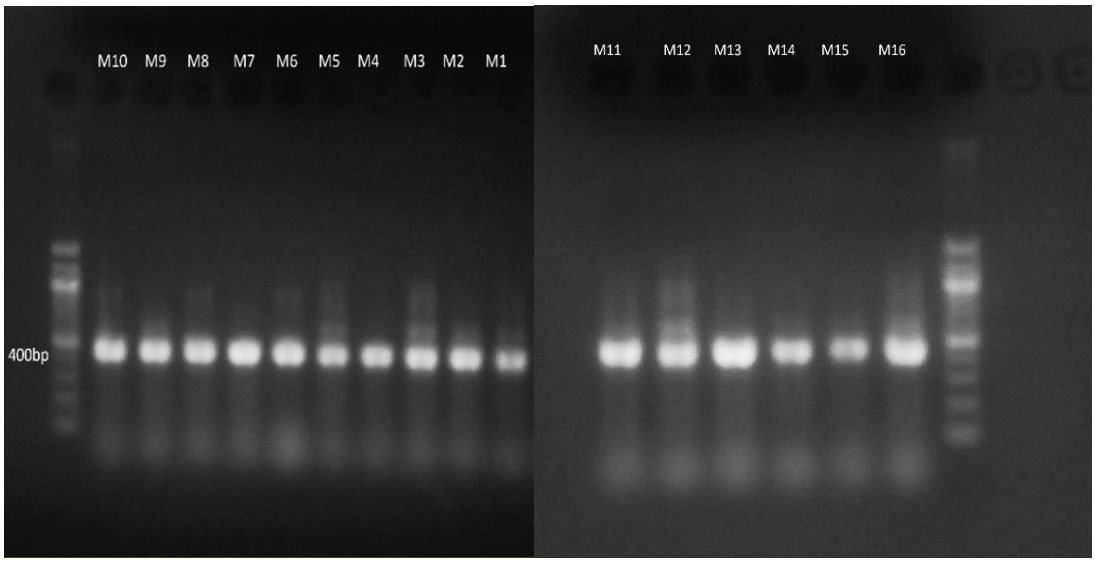
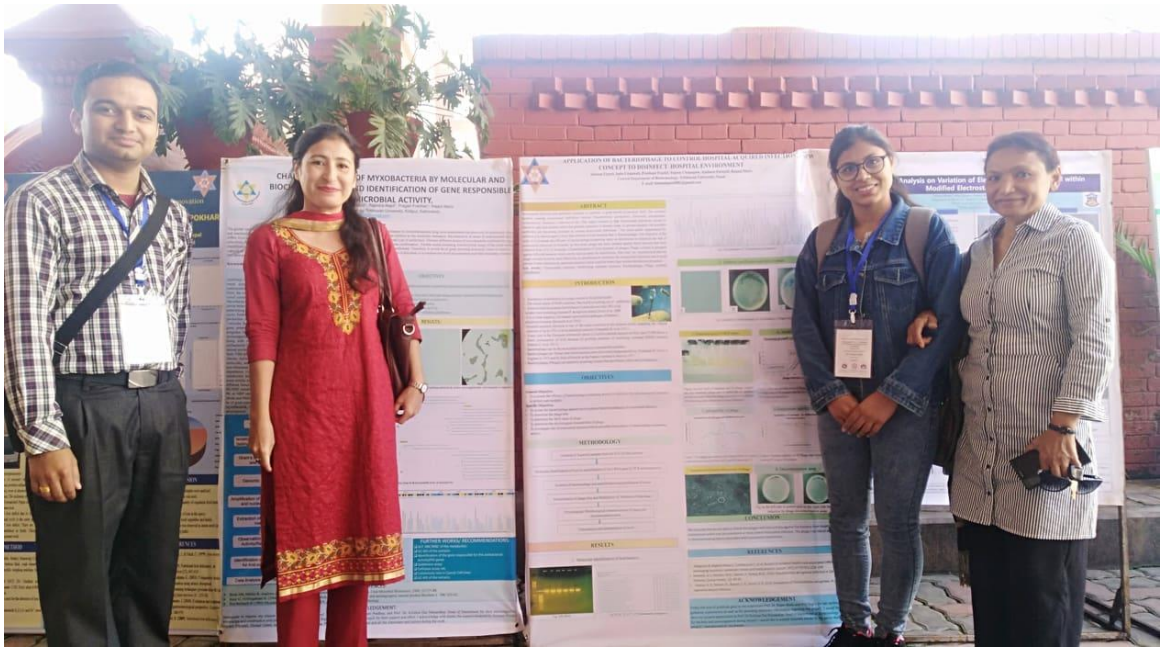


Figure 24: Agarose Gel (1%) electrophoresis of 16 S' rDNA amplified sequences compared with DNA marker of 100 basepair.

Concentration of the extracts using rota-evaporator

Sample dilution



@ Poster presentation (International Youth Conference on Science, Technology and Innovation 2019, 21-23 Oct, 2019, Kathmandu)

Receiving the Best poster award in IYCSTI-2019