BIOPROSPECTING MYXOBACTERIA FROM KASHMIR VALLEY FOR PRODUCTION OF ENZYMES AND SECONDARY METABOLITES

Thesis Submitted for the Award of the Degree of

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in

Biotechnology

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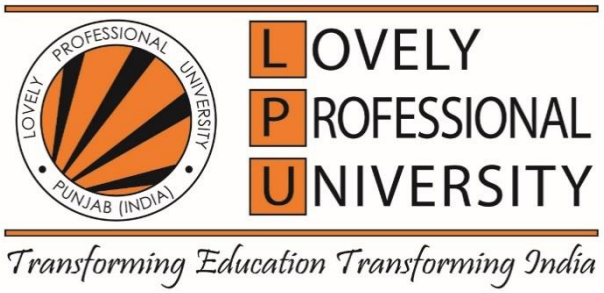

LOVELY PROFESSIONAL UNIVERSITY
PUNJAB
2024

DECLARATION

I, hereby declared that the presented work in the thesis entitled **"BIOPROSPECTING MYXOBACTERIA FROM KASHMIR VALLEY FOR PRODUCTION OF ENZYMES AND SECONDARY METABOLITES"** in fulfilment of degree of **Doctor of Philosophy (Ph. D.)** is outcome of research work carried out by me under the supervision **Dr. Reena Singh**, working as **Associate Professor** in the **School of Bioengineering and Biosciences** of Lovely Professional University, Punjab, India. In keeping with general practice of reporting scientific observations, due acknowledgements have been made whenever work described here has been based on findings of other investigator. This work has not been submitted in part or full to any other University or Institute for the award of any degree.

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CERTIFICATE

This is to certify that the work reported in the Ph. D. thesis entitled **"BIOPROSPECTING MYXOBACTERIA FROM KASHMIR VALLEY FOR PRODUCTION OF ENZYMES AND SECONDARY METABOLITES"** submitted in fulfillment of the requirement for the reward of degree of **Doctor of Philosophy (Ph.D.)** in the **Department of Biotechnology** of **School of Bioengineering and Biosciences**, is a research work carried out by **Daljeet Singh Dhanjal, 11719581**, is bonafide record of his/her original work carried out under my supervision and that no part of thesis has been submitted for any other degree, diploma or equivalent course.

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ABSTRACT

Myxobacteria are gram-negative, unicellular, rod-shaped, and obligate aerobic bacteria belonging to the δ-proteobacteria family. These microbes are known for inhabiting diverse environments such as soil, tree bark, herbivore dung, marine environments, dumping areas, and decaying plant material. These microbes are considered to be mesophilic as they usually grow within the pH range of 5-9 and temperature 6-38°C. Additionally, the microbes are well comprehended for their ability to produce industrially important enzymes like polysaccharidases, lipases, proteases and synthesize diverse bioactive compounds that exhibit antibacterial, antifungal, antiparasitic, and cytotoxic potential. The current study focuses on exploring the wetlands of Kashmir Valley, serving as an ideal habitat for the proliferation of myxobacteria. This location was selected as it is a highly unexplored area in relation to Indian Habitat to unravel the biodiversity of myxobacteria. In the present work, three myxobacterial isolates were isolated from the Hokersar and Mirgund Wetland, respectively that showed the characteristic features of myxobacteria like fruiting bodies formation and growth in swarming pattern. The molecular identification and sequencing revealed these three isolates, Hok1, Mir1, and Mir2, to share similarity with Uncultured *Anaeromyxobacter* sp. (86.5%), *Pyxidicoccus fallax* (99.93%) and *Myxococcus* sp. (100%) respectively.

Out of these isolates, Mir2 mainly showed significant extracellular protease production and antibacterial activity against *Bacillus cereus* MTCC 8714*, Escherichia coli* MTCC 1679, and *Pseudomonas aeruginosa* MTCC 2582 by the crude extract. This research further delves into the aspect of molecular biology, which involves the identification and sequencing of genes responsible for encoding industrially important enzymes like protease and polyketide synthase (PKS). Further, these genes were cloned into the pENTR-D-Topo, expressed in the pEZY19 vector, and purified by affinity chromatography (His-tagged). The SDS-PAGE of the purified protease (Pro) showed the molecular weight of ~63 kDa. The biochemical assessment of purified protease (Pro) obtained from Mir2*Pro* revealed its extracellular nature and optimum activity at temperature and pH of 40°C and 7.0 respectively. Moreover, the enzyme activity increased in the presence of metal ions like Mg^{2+}, $Ca^{2+,}$ and Fe^{2+} at both the concentration, i.e., 2mM and 5mM. 5mM Mg^{2+} increased the enzyme activity by 247% and 2mM Ca^{2+} increased the activity by 193%. However, Cu^{2+}, Co^{2+} and Zn^{2+} showed the inhibition of enzyme activity at 5mM concentration by 12.5, 16.5 and 3.08% respectively. Further, the presence of non-ionic detergent i.e., Tween-20 (2% and 5%) and Triton X-100 (2% and 5%) showed the increase in the activity of the enzyme. 5% Triton X 100 increased the activity by 538% and 5% Tween 20

increased the activity by 234% Whereas 2% Triton X 100 increased the activity by 201% and 2% Tween 20 increased the activity by 146%. However, the reduced enzyme activity was accorded for 2% and 5% SDS i.e., 13% and 25% respectively

Additionally, the investigation into PKS allowed successful identification and cloning into the pENTR-D-Topo and pEZY19 vectors and purified by affinity chromatography (His-Tagged), but the clone did not show antibacterial activity against tested strains like *Bacillus cereus* MTCC 8714*, Escherichia coli* MTCC 1679, and *Pseudomonas aeruginosa* MTCC 2582. Further, in-silico sequence analysis via the STRING database didn't show discernible interaction, and Clustal Omega assessment revealed the substantial differences in amino acid sequences of PKS, ACP, and AT proteins between *Myxococcus* and *E. coli,* highlighting the insufficiency of cloning a single PKS gene for recombinant protein synthesis.

Hence, the findings of the study highlight the potential of exploring unique niches for isolating more strains of myxobacteria to obtain industrially important enzymes and bioactive molecules for various industrial applications. Additionally, this work provides the avenue for exploring the challenges and complexities involved in obtaining bioactive molecules through genetic and recombinant DNA technology.

ABBREVIATIONS

1. % - Percent
2. °C - Degree Celsius
3. µg/mL - microgram per millilitre
4. µL - microliter
5. µm – micrometre
6. µM - micromolar
7. 16S – 16 Svedberg
8. ACP - acyl carrier protein
9. AT - acyltransferase
10. BLAST - Basic Local Alignment Search Tool
11. BSA - Bovine Serum Albumin
12. Ca^{2+} - Calcium ions
13. $CaCl_2.2H_2O$ - Calcium Chloride Dihydrate
14. CFE – Cell Free Extract
15. CMC - Carboxymethyl cellulose
16. Co^{2+} - Cobalt ions
17. $CoCl_2$ - Cobalt Dichloride
18. Cu^{2+} - Copper ions
19. $CuSO_4$ - Copper Sulfate
20. DMSO - Dimethyl sulfoxide
21. DNA - Deoxyribonucleic Acid
22. DTT – Dithiothreitol
23. EDTA - Ethylenediaminetetraacetic Acid
24. FAs – Fatty acids
25. Fe^{2+} - Ferrous cation
26. $FeCl_3$ – Ferric Chloride/Iron Trichloride
27. FTC - Fluorescein Thiocarbamoyl
28. g/L - grams per liter
29. GFP - green fluorescent protein
30. H_3BO_3 – Boric Acid
31. H_3PO_4 – Phosphoric Acid
32. HPLC - High-Performance Liquid Chromatography

33. IL-8 - Interleukin 8
34. IPTG - Isopropyl-1-thio-β-D-galactopyranoside
35. K_2HPO_4 - Dipotassium Phosphate
36. kb - Kilobases
37. KBr - Potassium Bromide
38. KCl - Potassium Chloride
39. kHz - Kilohertz
40. KI – Potassium Iodide
41. KNO_3 – Potassium Nitrate
42. KS - Ketosynthase
43. LB – Luria Bertani
44. LiCl – Lithium Chloride
45. m - metre
46. M – Molar
47. Mb - Megabase
48. MCA - 4-methyl-coumaryl-7-amide
49. MDR - Multiple Drug Resistance
50. Mg^{2+} - Magnesium ions
51. $MgCl_2$ - Magnesium Chloride
52. $MgSO_4$ - Magnesium Sulfate
53. $MgSO_4.7H_2O$ - Magnesium Sulfate Heptahydrate
54. min(s) – minute(s)
55. mL - Milliliter
56. mM - Millimolar
57. Mn^{2+} - Manganese ions
58. $MnCl_2.4H_2O$ - Manganese Chloride Tetrahydrate
59. $MnSO_4.7H_2O$ - Manganese Sulfate Heptahydrate
60. mol% - moles percentage
61. $Na_2MoO_4.2H_2O$ - Sodium Molybdate Dihydrate
62. NaOH - Sodium Hydroxide
63. NCBI - National Center for Biotechnology Information
64. nm - Nanometer
65. OD – Optical Density
66. PCR – Polymerase Chain Reaction

67. PEG - Polyethylene Glycol
68. pH – Potential of Hydrogen
69. PIC - Protease Inhibitor Cocktail
70. PKS - Polyketide Synthase
71. rDNA - Ribosomal Deoxyribonucleic Acid
72. RNA - Ribonucleic Acid
73. rpm - Revolutions Per Minute
74. SDS - Sodium Dodecyl Sulfate
75. SDS-PAGE - Sodium Dodecyl-sulfate Polyacrylamide Gel Electrophoresis
76. sec(s) – second(s)
77. $SnCl_2.2H_2O$ - Tin (II) Chloride Dihydrate/ Stannous Chloride Dihydrate
78. TCA - Trichloroacetic acid
79. TE – Tris-EDTA
80. TEMED - N, N, N′, N′ -Tetramethylethylenediamine
81. TLC – Thin-Layer Chromatography
82. Tris HCl - Tris Hydrochloride
83. Tris-Cl – Tris Chloride
84. WCX - water cycloheximide
85. w/v – Weight by Volume
86. Zn^{2+} - Zinc ions
87. $ZnCl_2$ - Zinc Chloride
88. ZOI – Zone of Inhibition

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everything and guardian who has gone through various hardships, ups and down in life but has always supported me at every step and energized me towards academic excellence. My wife, ***Mrs Hardeep Kaur,*** *for being my constant companion, providing invaluable assistance and encouragement during the highs and lows of my Ph.D. journey. My younger brother,* ***Tarundeep Singh****, for being a source of support whenever I needed it.*

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Daljeet Singh Dhanjal

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CHAPTER 1

INTRODUCTION

1.1. General Introduction

Microbes have been recognized for a long time as prolific producers of enzymes, catalyzing fundamental biochemical processes that have a wide range of applications in different industries and biological systems (Ali *et al.,* 2023). Different enzymes distinguish themselves by their unique properties, serving as organic catalysts in different industrial applications (Robinson, 2015). Moreover, enzymes derived from microbes are considered to be superior catalysts, surpassing the enzymes derived from plants and animals. As enzymes can be obtained in large quantities within a short period of time from the microbes. This property makes them suitable for commercial-scale applications in different industries (Adrio and Demain, 2014; Ariaeenejad *et al.,* 2024). In fact, the enzymes global market is expected to inflate at a compound annual growth rate (CAGR) of 6% starting from 2021 to 2031 and is anticipated to reach the value of USD 10.2 billion by the end of the predicted period (Janusz *et al.,* 2023). This growth is driven by the increasing demand for enzymes in different sectors, such as food and beverages, bioenergy, household care, and pharmaceuticals, reflecting their indispensable application across different industries (Golgeri *et al.,* 2024).

The discovery of enzymes from microbes dates back to the 20th century. However, ongoing research endeavours have continuously advanced their isolation, biochemical characterization, and scalable production—from bench-scale to pilot-scale. This progressive research evolution enhances the potential of their application in biotechnology (Nigam, 2013; Wu *et al.,* 2021). In recent years, myxobacteria has been reported for synthesizing diverse enzymes named cellulases, lipases, proteases, phosphatases, carboxylesterases (Sharma *et al*., 2021; Yuan *et al*., 2023)

Besides that, pathogens are increasingly becoming resistant to multiple drugs, and standard therapies are insufficient. Even second as well as third-line drugs are not found to be effective against multi-drug resistant strains (Nwobodo *et al.,* 2022). Since anti-microbial drugs are becoming ineffective in curbing the growth of pathogens, exploration for novel bioactive natural products has become more critical (Terreni *et al.,* 2021). Natural products are known to provide a variety of bioactive compounds, it is imperative to characterize novel natural compounds (Thomford *et al.,* 2018). As per the published literature from 2000 to 2008, around 300 secondary metabolites have been discovered and reported for exhibiting anti-microbial

activity (Saleem *et al.*, 2010). In the last 3 decades, 9000 myxobacterial strains have been isolated and reported for synthesizing 100 secondary metabolites with 600 analogues (Bhat *et al.*, 2021).

1.2. Characteristics feature of myxobacteria

Myxobacteria, gram-negative bacteria having rod-shaped morphology, are a member of δ-proteobacteria, order *Myxococcales*, found in the aquatic and terrestrial environment (Shimkets *et al.*, 2006; Saggu *et al.*, 2023a). Most myxobacteria found in the environment are obligate aerobes except *Anaeromyxobacter* sp. and *Anaeromyxobacter dehalogenans*, which are anaerobic and facultative anaerobic myxobacteria, respectively (Sanford *et al.*, 2002; Yamamoto *et al.*, 2014). Primarily, myxobacteria are mesophilic in nature and obtain their nutrition by preying on other microbes. They also have the ability to survive in a narrow range of pH (Reichenbach, 1999; Bhat *et al.*, 2021).

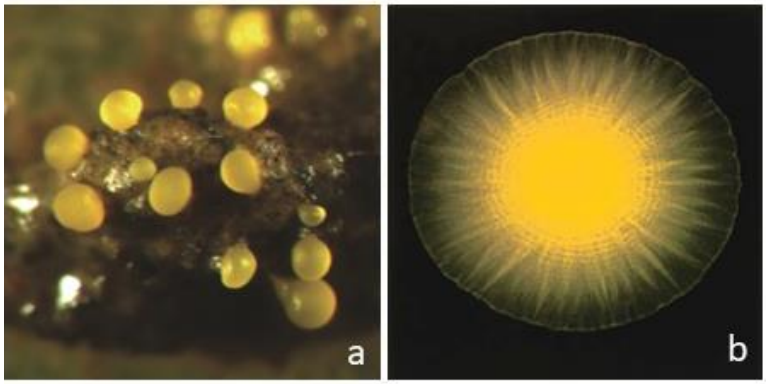


Figure 1.1: Growth of myxobacteria a) Fruiting bodies and b) Swarming body (Kaiser, 2006; Kaiser *et al.*, 2010)

Generally, myxobacteria grow in a swarm pattern by forming a thin film on the solid surface (Figure 1.1) and secrete enzymes to lyse the preying microbes to obtain nutrients (Livingstone *et al.*, 2017). Myxobacteria cells are known to be surrounded by a slime layer, which helps in coordinating their social behaviour and motility (Dworkin, 1996; Rajesh *et al.*, 2021). The unique life cycle of these bacteria separates them from other prokaryotes. Under normal conditions, these bacteria grow and proliferate themselves by transverse fission. Whereas, under stress conditions, vegetative cells aggregate themselves to form mushroom-type colonies, named fruiting bodies, as illustrated in Figure 1.2 (Muñoz-Dorado *et al.*, 2016). In most cases, vegetative cells form dry-resistant myxospores in unfavourable conditions (Figure 1.2). The myxospores formed by myxobacteria are highly resistant to severe environmental

conditions like desiccation, temperature, and UV radiation. These spores possess the ability to get reactivated under suitable conditions or solid media even after decades (Dawid, 2000; Kaiser, 2006; Kaiser *et al.*, 2010; Ramírez *et al.*, 2023). These soil-dwelling bacteria are found in a broad range of habitats, like decomposing plant material, under the roots of old trees, and in the soil where medical waste is dumped (Zhang *et al.*, 2013; Dahm, 2015; Kumar *et al.*, 2017).

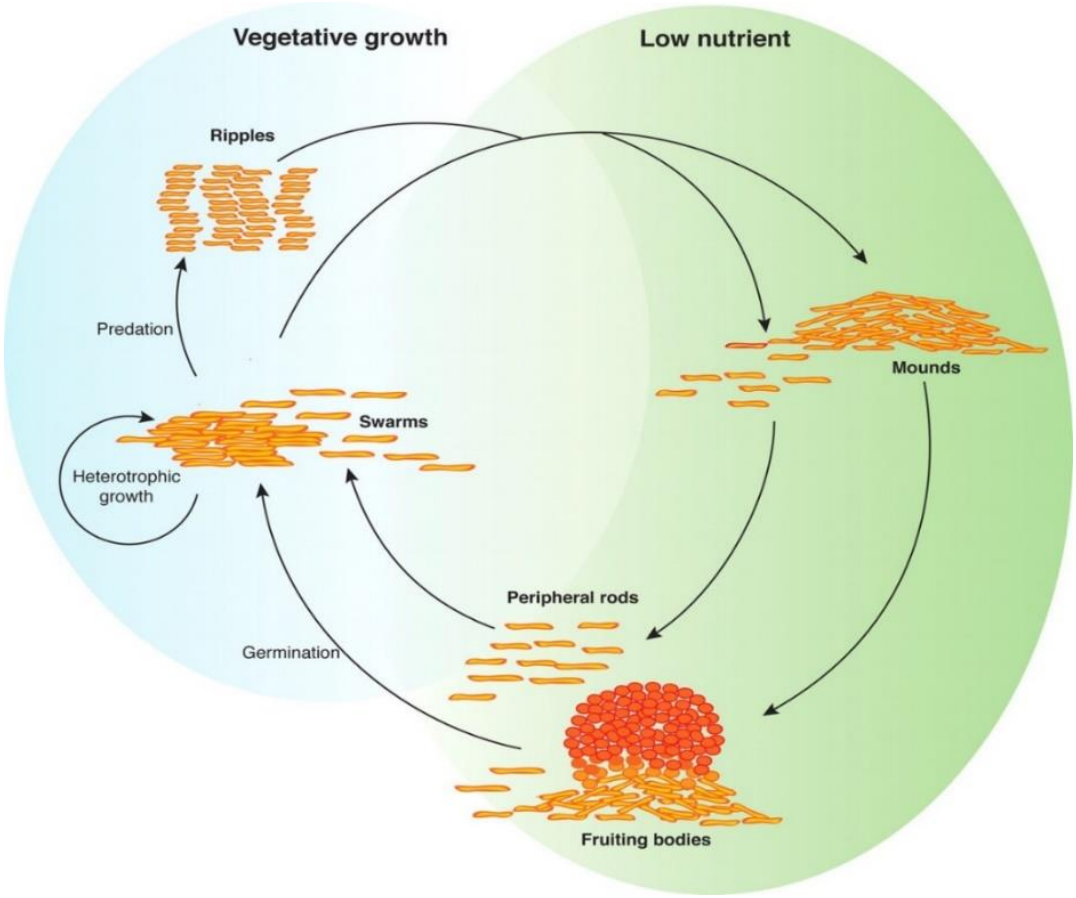


Figure 1.2: Myxobacteria life cycle and fruiting bodies formation under stress conditions (Courtesy: Mauriello *et al.*, 2010)

Based on 16s rRNA sequences, myxobacteria have been placed in δ-proteobacteria under the order *Myxococcales*. Moreover, on the basis of their biochemical, molecular and morphological characteristics, the *Myxococcales* order is further categorized into three suborders, i.e., *Cystobacterineae*, *Sorangiineae*, and *Nannocystineae*. These three suborders are further divided into eleven families and twenty-eight genes as illustrated in Figure 1.3 (Reichenbach, 2004; Shimkets *et al.*, 2006; Mohr *et al.*, 2012; Yamamoto *et al.*, 2014; Lang *et al.*, 2015; Wang *et al.*, 2021)

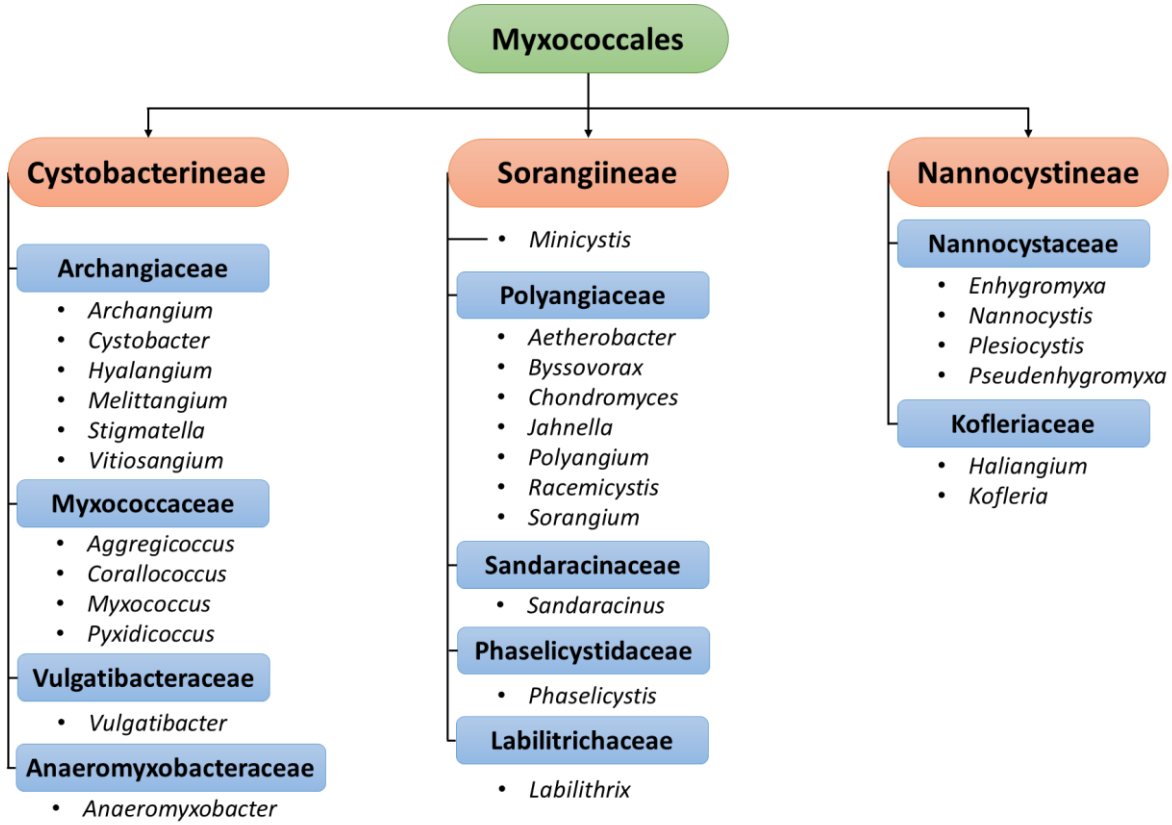


Figure 1.3: Schematic illustration of taxonomy distribution of order *Myxococcales* of δ-proteobacteria

1.3. Myxobacteria: a producer of enzyme and bioactive compounds

Myxobacteria are known as micro-predators as they synthesize a variety of enzymes for cell lysis and other distinct functions (Wrótniak-Drzewiecka *et al.*, 2016). For instance, *Sorangium* sp., is known for its efficient decomposition of biomacromolecules such as cellulose (Hou *et al.*, 2006; Mohr *et al.*, 2018b). Moreover, myxobacteria, both cellulolytic and predatory types, produce proteolytic enzymes. These enzymes serve multiple functions, including supplying amino acids, disrupting the cell membranes of eubacteria, and contributing to the lysis of prey microbes. Examples include serine proteases and extracellular alkaline/acid proteases (Sharma *et al.*, 2021). Conclusively, myxobacteria demonstrate a noteworthy enzymatic activity essential for their predatory behaviour, developmental intricacies, and prospective applications in different domains, especially in biotechnology and medicine (Wrótniak-Drzewiecka *et al.*, 2016).

Furthermore, the bioactive compounds synthesized by myxobacteria are generally strain-specific. Thus, isolating different strains from diverse niches has become the primary approach (Mohr, 2018). In addition, myxobacteria synthesize various secondary metabolites instead of single secondary metabolites (Dawid, 2000; Bhat *et al.*, 2021). The major secondary

metabolites synthesized by myxobacteria are alkaloids, non-ribosomal peptides, phenylpropanoids, polyketides, and steroids (Bhat *et al.*, 2021). Along with these, compounds like ceramides, cerebrosides, cholestenols, and lanosterol have also been isolated from myxobacterial species. Interestingly, each secondary metabolite produced by myxobacteria shows a unique action mechanism (Diez *et al.*, 2012).

Owing to the challenges associated with the culturing of myxobacteria due its complex genetic system, lesser reports have been reported till date from Indian habitat (Kumar *et al.*, 2017; Thakur *et al.*, 2017). India is a highly diverse region, there is a massive potential for getting novel myxobacterial species that can be exploited to obtain different industrially important enzymes and secondary metabolites. Hence, the current study focuses on addressing this gap by investigating the wetlands of Kashmir Valley for the production of industrially important enzymes and secondary metabolites. More specifically, the study aimed at the isolation of promising strains of myxobacterial species from diverse niches of the Kashmir Valley followed by screening for the production of enzymes and secondary metabolites. and the cloning of the genes related to these bio-activities. To accomplish the same, three myxobacterial strains were isolated and identified through sequencing of the ribosomal RNA gene. The identified isolates were screened for the production of hydrolytic enzymes and antimicrobial activity against *Bacillus cereus*, *Escherichia coli* and *Pseudomonas aeruginosa*. The genes coding for the protease as well as the polyketide synthase (PKS) subunit for antibacterial activity, were cloned first using the Gateway Cloning method, followed by subcloning in the expression vector for purification. The purified protease was also characterized for physico-chemical characteristics such as temperature optima, pH optima, effect of metal ions, and the effect of detergents to understand the enzyme activity. To explain the functionality of the PKS enzyme, in-silico sequence analysis via the STRING database and Clustal Omega assessment were carried out to study the interactions and amino acid constitution of PKS, ACP (acyl-carrier protein), and AT proteins between *Myxococcus* and *E. coli*. The study revealed the North-Western Himalayas, being relatively untapped ecological niche, offer a vast potential for discovering new strains of myxobacteria. In particular, the wetlands of Kashmir Valley, characterized by ecological richness and under exploration, serve as the ideal sites for the isolation and study of myxobacterial species.

CHAPTER 2
REVIEW OF LITERATURE

Myxobacteria, gram-negative bacteria (having morphology like rod-shaped) are the member of class δ-proteobacteria that are inhabitants of both marine and terrestrial ecosystem (Hook, 1977; McCurdy, 1989; Ivanova *et al.,* 2010; Mohr *et al.,* 2012; Iizuka *et al.,* 2013; Garcia *et al.,* 2014; Shrivastava and Sharma, 2021). These species of these bacteria exhibit remarkable characteristics like fruiting bodies formation, gliding ability and complex developmental cycle that separates it from other bacterial species (Dawid, 2000; Saggu *et al.,* 2023a). Additionally, they have been believed to have evolved in a similar fashion as purple bacteria (Shimkets and Woese, 1992; Kaiser 1993).

Due to the growing recognition as producer of distinctive bioactive molecules in past 3-4 decades, myxobacteria have drawn the attention of various researchers. In fact, they are considered to be a potent source for discovering novel drugs as bioactive molecules synthesized by myxobacteria acts on the unique targets of the cells that have not been targeted by secondary metabolites of other bacteria (Reichenbach, 2001; Gerth *et al.,* 2003; Bader *et al.,* 2020).

2.1. Historical background

The first myxobacterium "*Polyangium vitellinum"* was discovered by H.F. Link (German Botanist), who considered it to be gastromycete (Link, 1809; Dawid, 2000). Later, two additional species, i.e., *Chondromyces crocatus* and *Stigmatella aurantiaca* were identified and grouped into *Hyphomycetes* by M.J Berkeley in 1857. In 1892, Roland Thaxter first described about the macroscopic fruiting bodies, vegetative and differentiated cells and identified the *Stigmatella aurantiaca* and *Chondromyces crocatus* as myxobacteria (Thaxter, 1892). Prior to that, these species were classified as fungi (Berkeley, 1857) till E. Jahn (Another German botanist) illustrated the morphology, nutrition, motility and life cycle of these species (Jahn, 1924; Dawid, 2000).

In late 1980s, progressive development in the field of genetics and molecular biology resulted as remarkable step forward in understanding biology of model organism of myxobacteria, *Myxococcus xanthus*. The development of methods for isolating and purifying myxobacteria shifted the focus of research towards molecular biology, taxonomy, molecular genetics and biotechnology (Dworkin and Gibson, 1964; White, 1975; Kaiser *et al.,* 1979; Kaiser, 1984; Dworkin and Kaiser, 1985; Reichenbach, 1986; Reichenbach, 1988; McCurdy, 1989; Inouye

and Inouye, 1993; Dworkin, 1996; Yue *et al.*, 2023). One of the outstanding accomplishments was the discovery of bio-active metabolites with unique structure, synthesized by myxobacteria which operate on exclusive biological targets and have distinctive action mechanisms (Reichenbach and Dworkin, 1970; Reichenbach and Höfle, 1989; Reichenbach, 1999; Whitworth *et al.*, 2021).

2.2. Geographical distribution and ecology

Until now, all myxobacteria isolated have been found to be obligate aerobes, except for *Anaeromyxobacter dehalogenans*, the only facultative anaerobic species discovered (Sanford *et al.*, 2002; Yamamoto *et al.*, 2014). In fact, within the terrestrial habitat myxobacteria isolated so far have showcase their prevalence within the pH value ranging from 5.0 to 8.0. However, some genera have been reported to flourish in alkaline soils (pH 8.0-9.2) and acidic soils (pH 2.5) (Brockman and Boyd, 1963; Hook, 1977; Dawid, 2000; Wang *et al.*, 2022). The extensive investigation on myxobacteria has provided the evidence that they naturally grow in air, bogs, decomposing wood (decaying bark and wood), deep caves, herbivores dung and insects (Wu *et al.*, 1968; Hook, 1977; Hook *et al.*, 1980; Menne and Rückert, 1988; Reichenbach, 1999; Dawid, 2000; Saggu *et al.*, 2023a). In general, myxobacteria are proclaimed to be mesophilic in nature as they prefer to grow at the optimum temperature of 30°C. However, there are exceptions like *Polyangium* and *Nannocystis* species which have been isolated from the Antartica soil and stated as psychrophilic species of myxobacteria (Menne and Rückert, 1988; Dawid, 2000). Besides that, few species of myxobacteria are capable of growing at 38–40°C (McCurdy, 1969; Gerth *et al.*, 1994; Rouhizohrab and Mohammadipanah, 2021). Remarkably, spores of myxobacteria are reported to withstand at 58–60°C and this ability to survive high temperature have been used to purify them (Reichenbach and Dworkin, 1992; Rouhizohrab and Mohammadipanah, 2022). Lately, *Nannocystis konarekensis* (a new *Nannocystis* species) has been isolated from desert soil of iran which showed effective growth at 37°C, surpassing other *Nannocystis* species like *N. exedens* and *N. pusilla which* show optimum growth at 30°C (Mohr, 2018). In the group of myxobacteria, only *Chondromyces crocatus* has been reported to shows symbiotic relationships with *Sphingobacterium.* Usually, myxobacteria are non-pathoegnic in nature, but *Pajaroellobacter abortibovis* is the only pathogenic myxobacterium responsible for epizootic bovine abortion in cattle (Kunze *et al.*, 1994; Kunze *et al.*, 1995; Brooks *et al.*, 2016).

Based on myxobacterial diversity assessment, the average number of myxobacterial species per sample is determined to be 3.8 and 4.5 for Mediterranean regions and tropical rain forests respectively (Dawid, 2000). According to published literature, myxobacteria have been isolated for diverse habitats like deserts, marine environment and mountains (Li *et al.,* 2002; Zhang *et al.,* 2002; Zhang *et al.,* 2013). *Enhygromyxa salina, Haliangium tepidum, Haliangium ochraceum, Plesiocystis pacifica and Pseudoenhygromyxa salsuginis* are few myxobacterial species isolated from marine environment (Fudou *et al.,* 2002; Iizuka *et al.,* 2013; Garcia and Müller, 2020).

2.3. Structure and size

The extensive investigation on these rod-shaped myxobacteria unveiled their size to be 3-15 μm (in length) and 0.6-1.2 μm (in width) (Reichenbach, 1999). The vegetative cells of these myxobacteria are generally found in two forms, i.e., cylindrical rods (rigid) having rounded ends and cylindrical rods (flexible) with tapered ends. Further, their colonies appear red, orange and yellow attributed to the presence of carotenoid derivatives (Sánchez *et al.,* 2021).

Generally, myxobacteria cells are enclosed by slime layer composed of proteins, lipids and polysaccharides, which is also involved in the proteolytic activities of this microbe (Gnosspelius, 1978a; Gnosspelius, 1978b). The fimbriae extending from slimy layer of myxobacteria aid in cell motility. Interestingly, the organization of peptidoglycan in the cell wall of myxobacteria are found to be in patches which confer more flexibility to them (Reichenbach and Dworkin, 1969; Balagam *et al.,* 2021). Another interesting fact about myxobacteria is the outer membrane tubes formation, which is composed of lipid and outer membrane components, that has been reported in *M. fulvus* and *M. xanthus* (Hartzell, 2014; Wei *et al.,* 2014). The formation of outer membrane tubes is considered to be the typical trait of myxobacteria; however, these tubes do not facilitate the transfer or exchange of outer membrane proteins and lipids between cells (Nudleman *et al.,* 2005). In fact, formation of these outer membrane tubes has also been reported in other bacteria like *Bacillus subtilis*, *Deftia* sp. strain Cs1-4, *Francisella novicida* and *Salmonella enterica* serovar *typhimurium* (Dubey and Ben-Yehuda, 2011; Galkina *et al.,* 2011; Shetty *et al.,* 2011; McCaig *et al.,* 2013). Moreover, myxobacteria contains a contractile fibril system beneath the outer membrane, which is a chain-like structure composed of rings that aid myxobacteria in gliding (Lunsdorf and Reichenbach, 1989; Sánchez *et al.,* 2021).

2.4. Motility and swarming pattern

Myxobacteria are known for aggregating during formation of fruiting bodies and show gliding or swarming pattern on any surface for uptake of food (Shimkets, 1990; Dworkin, 1996; Kaiser, 2006; Furness *et al.,* 2020). During gliding motility, the cells of myxobacteria moves in the direction of long axis without rotating (Burchard, 1970). For this, myxobacteria secretes slime-like material for easy gliding on any solid surface, on other cells or water-air interface (Lunsdorf and Reichenbach, 1989; Yu and Kaiser, 2007; Rosenberg, 2012; Bondoc-Naumovitz *et al.,* 2023).

Studies on the surface motility or gliding in *M. xanthus* has revealed about the involvement of two multiple gene systems. One is the A (adventurous) motility system that controls the gliding motility of an individual cell and the S (social) motility system, that helps in the movement of cell during swarming or fruiting bodies formation (Hodgkin and Kaiser, 1979a; Hodgkin and Kaiser, 1979b; Chen *et al.*, 2024). For the matter of fact, the motility of cells during swarming movement is regulated by either A or S motility systems or by both systems simultaneously (Figure 2.1).

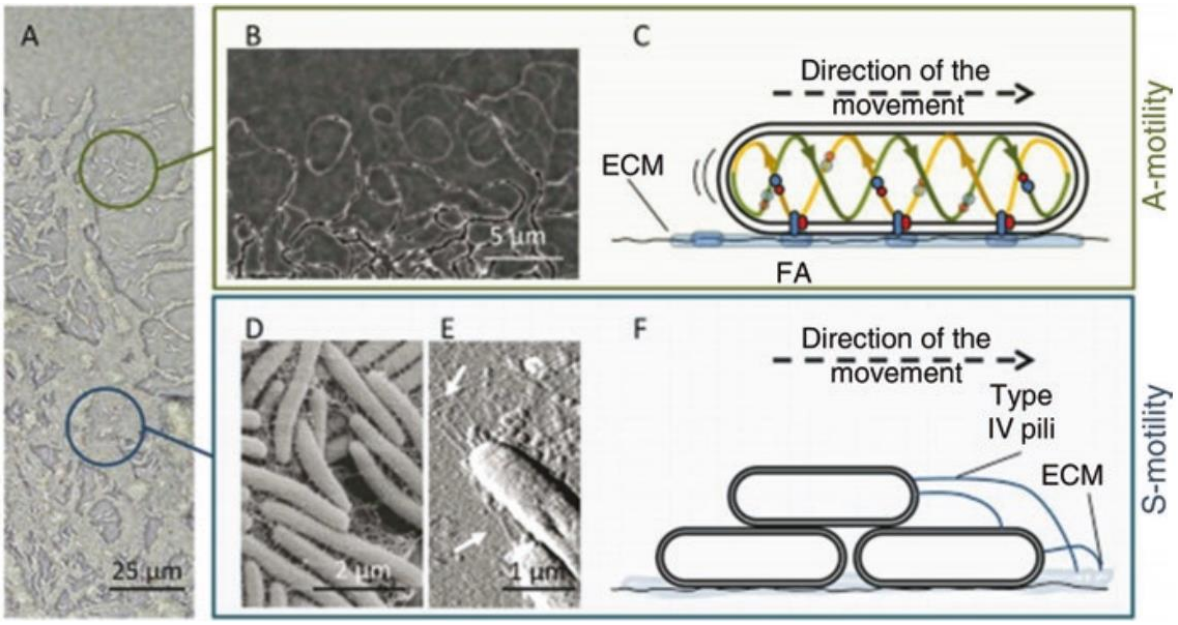


Figure 2.1: Depiction of A- and S-motility in myxobacterial strain i.e., *Myxococcus xanthus.* (a) Circle in green colour depicts cells with A-motility, circle in blue colour depicts cells with S-motility; (b) Phase contrast microscopy view of A-motility trail; (c) Gliding motility model; (d) Depiction of fibril components network for maintaining cell adhesion; (e) Atomic force microscopy view of T4 present at the pole of the cell; (f) S-motility model (Muñoz-Dorado *et al.*, 2016)

2.5. Fruiting bodies and myxospores

In spite of the fact that cellular differentiation and multicellular morphogenesis are characteristic feature of eukaryotes. The typical myxobacterial species exhibit multicellular morphogenesis by forming fruiting bodies during its development. Moreover, the size of fruiting bodies synthesized by myxobacteria ranges from 100 to 600 μm (Shimkets *et al.,* 2006; Stackebrandt *et al.,* 2007; Chen *et al.,* 2022). The most distinctive feature of *Myxococcales* order is the formation og fruiting bodies, also known as spore-forming bodies that emerge in response to starvation. These fruiting bodies are stated to be highly resistant to various physical and chemical stresses which ensures the myxobacteria survival in prolonged interval of starvation (Shimkets and Brun, 1999; McLaughlin and Higgs, 2023). Besides that, colour, shape and size of the fruiting bodies are genus and species specific, which serve as frame of reference during myxobacteria identification (Dawid, 2000; Wang *et al.,* 2021). Fruiting bodies of different colours of different myxobacterial strains have been illustrated in Figure 2.2.

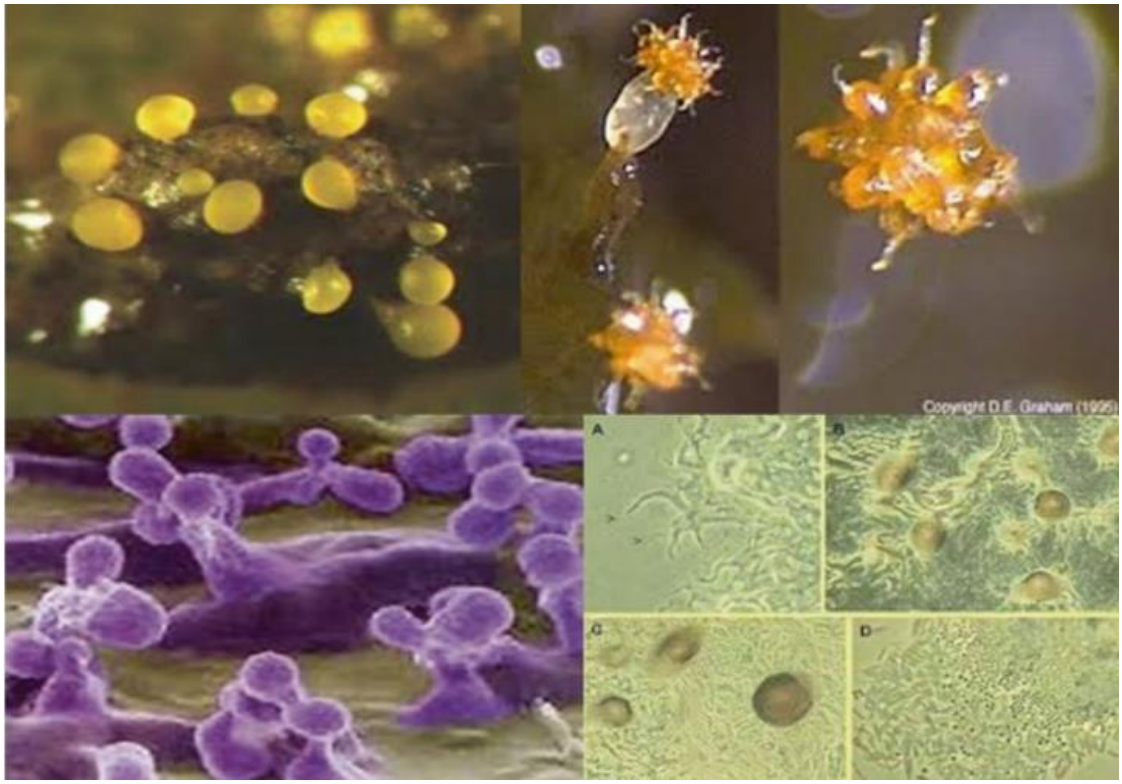


Figure 2.2: Pictorial depiction of diversity based on colours of fruiting bodies of different myxobacterial strains (Thakur *et al.,* 2018)

2.6. Taxonomy and phylogeny

Initially, different types of myxobacteria are classified based on their fruiting bodies' morphology. Based on their 16S rDNA sequences, the order "*Myxococcales*" was classified

under the δ-proteobacteria class (Oyaizu and Woese, 1985; Stackebrandt *et al.,* 1988; Brenner *et al.,* 2005). Further, the order *Myxococcales* is subdivided into three suborders, 11 families, 28 genera encompassing approximately 55 species. Among the *Cystobacterineae* suborder, there are three families i.e., *Archangiaceae, Myxococcaceae*, and *Vulgatibacteraceae* (Brenner *et al.,* 2005; Yamamoto *et al.,* 2014; Lang *et al.,* 2015). The *Nannocystineae* suborder encompasses three families i.e., *Nannocystaceae, Haliangiaceae* and *Kofleriaceae* (Brenner *et al.,* 2005; Ivanova *et al.,* 2010). Whereas, the suborder *Sorangiineae* comprises five families i.e., *Labilitrichaceae, Minicystidaceae, Phaselicystidaceae, Polyangiaceae* and *Sandaracinaceae* (Brenner *et al.,* 2005; Mohr *et al.,* 2012; Garcia *et al.,* 2014; Yamamoto *et al.,* 2014). The taxonomic placement of some myxobacteria genera is still under debate, such as *Anaeromyxobacter,* which Yamamoto and co-workers considered them that they belong to the *Anaeromyxobacteraceae* family. On the other hand, Lang and co-workers considered them to belong to the *Archangiaceae* family (Yamamoto *et al.,* 2014; Lang *et al.,* 2015).

The phylogenetic analysis based on 16S rDNA sequences affirms the relation amongst phylogenetic and morphological classifications of myxobacteria (Mohr *et al.,* 2017). According to Garcia *et al.,* (2011), 16s rDNA clustering of myxobacteria (aerobic in nature) in the phylogenetic tree was done by correlating the abundance of straight-chain as well as branched-chain fatty acids (FAs) (Garcia *et al.,* 2011). Further, on the assessment of the member of *Sorangiineae* family, predominance of straight-chain FAs (57-84%) was observed. Whereas, *Cystobacterineae* family showed the predominance of branched-chain fatty acids (FAs) (53-90%). However, the member of the *Nannocystineae* family showed the presence of both straight-chain as well as branched-chain FAs without the presence of hydroxy acids (Garcia *et al.,* 2011; Mohr *et al.,* 2017). Figure 2.3 illustrates the phylogenetic tree of the different myxobacterial strains of the order *Myxococcales*.

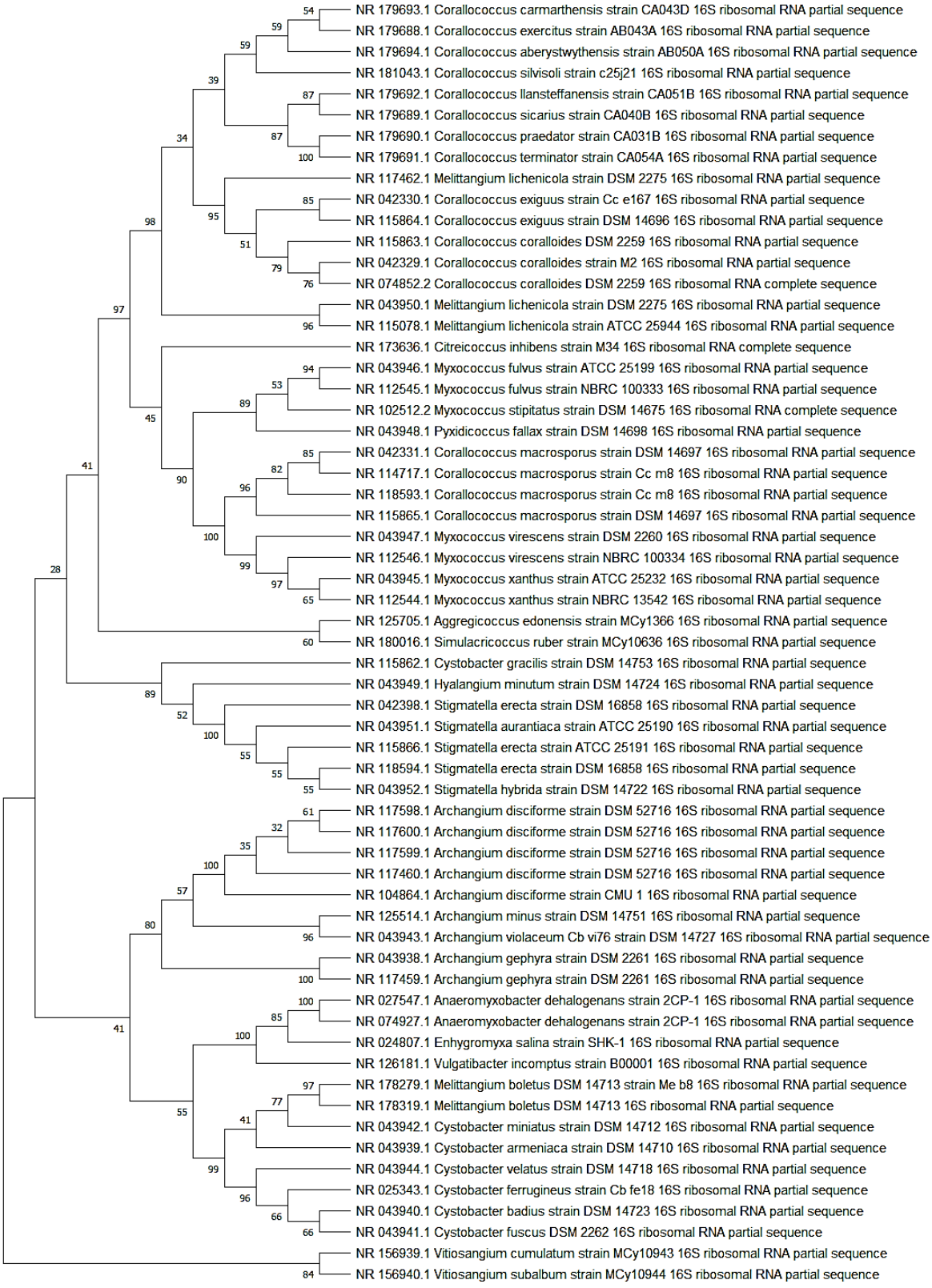


Figure 2.3: Neighbour-Joining inferred phylogenetic tree from 16S rRNA gene sequence derived from different myxobacterial strains belonging to the order *Myxococcales*. The number at branch point represent the percentage level of bootstrap based on 1000 replicates

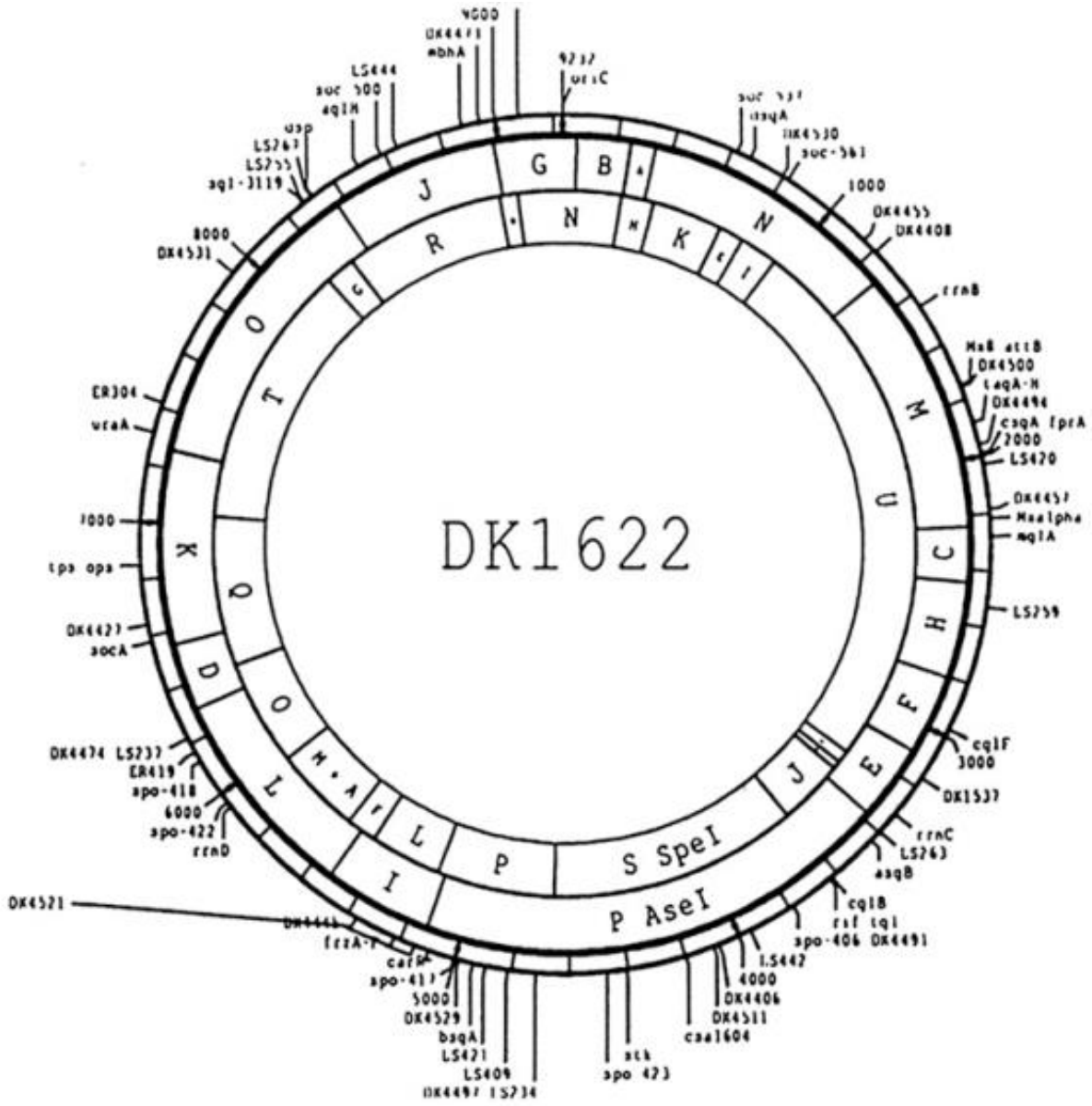


Figure 2.4: Genetic map of *Myxococcus xanthus* genome (Thakur *et al.,* 2018)

2.7. Genome

Myxococcus xanthus DK1622 was the first fruiting myxobacteria who genome was sequenced and which gave insight deep-insight about the genetic features aiding in sustaining the multi-cellular lifestyle (Figure 2.4) (Goldman *et al.,* 2006; Whitworth and Swain, 2020). Presently, the 13 whole genome sequence (WGS), 31 Contigs and 6 scaffolds of myxobacterial species, is available in online databases (https://www.ncbi.nlm.nih.gov/genome/?term=Myxobacteria) (Table 2.1). Generally, genome of myxobacteria have high GC content ranging from 65-75 mol%, with the exception of new isolated *Pajaroellobacter abortibovis*, which has a GC content of 47.5 mol% (Welly *et al.*, 2017). Moreover, the size of genome of myxobacterial

species varies from 1.8 Mb (*Pajaroellobacter abortibovis*) to 16.04 Mb (*Minicystis rosea* DSM 24000) (https://www.ncbi.nlm.nih.gov/genome/?term=Myxobacteria). Aerobic myxobacteria that produce fruiting bodies typically have genome size of 9 Mb or above. According to Han *et al.,* (2013), the large genomes of myxobacteria are likely the result of horizontal gene transfer, gene rearrangement and gene duplication. Reflecting the need for additional genes to support their complex life cycle and adaptation to diverse environments (Han *et al.,* 2013). In myxobacteria, the genome size is also correlated with the number of proteins encoded. For instance, the genome of *Myxococcales* encodes 7000-10500 proteins compared to 4000-5000 proteins encoded by non-*Myxococcales* deltaproteobacteria (Goldman *et al.,* 2007; Han *et al.,* 2013). The genome expansion in myxobacteria indicates the evolution of complex and socialized living patterns as well as adaptation to diverse environments (Stepkowski and Legocki 2001; Han *et al.,* 2013). An example of aerobic myxobacteria that doesn't produce fruiting bodies is *Vulgatibacter incomptus* DSM 27710 that has the genome size of 4.35 Mb. While, the genome size of anaerobic myxobacteria has determined to be approximately 5 Mb. The myxobacteria genome size is comparable to those that belong to non-*Myxococcales, a* deltaproteobacteria family, which is around 2-7 Mb in size (Bhat *et al.,* 2021).

Table 2.1: Summary of distinct attributes of the sequenced genomes of myxobacteria (https://www.ncbi.nlm.nih.gov/genome/?term=Myxobacteria**)**

Species Name	**Family**	**Assembly Level**	**GC%**	**Size (Mb)**	**Total number of genes**	**rRNA genes**	**tRNA genes**	**Other RNA genes**	**Pseudogenes**	**Predicted Proteins**
Myxococcus xanthus	M	Complete Genome	68.9`	9.14	7364	12	66	4	83	7199
Anaeromyxobacter dehalogenans	An	Complete Genome	74.7	5.03	4574	6	49	4	49	4466
Myxococcus fulvus	M	Scaffold	70.0	10.82	8622	3	79	4	109	8427
Corallococcus macrosporus	M	Complete Genome	70.6	8.97	7364	12	66	4	116	7166
Stigmatella aurantiaca	Ar	Contig	69.1	9.37	7484	3	50	3	109	7319
Archangium violaceum	Ar	Contig	68.9	12.54	10516	4	88	4	286	10134
Melittangium boletus	Ar	Complete Genome	68.4	9.91	8234	12	60	4	160	7998
Myxococcus stipitatus	M	Complete Genome	69.2	10.35	8149	9	82	4	114	7940
Cystobacter fuscus	Ar	Contig	68.6	12.28	10089	6	57	4	193	9829
Corallococcus coralloides	M	Complete Genome	69.9	10.08	8198	9	60	4	144	7981
Myxococcus guangdongensisi	M	Contig	69.8	11.2	8957	5	79	4	256	8614
Myxococccus dinghuesis	M	Contig	70.8	10.41	8570	4	80	4	328	8154
Myxococcus ginghaiensis	M	Contig	69.1	11.52	9222	5	76	4	294	8843
Pyxidicoccus xibeiensis	M	Contig	70.7	12.7	9924	5	76	3	295	9545
Paraliomyxa miuraensis	-	Scaffold	69.7	11.85	9280	3	77	4	40	9156
Anaeromyxobacter paludicola	An	Complete Genome	74.1	4.59	4086	6	50	4	47	3979
Anaeromyxobacter oryzae	An	Complete Genome	73.5	6.72	5931	6	49	4	43	5829

Anaeromyxobacter diazotrophicus	An	Contig	74.5	4.83	4334	3	48	4	53	4226
Corallococcus soli	M	Contig	70.6	9.44	7503	3	50	4	187	7259
Corallococcus silvisoli	M	Contig	70.7	9.23	7450	3	53	4	138	7752
Citreicoccus inhibens	M	Contig	69.5	9.05	7323	6	51	4	195	7067
Stigmatella hybrid	Ar	Contig	69.1	9.16	7422	3	51	3	118	7247
Cystobacter gracilis	Ar	Contig	69.3	11.73	9374	5	76	4	170	9119
Archangium primigenium	Ar	Contig	70.7	9.49	7794	12	63	4	157	7558
Myxococcus vastator	M	Contig	69.9	8.99	7703	7	59	3	355	7279
Myxococcus eversor	M	Scaffold	68.9	11.39	9130	3	78	4	127	8918
Pyxidicoccus trucidator	M	Scaffold	70.3	12.67	9736	7	81	3	159	9486
Pyxidicoccus caerfyrddinensis	M	Scaffold	70.2	13.43	10403	6	77	3	163	10154
Myxococcus llanfairpwllgwyngyllgogerychwyrndrobwllllantysiliogogogochensis	M	Contig	68.7	12.41	10323	7	84	4	321	9907
Pyxidicoccus fallax	M	Contig	70.5	13.53	10846	9	85	4	415	10333
Corallococcus interemptor	M	Contig	70	9.47	7903	7	53	4	158	7681
Corallococcus praedator	M	Contig	69.7	10.51	8889	9	55	3	341	8481
Corallococcus aberystwythensis	M	Contig	70	9.98	8334	6	54	4	274	7996
Corallococcus llansteffanensis	M	Contig	70.3	10.53	8774	3	52	4	404	8311
Corallococcus sicarius	M	Contig	70.2	10.39	8428	6	53	5	321	8043
Corallococcus carmarthensis	M	Contig	69.9	10.79	8975	6	53	4	253	8659
Corallococcus exercitus	M	Contig	70.3	10.15	8567	7	58	4	337	8161
Corallococcus terminator	M	Contig	69.5	10.35	8487	3	54	4	293	8133
Corallococcus exiguous	M	Contig	69.7	10.54	8491	10	59	4	158	8260
Cystobacter ferrugineus	Ar	Contig	68.5	12.05	10046	13	65	4	229	9735
Stigmatella erecta	Ar	Contig	69.4	9.22	7472	3	51	3	141	7274
Myxococcus virescens	M	Scaffold	69.2	9.24	7596	7	59	4	99	7427
Myxococcus hansupus	M	Complete Genome	69.2	9.49	7641	12	70	4	103	7452
Vulgatibacter incomptus	V	Complete Genome	68.9	4.35	3683	10	48	4	51	3570

Archangium gephyra	Ar	Complete Genome	69.4	12.49	10050	9	90	4	214	9733
Hyalangium minutum	Ar	Contig	68	11.19	8940	3	74	3	135	8725
Enhygromyxa salina	-	Contig	68.2	10.6	8245	8	51	4	72	8110
Sorangium cellulosum	P	Complete Genome	71.4	13.03	9702	12	65	3	134	9488
Haliangium ochraceum	K	Complete Genome	69.5	9.45	6941	6	47	4	141	6743
Plesiocystis pacifica	N	Contig	70.7	10.59	8366	6	92	4	141	8123

-; Not Available; An: Anaeromyxobacteraceae; Ar: Archangiaceae; K: Kofleriaceae; M: Myxococcaceae; N: Nannocystaceae; P: Polyangiaceae; V: Vulgatibacteraceae

Typically, myxobacteria lacks extra-chromosomal genetic material. However, *Myxococcus fulvus* 124B02 is an exception as it contains the plasmid pMF1(low-copy number), which is of 18.6 kb size and has a GC content of 68.7% (Zhao *et al.,* 2008; Chen *et al.,* 2016; Chen *et al.,* 2020). This plasmid has been identified to harbour 23 open reading frames, yet the genes it carries does not provide any discernible advantage to the host organism. However, leveraging the plasmid replication locus, shuttle vectors have been engineered to maintain stability of the genetic material in both *Escherichia coli* and Myxobacteria (Zhao *et al.,* 2008). Few, *M. xanthus* strains that are resistant to chloramphenicol have been reported to contain an extra-chromosomal DNA having resistance factor. This extra-chromosomal DNA is believed to be transferred by conjugation process with the help of *E. coli* phages (Morris *et al.,* 1978). Thus, it is believed that these extraneous plasmids maintained in myxobacteria gets integrated and express themselves in genome.

Retrons (retroelements) synthesized by multicopy single-stranded DNA (msDNA), was first seen in *M. xanthu*s (Yee *et al.,* 1984). Later, msDNA was determined to be present in other ten myxobacteria genera (Rice and Lampson, 1995). The phylogenetic analysis of myxobacterial retron species suggests their vertical transmission originating from a common ancestor carrying retro-elements (Lampson *et al.,* 1989; Lim and Maas, 1989; Inouye and Inouye, 1992; Wang *et al.,* 2024). However, no distinct physiological function has been identified to be associated with these retro-elements.

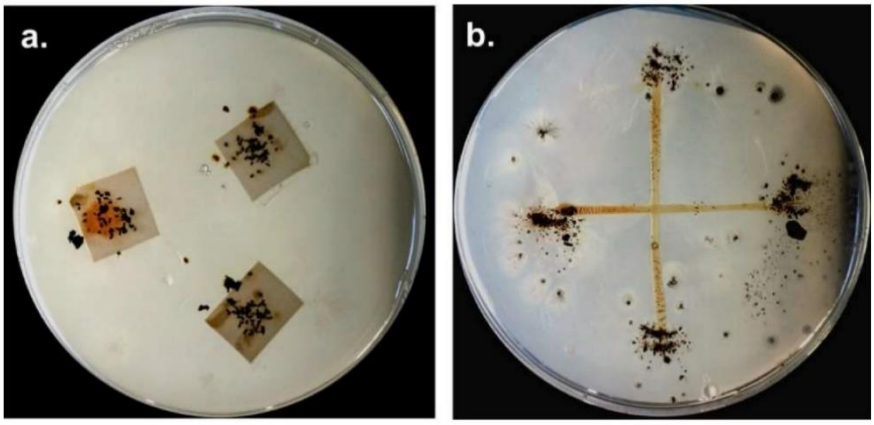


Figure 2.5: Method for myxobacteria isolation. a) Filter paper method; b) *E. coli* baiting method (Mohr, 2018)

2.8. Methods for isolation and purification of myxobacteria

According to the published literature, myxobacteria are generally found in animal dung, decaying plant material, freshwater, pharmaceutical dump yard, soil and marshy as well as marine environment. The method selected for myxobacterial isolation is chiefly influenced by

the habitat of isolation. The highly used method for myxobacteria isolation has been discussed as follows:

2.8.1. Escherichia coli baiting method

In this method, *E. coli* cells are grown on Luria Broth. The cells are harvested by centrifugation and resuspended in nuclease free water encompassing cycloheximide (50-250 µg/mL). This mixture is mixed to obtain thick slurry and cross-streaked on surface of water agar containing 50-250 µg/mL of cycloheximide and 12 g/L Agar (Figure 2.5). The center of the cross-streaked *E. coli* is inoculated with pea-sized soil sample. After inoculation, the plates are incubated at 32°C for 1-4 week and examined for the formation of fruiting bodies and swarming pattern (Reichenbach and Dworkin, 1992). Different genera like *Archangium* spp., *Corallococcus* spp. and *Myxococcus* spp., have been isolated via this method (Gaspari *et al.,* 2005; Saadatpour *et al.,* 2024).

2.8.2. Dung pellets baiting method

In this method, CY media (3g/L Casitone, 1g/L Yeast Extract, 1.36g/L $CaCl_2.2H_2O$, 15g/L Agar; pH 7.2) is prepared and a mound of soil moistened with 50-250 µg/mL cycloheximide stock solution is placed and spread over the surface of CY media. Subsequently, on each plate five dried and autoclaved dung pellets from rabbit or goat are positioned in such a manner that half of each dung pellet remains embedded in the soil while the other half remains exposed from the soil. After, 1-3 week of incubation within the range of 25-32°C, fruiting bodies of myxobacteria that have migrated from the soil to dung pellet is observed under the stereomicroscope (Reichenbach and Dworkin, 1992). Different genera like *Archangium* spp., *Corallococcus* spp., *Cystobacter* spp*., Myxococcus* spp. and *Nannocystis* spp. have been isolated via this method (Gaspari *et al.,* 2005; Saadatpour *et al.,* 2024).

2.8.3. Filter paper method

This method is predominantly used for isolating cellulose-degrading myxobacteria. In this method, sterile Whatman filter is used as the energy and carbon source, which is placed on the ST21CX agar [1g/L CaCl2.2H2O, 0.2g/L $FeCl_3$, 1g/L K_2HPO_4, 1g/L KNO_3, 1g/L $MgSO_4.7H_2O$, 0.1g/L $MnSO_4.7H_2O$, 0.02g/L Yeast extract, 10g/L Agar; cycloheximide and 1ml/L trace element solution; 10X Trace element solution: 0.02g/L $CoCl_2$, 0.01g/L $CuSO_4$, 8g/L EDTA trihydrate Na-Fe^{+3} salt, 0.01g/L H_3BO_3, 0.02g/L KBr, 0.02g/L KI, 0.005g/L LiCl, 0.1g/L $MnCl_2.4H_2O$, 0.01g/L $Na_2MoO_4.2H_2O$, 0.005g/L $SnCl_2.2H_2O$, 0.02g/L $ZnCl_2$; pH 7.4]

surface. On the filter paper, a clump of soil about the size of a pea treated with cycloheximide (50-250 µg/mL) is placed and the plates are incubated at 32°C and observed for swarming pattern and fruiting bodies after 1-4 weeks (Figure 2.5) (Reichenbach and Dworkin, 1992; Tianmei *et al.,* 2023).

2.8.4. Moist chamber method

In this method, cycloheximide (50-250 µg/mL) is applied to an autoclaved Whatman filter paper and sections of bark are fully immersed in cycloheximide (50-250 µg/mL) before placing them on sterilized petri plate. After the incubation for 1-4 weeks at 32°C, the bark is observed for fruiting bodies (Peterson 1969). Different genera like *Myxococcus* spp. *Archangium* spp., *Cystobacter* spp., *Chondromyces* spp., *Corallococcus* spp., *Melittangium*spp., and *Stigmatella* spp. have been isolated by this approach (Gaspari *et al.,* 2005; Novozhilov *et al.,* 2022).

2.8.5. Isolation from spores

It is an extensively used method for isolating myxobacteria that form spores, as myxospores are heat resistant. In this methods, soil (air-dried) is diluted by addition of distilled water (sterile) and subjected to heating at 55-60°C for 10-15 mins and then placed on CY-C10 medium (1% Casitone supplemented with CY media containing 3g/L Casitone, 1g/L Yeast Extract, 1.36g/L $CaCl_2.2H_2O$, 15g/L Agar; pH 7.2) containing cycloheximide (50 µg/mL). The antibiotics like neomycin, gentamycin, kanamycin, *etc*., are added in the media to stop the growth of other bacteria (Karwowski *et al.,* 1996). This method is stated to be effective in isolating member of *Sorangiineae* family as they are resistant to various antibiotics.

To obtain a pure culture of myxobacteria, cells are picked from the swarms' edge and sub-cultured several times until a pure culture is obtained. After visualization under a stereomicroscope, several species of myxobacteria have been purified from fruiting bodies with the help of sterile needle. Followed by their culturing on CY agar media, SP agar medium 2.5g/L Casitone, 1g/L Galactose, 0.25g/L K_2HPO_4, 0.5g/L $MgSO_4.7H_2O$, 1g/L Raffinose, 5g/L starch (soluble), 1g/L Sucrose and 15g/L Agar; pH 7.4.), CNST-CMC agar medium [0.5g/L KNO_3, 0.25g/L $Na_2HPO_4.12H_2O$, 1g/L $MgSO_4.7H_2O$, 0.01g/L $FeCl_3.6H_2O$, 2g/L Carboxymethyl Cellulose powder, 15g/L Agar; and 1ml/L trace element solution; 10X Trace element solution: 0.02g/L $CoCl_2$, 0.01g/L $CuSO_4$, 8g/L EDTA trihydrate Na-Fe^{+3} salt, 0.01g/L H_3BO_3, 0.02g/L KBr, 0.02g/L KI, 0.005g/L LiCl, 0.1g/L $MnCl_2.4H_2O$, 0.01g/L $Na_2MoO_4.2H_2O$, 0.005g/L $SnCl_2.2H_2O$, 0.02g/L $ZnCl_2$] (Reichenbach and Dworkin, 1992). Moreover, Myxospores have been used for the purification of *Melittangium* spp., *Stigmatella*

spp., *Cystobacter* spp., and *Archangium* spp. (all member of *Archangiaceae* family) and *Chondromyces* spp. (*Polyangiaceae*), due to their ability to withstand the exposure to ultrasonic stimulation for 10-60 sec via ultrasonic probe of 20 kHz (Sutherland, 1976; Rouhizohrab and Mohammadipanah, 2022).

2.9. Exploration of myxobacteria for enzyme

In current scenario, procurement of improved, robust, and high-yielding enzymes has become the need of industries to render industrial processes more cost-effectively. The published literature has provided evidence that microbes are the viable source of important industrial enzymes. Enzymes like amylases, proteases, cellulases, and so on, which belong to the group of enzymes known as hydrolases are generally obtained from the microbes. These enzymes are well-known to participate in reactions like hydrolysis, condensation, and alcoholysis (Adrio and Demain, 2014; Liu and Kokare, 2023). Even though microbial enzymes were discovered in the 20^{th} century. Studies are still being conducted continuously on the isolation, characterization of enzyme properties, scale-up to bench- and pilot-scale and their utilization in bio-industries have constantly progressed. Additionally, this knowledge is regularly updated in the form of scientific literature (Nigam, 2013).

The primary reason for exploring microbial enzymes is that they possess unique properties like their ability to function under abnormal conditions, primarily those of temperature and pH. This is why some enzymes are classified as acidophilic, alkalophilic, thermophilic or psychrophilic (Kochhar *et al.,* 2022). For instance, the presence of thermostable enzyme-producing microorganisms capable of performing the biological function at a higher temperature than normal reaction temperature. This reduces the chances of microbial contamination during prolonged industrial reactions at the industrial scale. In fact, enzymes having high thermostability improves the efficiency of the breakdown and digestion of raw material. Additionally, high reaction temperature also enhances the penetration ability of enzymes (Turner *et al.,* 2007). Saccharification and hydrolysis of agricultural residues containing polysaccharides require a longer processing time, which increases the risk of contamination during this period (Tse *et al.,* 2021). Thus, due to the ability of hydrolases to remain stable and active at varied temperatures, it is in high demand (de Lourdes Moreno *et al.,* 2013). Moreover, during the hydrolysis of substrates or raw materials in industrial processes, the high-temperature enzymes improve the mass transfer and reduce the viscosity of the substrates. These abilities can also be ascribed to unculturable microbes inhabiting in

extreme niches. These unculturable microbes serve as a reservoir for specialized enzymes that exhibit exceptional specificity, allowing them to effectively operate under varying pH and temperature conditions (Cruz-Casas *et al.*, 2021). This significant diversity in enzymes lies in their relevance to different industrial applications, encompassing beverages, cosmetics, confectioneries, detergent, food, paper, pharmaceutical, leather processing and textile industries (Chapman *et al.*, 2018).

2.9.1. Hydrolases

Notably, Hydrolases also known as "substratease" or "substrate hydrolases" has gained significant attention and accorded to play a significant role in various industrial applications. In general, hydrolases are the enzyme that are characterized by their ability to catalyze the hydrolytic cleavage of anhydride bond. This activity is facilitated by the involvement and presence of water molecules (Busto *et al.,* 2010). The hydrolases enzymes have been well-comprehended for its immense practical importance in different industries like cosmetics, food, beverages, pharmaceutical and paper industries (Chapman *et al.,* 2018). The highly notable and commercially significant hydrolases are amylases, cellulases and proteases. For instance, proteases have been comprehended to play significant role as key components in variety of detergent formulation (Niyonzima and More, 2014; Robinson, 2015). On the other hand, cellulases are utilized in the field of bioethanol research (Ejaz *et al.,* 2021; Ranganathan *et al.,* 2022; Ilić *et al.,* 2023). Amidst several applications in the food industry, amylases play a crucial role in processes that involve the transformation of starch into sugar syrup and the liquefaction of starch (de Souza and de Oliveira Magalhães, 2010; Ashok *et al.,* 2023). These examples highlight the significant importance of hydrolases in a wide range of industrial applications. Therefore, motivating to explore new location for isolating these enzymes and understand their distinct functions and contributions.

2.9.1.1. Amylases

Amylases are a class of enzymes that are specialized in the process of hydrolyzing starch. These enzymes can be classified into three main types:

1. α-Amylase: α-amylases refer to α-endo-amylases, which explicitly hydrolyze the α-1,4 glycosidic bonds present in starch molecules. The internal bonds of starch are disrupted by α-amylase which results in the production of smaller, soluble sugar molecules (de Souza and de Oliveira Magalhães, 2010).

2. β-Amylase: β-amylases are classified as exo-amylases and are primarily involved in the hydrolysis of α-1,4 glycosidic bonds present at the nonreducing ends of starch molecules. The aforementioned procedure progressively liberates maltose molecules (Das and Kayastha, 2019).

3. γ-Amylase: γ-amylases, also referred as glucoamylases, exhibit the ability to enzymatically break down starch by cleaving both α-1,4 and α-1,6 glycosidic bonds. The process of enzymatic action is highly efficient in the hydrolysis of starch molecules, resulting in the cleavage of these complex polysaccharides into individual glucose units (Kumar and Chakravarty, 2018).

These three distinct forms of amylases play indispensable role in wide range of industrial applications, particularly in the food sector. The microbes synthesizing these enzymes play a crucial role in diverse processes like saccharification, baking, brewing and starch conversion. This process involves the transformation of starch into simpler digestible sugars and other valuable products (Khan *et al.,* 2011).

The utilization of starch as a carbon and energy source is prevalent throughout several microbes, plants and animals. The α-amylases derived from microbes exhibit a wide range of diversity encompassing bacterial species like *B. amyloliquefaciens, B. acidocaldarius* and *B. cereus;* fungal species like *A. niger, H. lanuginosus* and *F. oxysporum*; and yeast species like *L. starkeyi* and *E. jibuligera*. Amylases, those exhibiting thermophilic characteristics have notable utility in different industrial applications (Mehta and Satyanarayana, 2016). An exemplary use is in the starch processing, wherein the elevated temperatures ranging from 100-110°C is required. Thermostable amylases have demonstrated their significance in this particular context, as they are widely used at industrial scale for the processing of starch (Gomes *et al.,* 2003).

Applications of Amylases

Starch Processing Industry: In this industry, the starch processing heavily relies on the utilization of α-amylases which play a crucial role in the liquefication of starch into glucose and fructose syrups. This method involves three fundamental steps namely saccharification, liquefaction and gelatinization, wherein a diverse range of enzymes are used at each respective stage. The optimization of this process is enhanced when high temperature steps are integrated, highlighting the significance of enzyme capable of functioning a extreme temperatures (de Souza and de Oliveira Magalhães, 2010).

Detergent Industry: The utilization of this enzymes in detergent formulations is prevalent in the detergent industry owing to their robustness, effectiveness, and eco-friendly attributes. Amylolytic enzymes have a substantial role in around 90% of detergent formulations (Hmidet *et al.,* 2019).

Biofuel Industry: The biofuel industry relies on the extraction of fermentable sugars from complex polysaccharides for the manufacturing of bioethanol, a commonly utilized biofuel. Starch, being a widely accessible polysaccharide from an economic standpoint, has been shown to undergo hydrolysis through the utilization of amylolytic enzymes. The hydrolysis results in the production of fermentable sugars (Ashok *et al.,* 2023).

Paper Industry: The paper industry is a sector that involves the production, distribution, and consumption of paper products. The high viscosity of natural starch renders it unsuitable for the manufacturing of high-quality paper. In this case, α-amylases are used for partial hydrolysis of starch in order to achieve the optimum viscosity of the slurry. This improves both the coating and paper quality (de Souza and de Oliveira Magalhães, 2010).

2.9.1.2. Cellulases

Cellulases, an enzyme class that are extensively involved in the hydrolysis of cellulose, specifically targeting the β-1,4 glycosidic linkages present within cellulose and other similar cellulosic macro-complexes. In terms of their structural organization, cellulases are composed of three discrete functional domains, namely the catalytic core domain, the cellulose binding domain and the linker domain (Paul *et al.,* 2021).

There are three basic types of cellulases, distinguished by their distinct hydrolytic properties:

1. Endo-1,4-β-D-glucanases: These enzymes are known to catalyze the cleavage of internal β-1,4 glycosidic linkages present in cellulose. This enzymatic activity serves as the initial step in the hydrolysis process. Thus, it is extensively involved in the breakdown of cellulose chains (Annamalai *et al.,* 2016).

2. Exo-1,4-β-glucanases: These enzymes are known to catalyze the cleavage on the terminal end of β-1,4 glycosidic linkages present in cellulose. Hence, facilitating the degradation of cellulose chains from their terminal ends (Jin *et al.,* 2023).

3. β-D-glucosidases: These enzymes play a crucial role in the hydrolysis of oligosaccharides generated by endo- and exo-cellulases, facilitating their conversion into individual glucose units (Woodward and Wiseman, 1982).

Cellulases are predominantly synthesized by a diverse microbial species, encompassing fungi, bacteria (both anaerobic and aerobic, exhibiting thermophilic and mesophilic characteristics), termites and protozoans. The hydrolysis of cellulose is significantly influenced by these enzymes (Ejaz *et al.,* 2021).

Applications of Cellulases

Laundry and Textile Industry: The laundry and textile industry utilize cellulases for procedure like bio-stoning of denim, which was conventionally accomplished through the manual rubbing of pumice stones. Cellulases are also used in the bio-polishing process, which involves the removal of lint from fabrics to improve the aesthetic and tactile qualities of clothing (Ito *et al.,* 1989; Niyonzima and More, 2014).

Paper Industry: The utilization of cellulases and hemicellulases in the paper pulping industry is integral to the production of paper, which is commonly known as "bio-pulping." These enzymes have the ability to improve the process of saccharification, enhance the drainage properties of the pulp and reduce viscosity. Consequently, these improvements result in speed up operations within paper mills. Cellulases are also used in the process of de-inking, in which it specifically removes the laser and xerographic inks (Basak *et al.,* 2021).

Beer and Wine Industry: The beer and wine industry heavily rely on the utilization of barley, a key ingredient of beer production. Moreover, Barley is known to possess a substantial quantity of cellulosic material. Cellulases are actively involved in the process of saccharification of cellulosic material. After saccharification, the resulted product is subject for the fermentation of sugars with the help of yeast or bacteria. During the malting process of barley, enzymes like amylases, carboxypeptidases, and endoglucanases comes into action which facilitates the breakdown of stored substances within barley seeds (Chakraborty *et al.,* 2016).

Extraction of Vegetables\Fruit Juices and Olive Oil: The clarification of fruit and vegetable juices involves the use of macerozymes, which encompass cellulases, hemicellulases, and pectinases. These enzymes facilitate the liquefaction of fruit pulp, hence enhancing the efficiency of juice extraction. Furthermore, they are also used in the process of extracting and enriching vitamin E and antioxidants in olive oil (Toushik *et al.,* 2017).

2.9.1.3. Proteases

Proteases/Peptidases have gained the significant interest of the researchers over an extended period of time owing to their critical role in metabolic processes and their broad range of industrial applications. Proteases are enzymes that have exhibited a wide distribution and considerable diversity within the microbial realm (Song *et al.,* 2023). The protease enzymes can be divided into two primary categories, namely exopeptidases and endopeptidases, based on the peptide bond they selectively target (Boon *et al.,* 2020). The proteolytic enzymes have been derived from various sources, encompassing animals, plants and microbes (Dhillon *et al.,* 2017). However, microbes are considered to be the most desirable source of proteases owing to their ease of genetic manipulation and protein expression abilities (Ibrahim *et al.,* 2015). According to Horikoshi (2008), microbial proteases constitute roughly 40% of the overall global enzyme output (Horikoshi 2008). Furthermore, Horikoshi (2011) states that these proteases hold a dominant position in nearly two-thirds of the global commercial production of proteases (Horikoshi *et al.,* 2011). Numerous studies have been conducted to investigate diverse types of proteases and their production employing various molecular biology techniques (Gupta *et al.,* 2002; Herman *et al.,* 2023). Proteases are classified into several categories based on the functional groups present in their active sites.

Endopeptidases:

Metalloproteases: Metalloproteases are a class of proteases that possess an active site consisting of a metal ion, commonly a divalent zinc ion. The ideal pH range for these enzymes is between 7.0 and 9.0. Matrix metalloproteases (MMPs) are a notable example of such enzymes. MMPs are known to be involved in crucial processes such as apoptosis and the composition of the extracellular matrix (Mansfeld, 2007).

Aspartic proteases: Aspartic proteases are the enzyme characterized by the presence of an aspartate residue inside their active sites. These proteases exhibit best enzymatic activity within an acidic pH range, with a pH optimum often observed around 2.0. Some examples of enzymes are chymosin, cathepsin D, pepsin, penicillopepsin and rennin (Dunn, 2013).

Cysteine proteases: Cysteine proteases are characterized by the presence of a thiol group derived from cysteine in their active sites. These enzymes function by employing nucleophilic assaults on substrate molecules. Prominent examples of these type of enzyme involves cathepsins B and H, calpains, papain, and streptococcal proteinases, which are classified as cytosolic calcium-activated proteases (Buttle and Mort, 2013).

Serine proteases: Serine proteases are distinguished by the presence of a nucleophilic serine residue within their active sites. The majority of serine proteases exhibit an optimal pH at a neutral level and can be classified into two main groups: bacterial serine proteases and

mammalian serine proteases. Several examples of enzymes are Chymotrypsin, Elastase, Kallikrein, Subtilisin and Trypsin (Stapels *et al.,* 2015).

Exopeptidases:

Aminopeptidases: Aminopeptidases are enzymes that hydrolyze polypeptides by cleaving them at the unbound N-terminal end, resulting in the liberation of amino acids, dipeptides, or tripeptides. These enzymes are synthesized by variety of bacterial and fungal species, either produced intracellularly and extracellularly (Gonzales and Robert-Baudouy, 1996; Nandan and Nampoothiri, 2017).

Carboxypeptidases: Carboxypeptidases are enzymes that catalyze the hydrolysis of peptide bonds at the carboxy terminal end of polypeptides, resulting in the removal of amino acids or dipeptides. Based on their ideal pH ranges, carboxypeptidases can be categorized as cysteine carboxypeptidases, serine carboxypeptidases and metallocarboxypeptidases (Song *et al.*, 2021).

Applications of Proteases

Food Industry: During the production of cheese, protease hydrolyse casein to prevent coagulation. Whereas, in case of baking, fungal protease (heat liable) partially hydrolyzes the gluten for robust preparation of dough. Alkaline microbial proteases are commonly used for preparation of protein hydrolysates having high nutritional value, that are important for beverages fortification and infant food (Ray, 2012).

Leather Industry: The increasing utilization of alkaline protease in developing leather industries is due to its strong elastolytic and keratinolytic properties important for processing of leather. Proteases are used in the bating, soaking, and dehairing processes of skin. Enzymatic treatments, particularly using microbial alkaline proteases are now preferred for processing leather owing to their effectiveness and specificity (Razzaq *et al.*, 2019).

Detergent Industry: Proteases are essential elements in detergents, accounting for approximately 20% of enzyme sales. They are utilized in home laundry, denture, and contact lens cleansers. Proteases such as BIO-40 and BIOTEX have become common products in the market. Progressive development in recombinant DNA technology has resulted in bioengineered proteases that possess improved stability. They are also used in dishwashing and cleaning detergents across many industries (Razzaq *et al.*, 2019).

2.10. Assay methods for proteases

One of the common approach used to evaluate proteolytic activity involves the utilization of the sulphanilamide azocasein substrate. Azocasein is a protein that has been modified chemically to add in the sulphanilamide groups, which covalently links with the peptide bonds in milk protein casein and develops an orange colour. This process involves the use of protease enzyme facilitating the hydrolysis of peptide bonds resulting in the release of smaller peptides and amino acids during a 1-hour incubation period. After incubation, the Trichloroacetic acid (TCA) is subsequently added for the precipitation of both the enzyme and natural azocasein, which are then removed via centrifugation. The low molecular weight oligopeptides and released amino acids that are not influenced by TCA precipitation persist in the solution resulting in development of an orange tint. The spectrophotometric quantification of this colour and its intensity enable us to determine the level of protease activity (Coêlho *et al.*, 2016).

Additional approaches used for the determination of protease activity involves the utilization of gelatin-agar diffusion method (Ammar *et al.,* 1986). Another alternative method used for the evaluation of proteinase activity against casein involves the usage of Folin–Ciocalteu (FC) reagent and is measured at a wavelength of 600nm (Kim *et al.,* 2001). In addition, the activity of proteinase against casein can also be determined by evaluating the amount of tyrosine using a Folin-phenol technique and measuring it at a wavelength of 578 nm (Guangrong *et al.,* 2006). An alternative approach involves the process of incubating azocasein along with an enzyme solution and then measuring the absorbance at a wavelength of 440 nm (Olajuyigbe and Ajele 2005).

Furthermore, another technique employing a protein A substrate tagged with a green fluorescent protein (GFP) has been formulated. In this method, when a protease cleaves this substrate, it emits fluorescence that can be quantitively measured. Thus, fluorescence serves as a sensitive test to measure the release of tyrosine (in picograms). The resulting relative fluorescence intensity allows the reliable assessment of the protease activity (Fujino *et al.,* 2002). Another method involves the utilization of a fluorescence zymogram in-gel experiment, where an SDS polyacrylamide gel is combined with a peptide-MCA (4-methyl-coumaryl-7-amide) substrate. This allows the assessment of both the specificity and molecular weight of the protease enzyme (Yasothornsrikul and Hook 2000). Furthermore, a fluorescence polarization method has been established to quantify alterations in molecular size caused by the cleavage of the intact fluorescein thiocarbamoyl (FTC)-casein molecules into smaller FTC peptides. This assay, exhibits a high level of sensitivity. Moreover, it does not involve the use of separations, precipitations, or transfers of reaction mixtures. Therefore, it serves as a non-radioactive alternative for determining the protease activity (Bolger and Checovich 1994).

2.11. Enzymes synthesized by myxobacteria

Myxobacteria are known as micro-predators, which synthesize a variety of enzymes for cell lysis and other distinct functions. There are different enzymes synthesized by myxobacterial species (Wrótniak-Drzewiecka *et al.,* 2016). For instance, *Sorangium* sp., are known for their efficient decomposition of biomacromolecules such as cellulose (Hou *et al.,* 2006). Moreover, myxobacteria that are both cellulolytic and predatory types produce proteolytic enzymes. These enzymes serve multiple functions, including supplying amino acids to myxobacteria, disrupting cell membranes of eubacteria, and contributing to the lysis of prey microbes. Examples include serine proteases, extracellular alkaline and acid proteases (Sharma *et al.,* 2021).

The significant role of lipids has been recorded in the *Myxococcus* life cycle, particularly during development and predation. Myxobacteria, such as *Myxococcus xanthus* possess a large number of putative lipase genes, including α/β hydrolases, lipases, GDSL lipases and patatin. Lipolytic enzymes are involved in breaking down membranes and releasing fatty acids and cytoplasmic contents from prey (Moraleda-Muñoz and Shimkets, 2007). Studies on *Myxococcus virescens* reveal the presence of extracellular protein complexes that inhibit the functioning of lysozyme and exhibit proteolytic activity against gelatin (Moraleda-Muñoz and Shimkets, 2007). These complexes may play a role in nutrition and serve as substrates for extracellular enzymes. *M. xanthus* synthesizes ApaH-like phosphatases. These phosphatases potentially serve as both Ap4A hydrolases and tyrosine protein phosphatases, respectively (Sasaki *et al.,* 2014). *M. virescens* synthesizes prolyl endopeptidases that have the ability to break down peptides (immunotoxic in nature) associated with celiac disease (Alvarez-Sieiro *et al.,* 2014). Conclusively, myxobacteria demonstrate a noteworthy enzymatic activity that plays a pivotal role in their predatory behaviour and developmental intricacies. These diverse activities highlights prospective applications across multidisciplinary domains, especially in biotechnology and medicine (Wrótniak-Drzewiecka *et al.,* 2016). Moreover, different enzymes obtained from different myxobacterial species have been comprehended in Table 2.2.

Table 2.2: Enlist of different enzymes synthesized by different myxobacterial species

Species Name	**Enzyme**	**References**
Corallococcus sp.	endo-chitinase	(Li *et al.,* 2019)
Sorangium cellulosum	Xylanase	(Wang *et al.,* 2012)
Archangium sp.	Protease	(Rouhizohrab and Mohammadipanah, 2021)

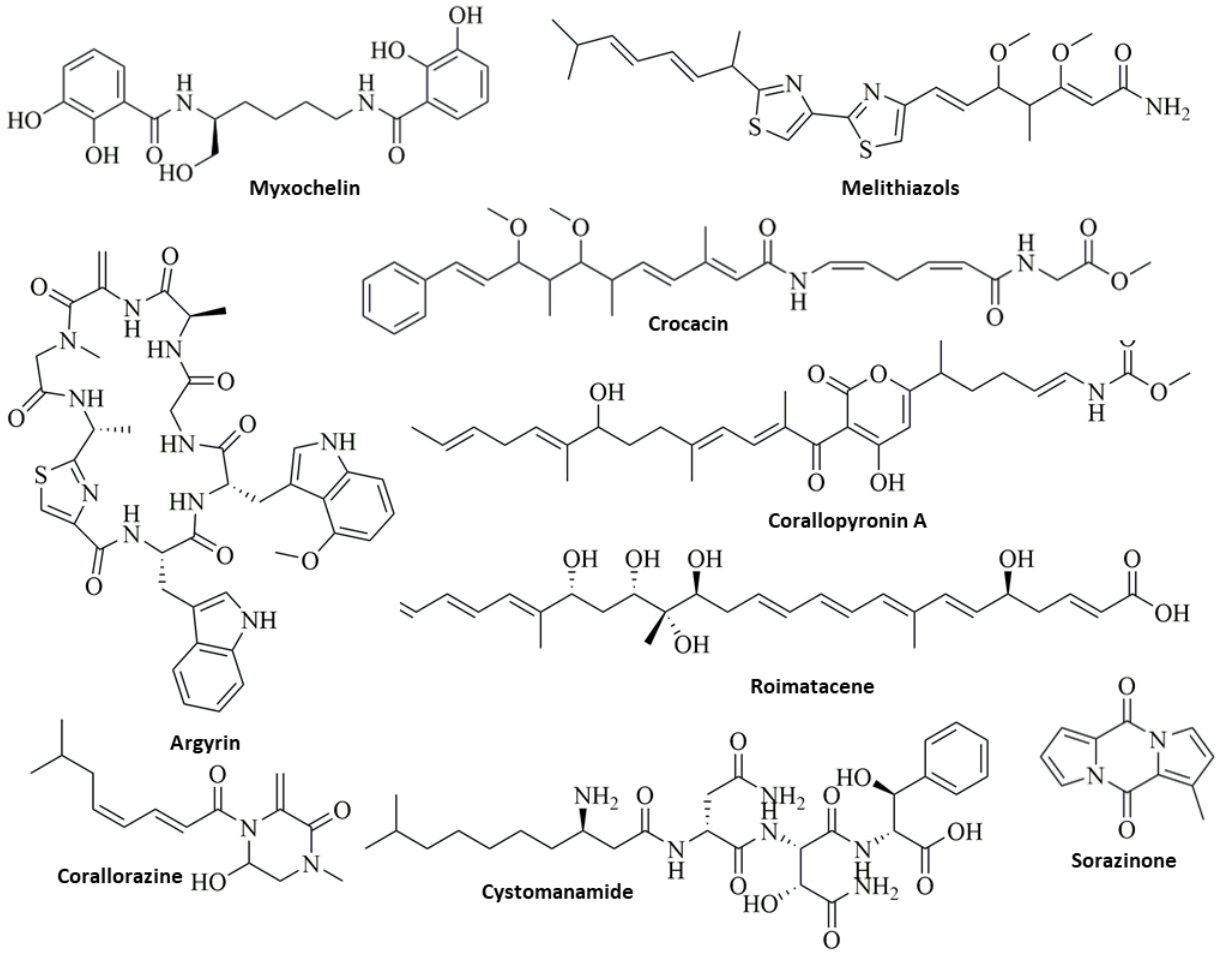
Myxococcus sp.	Protease	(Kim *et al.,* 2009)
Myxococcus xanthus	β-1,4-endoglucanase	(Quillet *et al.,* 1995)
Myxococcus xanthus	ApaH-like phosphatase	(Sasaki *et al.,* 2014)
Archangium lipolyticum sp. Nov	Lipase	(Zhou *et al.,* 2023)
Corallococcus coralloides	GH13 glycoside phosphorylase	(Franceus and Desmet, 2019)
Stigmatella aurantiaca	endo-N-acetyl-beta-D-glucosaminidase	(Bourgerie *et al.,* 1994)
Archangium sp.	Malto-Oligosaccharide-Producing Amylase	(Fan *et al.,* 2021)
Myxococcus sp.	GH6 Endoglucanase	(Huang *et al.,* 2023)

2.12. Bioactive molecules synthesized by myxobacteria

Approximately 47% of anticancer drugs and 70% of anti-infectives have been derived from natural products (Newman and Cragg, 2007; Newman and Cragg, 2020). In accordance with microbial genome sequences, microbes are likely to contribute to natural products. Microbes such as actinomycetes have been extensively exploited for discovering diverse bioactive molecules. As of now, myxobacteria are being studied for their ability to generate novel bioactive molecules with an unusual mode of action that is rarely found in other natural products. Nearly 100 core distinct structures and around 500 derivatives of bioactive molecules have been reported to be obtained from myxobacteria (Reichenbach, 2001; Bhat *et al.,* 2021). Myxobacteria produce secondary metabolites at the beginning of the growth phase rather than during the late logarithmic and stationary phases of growth as synthesized by other microbes (Weissman and Müller, 2009). Moreover, myxobacteria have the ability to produce a strain-specific class of bioactive molecules. Furthermore, a myxobacteria species usually produce diverse bioactive molecules. For instance, *Sorangium cellulosum* So ce12 (Bode and Müller, 2008; Wenzel and Müller, 2009a; Wenzel and Müller, 2009b) produces chivosazoles (anti-fungal) (Jansen *et al.,* 1997), disorazole (tubulin destabilizers) (Jansen *et al.,* 1994), sorangicins (RNA polymerase inhibitors) (Irschik *et al.,* 1987), and sulfangolids (anti-viral) (Bode and Müller, 2008). The major secondary metabolites synthesized by myxobacteria are alkaloids, non-ribosomal peptides, phenylpropanoids, polyketides, and steroids (Bhat *et al.,* 2021). Along with these, compounds like ceramides, cerebrosides, cholestenols, and lanosterol have also

been isolated from myxobacterial species. Interestingly, each secondary metabolite produced by myxobacteria shows a unique action mechanism (Diez *et al.*, 2012). For example, antibacterial compounds obtained from myxobacterial species target acetyl-CoA carboxylase, cellular electron-transport chain, DNA, type II signal peptidase, and viral RNA polymerase (Herrmann *et al.*, 2017). The chemical structure of the few antibacterial compounds synthesized by myxobacteria is illustrated in Figure 2.6. Furthermore, Table 2.3 provides a comprehensive list of different bioactive metabolites obtained from myxobacteria, along with their mode of action and biological activities.

2.12.1. Structural depiction of different antibacterial compounds synthesized by myxobacterial species



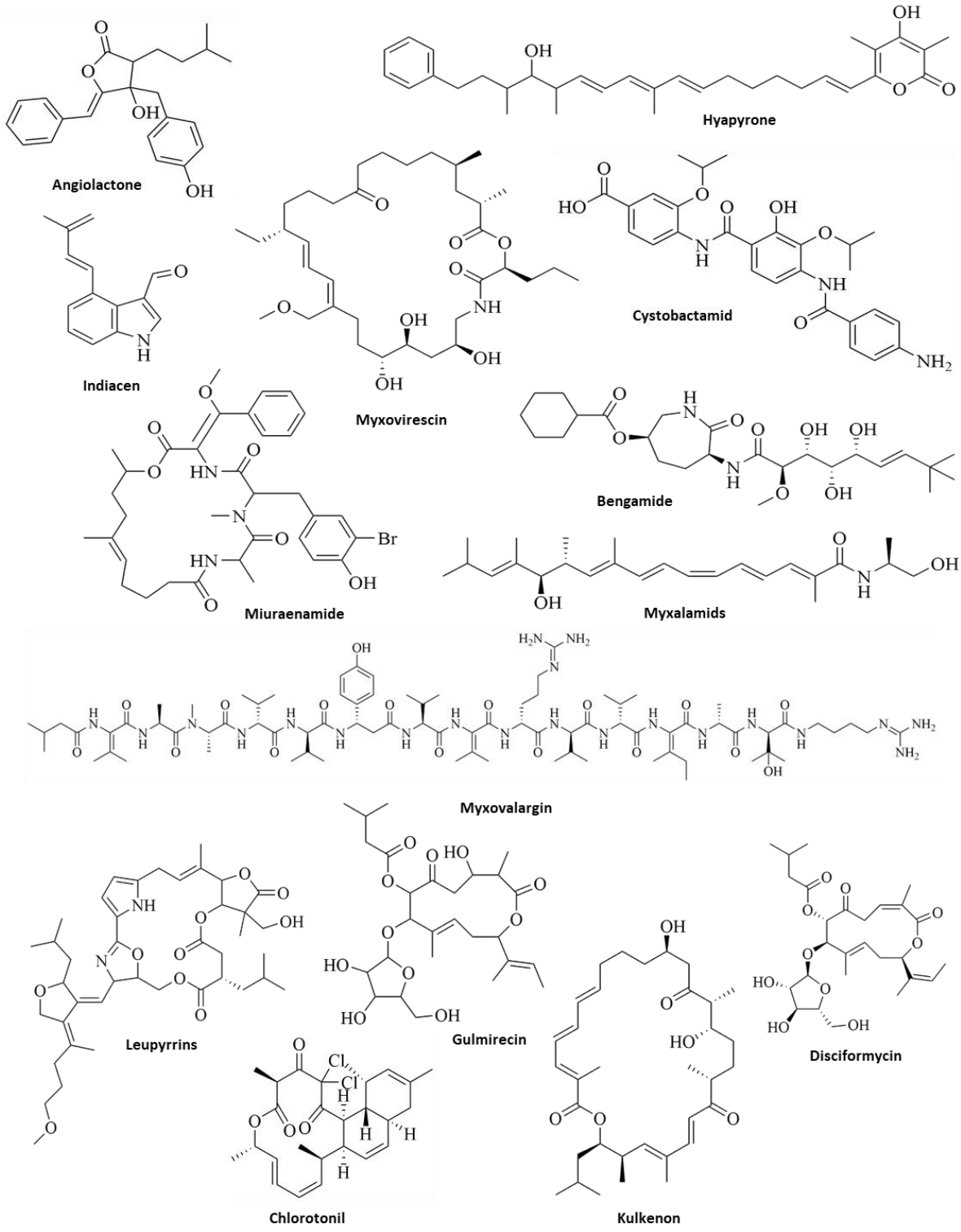


Figure 2.6: Chemical structure of different anti-bacterial compounds synthesized by myxobacterial species

Table 2.3: Summary of the reported secondary metabolites obtained from different myxobacterial strains along with their mechanism of action and biological activity

Source	Taxonomy (Suborder; Family; Genus)	Secondary Metabolite	Structural Class	Mechanism of Action	Biological Activity	References
Archangium disciforme	Cystobacterineae; Cystobacteraceae; *Archangium*	Angiolam	Macrolactone	Protein synthesis inhibition	Anti-bacterial	(Kunze *et al.*, 1985; Bhat *et al.*, 2021)
Archangium disciforme An d30	Cystobacterineae; Cystobacteraceae; *Archangium*	Myxochelin	Catechol siderophore	5-lipoxygenase inhibition	Anti-bacterial, Anti-tumor invasive, anti-viral, siderophore	(Kunze *et al.*, 1989; Silakowski *et al.*, 2000; Schieferdecker *et al.*, 2015; Schieferdecker *et al.*, 2017; Bhat *et al.*, 2021)
Archangium disciforme	Cystobacterineae; Cystobacteraceae; *Archangium*	Pretubulysin	Peptide	Microtubules Depolymerization	Anti-angiogenic, Cytotoxic	(Ullrich *et al.*, 2009; Herrmann *et al.*, 2012; Rath *et al.*, 2012; Braig *et al.*, 2014; Kretzschmann *et al.*, 2014; Bhat *et al.*, 2021)
Archangium disciforme An d48	Cystobacterineae; Cystobacteraceae; *Archangium*	Tubulysin	Peptide	Tubulin polymerization inhibition	Cytostatic	(Sasse *et al.*, 2000; Steinmetz *et al.*, 2004; Kaur *et al.*, 2006; Khalil *et al.*, 2006; Chai *et al.*, 2010; Murray *et al.*, 2015; Nicolaou *et*

						al., 2016; Bhat *et al.*, 2021)
Archangium violaceum	Cystobacterineae; Cystobacteraceae; *Archangium*	Vioprolide	Peptolide	-	Anti-fungal, Cytotoxic	(Schummer *et al.*, 1996; Sharma and Subramanian, 2017; Bhat *et al.*, 2021)
Archangium violaceum	Cystobacterineae; Cystobacteraceae; *Archangium*	Archazolid	Macrolactone	Vacuolar-type ATPases Inhibition	Cytotoxic	(Huss *et al.*, 2005; Menche *et al.*, 2007; Wiedmann *et al.*, 2012; Kubisch *et al.*, 2014; Schneider *et al.*, 2015; Sharma and Subramanian, 2017; Bhat *et al.*, 2021)
Archangium violaceum Cb vi76	Cystobacterineae; Cystobacteraceae; *Archangium*	Gephyronic acid	Aliphatic acid	Eukaryotic protein synthesis inhibition	Anti-fungal, Cytotoxic	(Stevens *et al.*, 2014; Sharma and Subramanian, 2017; Bhat *et al.*, 2021)
Archangium gephyra	Cystobacterineae; Cystobacteraceae; *Archangium*	Archazolid	Macrolactone	Vacuolar-type ATPases Inhibition	Cytotoxic	(Huss *et al.*, 2005; Menche *et al.*, 2007; Wiedmann *et al.*, 2012; Kubisch *et al.*, 2014; Schneider *et al.*, 2015; Sharma and Subramanian, 2017; Bhat *et al.*, 2021)
Archangium gephyra	Cystobacterineae; Cystobacteraceae; *Archangium*	Argyrin	Cyclic Peptide	Target Elongation factor G, Inhibits Antibody formation	Anti-bacterial, Anti-fungal, Cytotoxic, Immunosuppressant	(Sasse *et al.*, 2002; Nickeleit *et al.*, 2008; Nyfeler *et al.*, 2012; Sharma and

				and proteasome inhibition		Subramanian, 2017; Bhat *et al.*, 2021)
Archangium gephyra Ar 10844	Cystobacterineae; Cystobacteraceae; *Archangium*	Aurafuron	Furanone	Acetyl CoA carboxyl Inhibition	Anti-bacterial, Anti-fungal, Cytotoxic	(Kunze *et al.*, 2005; Frank *et al.*, 2007; Sharma and Subramanian, 2017; Bhat *et al.*, 2021)
Archangium gephyra	Cystobacterineae; Cystobacteraceae; *Archangium*	Cyrmenin	N-Acylpeptide	Respiration (Complex III) Inhibition	Anti-fungal, Cytotoxic	(Sharma and Subramanian, 2017; Bhat *et al.*, 2021)
Archangium gephyra Ar 3895	Cystobacterineae; Cystobacteraceae; *Archangium*	Gephyronic acid	Aliphatic acid	Eukaryotic protein synthesis inhibition	Anti-fungal, Cytotoxic	(Stevens *et al.*, 2014; Sharma and Subramanian, 2017; Bhat *et al.*, 2021)
Archangium gephyra Ar 315	Cystobacterineae; Cystobacteraceae; *Archangium*	Tubulysin	Peptide	Tubulin polymerization inhibition	Cytostatic	(Sasse *et al.*, 2000; Steinmetz *et al.*, 2004; Kaur *et al.*, 2006; Khalil *et al.*, 2006; Chai *et al.*, 2010; Murray *et al.*, 2015; Nicolaou *et al.*, 2016; Sharma and Subramanian, 2017; Bhat *et al.*, 2021)
Archangium gephyra	Cystobacterineae; Cystobacteraceae; *Archangium*	Melithiazol	Bithiazole	Respiration (Complex III) Inhibition	Anti-fungal, Cytotoxic	(Sasse *et al.*, 1999; Weinig *et al.*, 2003; Sharma and Subramanian, 2017; Bhat *et al.*, 2021)

Stigmatella aurantiaca DW4/3-1	Cystobacterineae; Archangiaceae; *Stigmatella*	Myxochromide	Depsipeptide	-	Anti-fungal, Cytotoxic	(Wenzel *et al.*, 2005; Ohlendorf *et al.*, 2008; Sharma and Subramanian, 2017; Bhat *et al.*, 2021)
Stigmatella aurantiaca DW4/3-1	Cystobacterineae; Archangiaceae; *Stigmatella*	Aurafuron	Furanone	Acetyl CoA carboxyl Inhibition	Anti-bacterial, Anti-fungal, Cytotoxic	(Kunze *et al.*, 2005; Frank *et al.*, 2007; Sharma and Subramanian, 2017; Bhat *et al.*, 2021)
Stigmatella aurantiaca MYX-030	Cystobacterineae; Archangiaceae; *Stigmatella*	Myxocoumarin	Polyphenol	-	Anti-fungal	(Gulder *et al.*, 2013; Sharma and Subramanian, 2017; Bhat *et al.*, 2021)
Stigmatella aurantiaca Sg a15	Cystobacterineae; Archangiaceae; *Stigmatella*	Aurachin	Quinolone	Respiration (complexes I and III) Inhibition	Anti-bacterial, Anti-fungal, Anti-malarial	(Kunze *et al.*, 2008; Sharma and Subramanian, 2017; Bhat *et al.*, 2021)
Stigmatella aurantiaca Sg a15	Cystobacterineae; Archangiaceae; *Stigmatella*	Myxochelin	Catechol siderophore	5-lipoxygenase Inhibition	Anti-bacterial, Anti-tumor invasive, Anti-viral, siderophore	(Kunze *et al.*, 1989; Silakowski *et al.*, 2000; Schieferdecker *et al.*, 2017; Sharma and Subramanian, 2017; Bhat *et al.*, 2021)
Stigmatella aurantiaca Sg a15	Cystobacterineae; Archangiaceae; *Stigmatella*	Stigmatellin	γ-Chromone	Respiration (complexes I and III) Inhibition	Anti-fungal, Cytotoxic	(Kunze *et al.*, 1984; Thierbach *et al.*, 1984; Sharma and Subramanian, 2017; Bhat *et al.*, 2021)

Stigmatella erecta strain Pd e32	Cystobacterineae; Archangiaceae; *Stigmatella*	Aurachin	Quinolone	Respiration (complexes I and III) Inhibition	Anti-bacterial, Anti-fungal, Anti-malarial	(Kunze *et al.*, 2008; Bhat *et al.*, 2021; Wang *et al.*, 2024)
Melittangium lichenicola Me l46	Cystobacterineae; Archangiaceae; *Melittangium*	Melithiazol	Bithiazole	Respiration (complex III) Inhibition	Anti-fungal, cytotoxic	(Sasse *et al.*, 1999; Weinig *et al.*, 2003; Bhat *et al.*, 2021; Wang *et al.*, 2024)
Cystobacter fuscus Cb 685	Cystobacterineae; Archangiaceae; *Cystobacter*	Althiomycin	Cyclic peptide	Peptidyl transferase reaction inhibition	Anti-bacterial, Cytotoxic	(Fujimoto *et al.*, 1970; Kunze *et al.*, 1982; Sharma and Subramanian, 2017; Bhat *et al.*, 2021)
Cystobacter fuscus AJ-13278	Cystobacterineae; Archangiaceae; *Cystobacter*	Cystothiazole	Bithiazole	Respiration (complex III) Inhibition	Anti-fungal, Cytotoxic	(Ojika *et al.*, 1998; Suzuki *et al.*, 2003; Sharma and Subramanian, 2017; Bhat *et al.*, 2021; Wang *et al.*, 2024)
Cystobacter fuscus MCy9118	Cystobacterineae; Archangiaceae; *Cystobacter*	Macyranone	Epoxyketone	Proteasome Inhibition	Anti-parasite, Cytotoxic	(Keller *et al.*, 2015; Sharma and Subramanian, 2017; Bhat *et al.*, 2021; Wang *et al.*, 2024)
Cystobacter ferrugineus Cb G35	Cystobacterineae; Archangiaceae; *Cystobacter*	p-hydroxyacetophenone amide	Amide	-	Anti-bacterial, Anti-fungal, Anti-algal	(Zander *et al.*, 2011; Sharma and Subramanian, 2017; Bhat *et al.*, 2021; Wang *et al.*, 2024)
Cystobacter ferrugineus Cb G35	Cystobacterineae; Archangiaceae; *Cystobacter*	Roimatacene	Polyene	-	Anti-bacterial	(Zander *et al.*, 2011; Sharma and Subramanian, 2017;

						Bhat *et al.*, 2021; Wang *et al.*, 2024)
Cystobacter armeniaca	Cystobacterineae; Archangiaceae; *Cystobacter*	Cyrmenin	N-Acylpeptide	Respiration (Complex III) Inhibition	Anti-fungal, Cytotoxic	(Sasse *et al.*, 2003; Bhat *et al.*, 2021; Wang *et al.*, 2024)
Cystobacter sp.	Cystobacterineae; Archangiaceae; *Cystobacter*	Cystobactamid	Peptide	Type IIa topoisomerase Inhibition	Anti-bacterial	(Baumann *et al.*, 2014; Hüttel *et al.*, 2017; Bhat *et al.*, 2021; Wang *et al.*, 2024)
Cystobacter sp.	Cystobacterineae; Archangiaceae; *Cystobacter*	Cystodienoic acid	Terpene	-	Cytotoxic	(Raju *et al.*, 2015; Bhat *et al.*, 2021; Wang *et al.*, 2024)
Cystobacter sp. SBCb004	Cystobacterineae; Archangiaceae; *Cystobacter*	Tubulysin	Peptide	Tubulin polymerization Inhibition	Cytostatic	(Sasse *et al.*, 2000; Steinmetz *et al.*, 2004; Kaur *et al.*, 2006; Khalil *et al.*, 2006; Chai *et al.*, 2010; Murray *et al.*, 2015; Nicolaou *et al.*, 2016; Bhat *et al.*, 2021; Wang *et al.*, 2024)
Hyalangium minutum	Cystobacterineae; Archangiaceae; *Hyalangium*	Hyaladione	Quinone	-	Anti-bacterial, Anti-fungal, Cytotoxic	(Okanya *et al.*, 2012; Bhat *et al.*, 2021; Wang *et al.*, 2024)
Hyalangium minutum	Cystobacterineae; Archangiaceae; *Hyalangium*	Hyalachelin	Catecholate siderophore	-	Cytotoxic	(Nadmid *et al.*, 2014; Bhat *et al.*, 2021; Wang *et al.*, 2024)

Hyalangium minutum	Cystobacterineae; Archangiaceae; *Hyalangium*	Hyafurone	Furanone, polyketide	-	Anti-bacterial, Anti-fungal	(Okanya *et al.*, 2014; Bhat *et al.*, 2021; Wang *et al.*, 2024)
Hyalangium minutum	Cystobacterineae; Archangiaceae; *Hyalangium*	Hyapyrroline	Furanone	-	Anti-bacterial, Anti-fungal	(Okanya *et al.*, 2014; Bhat *et al.*, 2021; Wang *et al.*, 2024)
Hyalangium minutum	Cystobacterineae; Archangiaceae; *Hyalangium*	Hyapyrone	Furanone	-	Anti-bacterial, Anti-fungal, Cytotoxic	(Okanya *et al.*, 2014; Bhat *et al.*, 2021; Wang *et al.*, 2024)
Myxococcus virescens	Cystobacterineae; Myxococcaceae; *Myxococcus*	Bengamide	Caprolactam	MetAP and NFkB pathway Inhibition	Anti-bacterial, Anti-helmintic, Anti-inflammatory, Cytotoxic	(Gnosspelius, 1978b; Johnson *et al.*, 2012; Wenzel *et al.*, 2015; Wang *et al.*, 2024)
Myxococcus virescens Mx v48	Cystobacterineae; Myxococcaceae; *Myxococcus*	Myxovirescin	Macrolide	Type II signal peptidase LspA inhibition, Hinders the synthesis of cell wall	Anti-bacterial	(Gnosspelius, 1978b; Zafriri *et al.*, 1981; Xiao *et al.*, 2012; Xiao and Wall, 2014; Wang *et al.*, 2024)
Myxococcus xanthus	Cystobacterineae; Myxococcaceae; *Myxococcus*	Xanthacin	-	-	Anti-bacterial	(McCurdy and MacRae, 1974; Qian *et al.*, 2017; Bhat *et al.*, 2021)
Myxococcus xanthus	Cystobacterineae; Myxococcaceae; *Myxococcus*	Myxalamid	Polyene	Respiration (Complex I) Inhibition	Anti-fungal	(Gerth *et al.*, 1983; Qian *et al.*, 2017; Bhat *et al.*, 2021; Wang *et al.*, 2024)

Myxococcus xanthus DK 1622	Cystobacterineae; Myxococcaceae; *Myxococcus*	Myxochromide	Depsipeptide	-	Anti-fungal, Cytotoxic	(Wenzel *et al.*, 2005; Ohlendorf, Kehraus, *et al.*, 2008; Qian *et al.*, 2017; Bhat *et al.*, 2021)
Myxococcus xanthus DSM504/15	Cystobacterineae; Myxococcaceae; *Myxococcus*	Saframycin Mx1	Quinone, heterocyclic	Induces breaking of single strand DNA due to covalent binding with DNA	Anti-bacterial, Cytotoxic	(Irschikn *et al.*, 1988; Qian *et al.*, 2017; Bhat *et al.*, 2021; Wang *et al.*, 2024)
Myxococcus fulvus DSM2549	Cystobacterineae; Myxococcaceae; *Myxococcus*	Myxopyronin	α-Pyrone	RNA polymerase Inhibition	Anti-bacterial	(O'Neill *et al.*, 2000; Mukhopadhyay *et al.*, 2008; Moy *et al.*, 2011; Srivastava *et al.*, 2011; Sahner *et al.*, 2015; Bhat *et al.*, 2021)
Myxococcus fulvus	Cystobacterineae; Myxococcaceae; *Myxococcus*	KR-025	Bithiazole	-	Cytotoxic	(Ahn *et al.*, 1999; Bhat *et al.*, 2021; Wang *et al.*, 2024)
Myxococcus fulvus Mx f50	Cystobacterineae; Myxococcaceae; *Myxococcus*	Myxopyronin	α-Pyrone	RNA polymerase Inhibition	Anti-bacterial	(O'Neill *et al.*, 2000; Mukhopadhyay *et al.*, 2008; Moy *et al.*, 2011; Srivastava *et al.*, 2011; Sahner *et al.*, 2015; Bhat *et al.*, 2021; Wang *et al.*, 2024)

Myxococcus fulvus	Cystobacterineae; Myxococcaceae; *Myxococcus*	Myxothiazol	Bithiazole	Respiration (Complex III) Inhibition	Anti-fungal, Cytotoxic	(Gerth *et al.*, 1980; Thierbach and Reichenbach, 1981; Ahn *et al.*, 1999; Ahn *et al.*, 2007; Shrivastava and Sharma, 2021)
Myxococcus fulvus strain Mx f147	Cystobacterineae; Myxococcaceae; *Myxococcus*	Pyrrolnitrin	Phenylpyrrole	Respiration (Complex I) Inhibition	Anti-bacterial, Anti-fungal	(Tripathi and Gottlieb, 1969; Shrivastava and Sharma, 2021)
Myxococcus fulvus	Cystobacterineae; Myxococcaceae; *Myxococcus*	Myxovalargin	Peptide	Damage Cell Membrane; Protein Synthesis Inhibition	Anti-bacterial, Anti-malarial, Cytotoxic	(Irschik *et al.*, 1983; Irschik and Reichenbach, 1985; Bhat *et al.*, 2021; Shrivastava and Sharma, 2021)
Myxococcus stipitatus Mx s40	Cystobacterineae; Myxococcaceae; *Myxococcus*	Phenalamide	Polyene	-	Anti-cancer, Anti-viral	(Kim *et al.*, 1991; Bhat *et al.*, 2021; Wang *et al.*, 2024)
Myxococcus stipitatus	Cystobacterineae; Myxococcaceae; *Myxococcus*	Rhizopodin	Macrodiolide	Actin Polymerization Inhibition	Anti-fungal, Cytotoxic	(Sasse *et al.*, 1993; Hagelueken *et al.*, 2009; Oku *et al.*, 2014; Zhang *et al.*, 2015; Bhat *et al.*, 2021; Shrivastava and Sharma, 2021; Wang *et al.*, 2024)
Myxococcus stipitatus AJ-12587	Cystobacterineae; Myxococcaceae; *Myxococcus*	Stipiamide	Polyene	-	Anti-viral, Reversal agent against Multi-Drug Resistance Strain	(Kim *et al.*, 1991; Andrus 2004; Bhat *et al.*, 2021; Shrivastava and

						Sharma, 2021; Wang *et al.*, 2024)
Myxococcus sp. Strain 131	Cystobacterineae; Myxococcaceae; *Myxococcus*	Myxotyroside	Rhamnoside	-	Anti-malarial, Cytotoxic	(Ohlendorf *et al.*, 2009; Bhat *et al.*, 2021; Shrivastava and Sharma, 2021; Wang *et al.*, 2024)
Corallococcus coralloides DSM 2550	Cystobacterineae; Myxococcaceae; *Corallococcus*	Corallopyronin	α-Pyrone	Eubacterial RNA polymerase Inhibition	Anti-bacterial, Anti-filarial	(Irschik and Reichenbach, 1985; O'Neill *et al.*, 2000; Mukhopadhyay *et al.*, 2008; Mariner *et al.*, 2011; Schiefer *et al.*, 2012; Schäberle *et al.*, 2015; Bhat *et al.*, 2021; Shrivastava and Sharma, 2021; Wang *et al.*, 2024)
Corallococcus coralloides strain M23	Cystobacterineae; Myxococcaceae; *Corallococcus*	Coralmycin	Peptide	-	Anti-bacterial	(Kim *et al.*, 2016; Bhat *et al.*, 2021; Shrivastava and Sharma, 2021; Wang *et al.*, 2024)
Corallococcus coralloides	Cystobacterineae; Myxococcaceae; *Corallococcus*	Myxalamid	Polyene	Respiration (Complex I) Inhibition	Anti-fungal	(Gerth *et al.*, 1983; Bhat *et al.*, 2021; Shrivastava and Sharma, 2021; Wang *et al.*, 2024)
Pyxidicoccus fallax	Cystobacterineae; Myxococcaceae; *Pyxidicoccus*	Disciformycin	Macrolide glycoside	-	Anti-bacterial	(Surup *et al.*, 2014; Bhat *et al.*, 2021;

						Shrivastava and Sharma, 2021)
Pyxidicoccus fallax	Cystobacterineae; Myxococcaceae; *Pyxidicoccus*	Gulmirecin	Macrolide	-	Anti-bacterial	(Schieferdecker *et al.*, 2014; Bhat *et al.*, 2021; Shrivastava and Sharma, 2021; Wang *et al.*, 2024)
Pyxidicoccus fallax HKI 727	Cystobacterineae; Myxococcaceae; *Pyxidicoccus*	Myxochelin	Catechol siderophore	5-lipoxygenase Inhibition	Anti-bacterial, Anti-tumor invasive, Anti-viral, Siderophore	(Kunze *et al.*, 1989; Silakowski *et al.*, 2000; Schieferdecker *et al.*, 2015; Schieferdecker *et al.*, 2017; Bhat *et al.*, 2021; Shrivastava and Sharma, 2021; Wang *et al.*, 2024)
Pyxidicoccus sp. MCy9557	Cystobacterineae; Myxococcaceae; *Pyxidicoccus*	Pyxipyrrolone	Pyrrole	-	Cytotoxic	(Kjaerulff *et al.*, 2017; Bhat *et al.*, 2021; Shrivastava and Sharma, 2021; Wang *et al.*, 2024)
Nannocystis pusilla B150	Nannocystineae; Nannocystaceae; *Nannocystis*	Phenylnannolone	Polyene	-	Reversal agent against Multi-Drug Resistance Strain	(Ohlendorf, Leyers, *et al.*, 2008; Bouhired *et al.*, 2014; Bhat *et al.*, 2021; Shrivastava and Sharma, 2021; Wang *et al.*, 2024)

Nannocystis exedens	Nannocystineae; Nannocystaceae; *Nannocystis*	Nannochelin	Hydroxamate	Chelation of Iron and Siderophore	Anti-bacterial, Anti-fungal	(Kunze *et al.*, 1992; Bhat *et al.*, 2021; Shrivastava and Sharma, 2021; Wang *et al.*, 2024)
Nannocystis sp DSM18870	Nannocystineae; Nannocystaceae; *Nannocystis*	Nannocystin	cyclic lactone	Elongation factor I (EF-1α) Inhibition	Anti-fungal, Cytotoxic	(Hoffmann *et al.*, 2015; Krastel *et al.*, 2015; Bhat *et al.*, 2021; Shrivastava and Sharma, 2021; Wang *et al.*, 2024)
Nannocystis sp. MB1016	Nannocystineae; Nannocystaceae; *Nannocystis*	Nannocystin	cyclic lactone	Elongation factor I (EF-1α) Inhibition	Anti-fungal, Cytotoxic	(Hoffmann *et al.*, 2015; Krastel *et al.*, 2015; Bhat *et al.*, 2021; Shrivastava and Sharma, 2021; Wang *et al.*, 2024)
Enhygromyxa salina	Nannocystineae; Nannocystaceae; *Enhygromyxa*	Salimabromide	Furano lactone	-	Anti-bacterial	(Felder *et al.*, 2013; Bhat *et al.*, 2021; Shrivastava and Sharma, 2021; Wang *et al.*, 2024)
Enhygromyxa salina	Nannocystineae; Nannocystaceae; *Enhygromyxa*	Salimyxin	Sterol	-	Anti-bacterial	(Felder *et al.*, 2013; Bhat *et al.*, 2021; Shrivastava and Sharma, 2021; Wang *et al.*, 2024)
Enhygromyxa salina	Nannocystineae; Nannocystaceae; *Enhygromyxa*	Enhygrolide	Furanone	-	Anti-bacterial	(Felder *et al.*, 2013; Bhat *et al.*, 2021; Shrivastava and Sharma, 2021; Wang *et al.*, 2024)

Haliangium ochraceum SMP-2	Nannocystineae; Haliangiaceae; *Haliangium*	Haliamide	Polyketide- non-ribosomal peptide hybrid	-	Anti-bacterial, Anti-fungal, Cytotoxic	(Sun *et al.*, 2016; Bhat *et al.*, 2021; Shrivastava and Sharma, 2021; Wang *et al.*, 2024)
Haliangium luteum	Nannocystineae; Haliangiaceae; *Haliangium*	Haliangicin	Polyene	Respiration (Complex III) Inhibition	Anti-fungal, Cytotoxic	(Fudou *et al.*, 2001; Bhat *et al.*, 2021; Shrivastava and Sharma, 2021; Wang *et al.*, 2024)
Aetherobacter sp.	Sorangiineae; Polyangiaceae; *Aetherobacter*	Aetheramide	Depsipeptide	-	Anti-fungal, Anti-viral, Cytostatic	(Plaza *et al.*, 2012; Bhat *et al.*, 2021; Shrivastava and Sharma, 2021; Wang *et al.*, 2024)
Byssovorax cruenta	Sorangiineae; Polyangiaceae; *Byssovorax*	Cruentaren	Benzolactone	F0-F1 mitochondrial ATPase Inhibition	Anti-fungal, Cytotoxic	(Kunze *et al.*, 2006; Kunze *et al.*, 2007; Huss and Wieczorek, 2009; Pérez-Sayáns *et al.*, 2009; Hall *et al.*, 2014; Bhat *et al.*, 2021; Shrivastava and Sharma, 2021; Wang *et al.*, 2024)
Chondromyces crocatus Cm c5	Sorangiineae; Polyangiaceae; *Chondromyces*	Thuggacin	Macrolide	Electron Transport Chain Inhibition	Anti-bacterial	(Irschik *et al.*, 2007; Steinmetz *et al.*, 2007; Buntin *et al.*, 2010; Zaburannyi *et al.*, 2016; Bhat *et al.*, 2021; Shrivastava and

						Sharma, 2021; Wang *et al.*, 2024)
Chondromyces crocatus Cm c5	Sorangiineae; Polyangiaceae; *Chondromyces*	Ajudazol	Isochromanone	Electron Transport Chain Inhibition	Anti-bacterial, Anti-fungal	(Jansen *et al.*, 2002; Kunze *et al.*, 2004; Buntin *et al.*, 2008; Bhat *et al.*, 2021; Shrivastava and Sharma, 2021; Wang *et al.*, 2024)
Chondromyces crocatus Cm c2	Sorangiineae; Polyangiaceae; *Chondromyces*	Chondramide	Depsipeptide	Actin Polymerization Inhibition	Anti-fungal, Anti-viral, Cytotoxic	(Kunze *et al.*, 1995; Sasse *et al.*, 1998; Waldmann *et al.*, 2008; Herrmann *et al.*, 2013; Foerster *et al.*, 2014; Menhofer *et al.*, 2014; Bhat *et al.*, 2021; Shrivastava and Sharma, 2021; Wang *et al.*, 2024)
Chondromyces crocatus Cm c5	Sorangiineae; Polyangiaceae; *Chondromyces*	Chondrochloren	Styrene	-	Anti-bacterial, Anti-fungal	(Jansen *et al.*, 2002; Rachid *et al.*, 2009; Bhat *et al.*, 2021; Shrivastava and Sharma, 2021; Wang *et al.*, 2024)
Chondromyces crocatus Cm c3	Sorangiineae; Polyangiaceae; *Chondromyces*	Crocacin	N-Acyldipeptide	Respiration (Complex III) Inhibition	Anti-bacterial, Anti-fungal, Cytotoxic	(Kunze *et al.*, 1994; Jansen *et al.*, 1999; Bhat *et al.*, 2021; Shrivastava and Sharma, 2021; Wang *et al.*, 2024)

Chondromyces pediculatus	Sorangiineae; Polyangiaceae; *Chondromyces*	Crocacin	N-Acyldipeptide	Respiration (Complex III) Inhibition	Anti-bacterial, Anti-fungal, Cytotoxic	(Kunze *et al.*, 1994; Jansen *et al.*, 1999; Bhat *et al.*, 2021; Shrivastava and Sharma, 2021; Wang *et al.*, 2024)
Chondromyces pediculatus	Sorangiineae; Polyangiaceae; *Chondromyces*	Pedein	Cyclic peptide	Disrupts integrity of membrane	Anti-fungal, Cytotoxic	(Kunze *et al.*, 2008; Bhat *et al.*, 2021; Shrivastava and Sharma, 2021; Wang *et al.*, 2024)
Chondromyces sp.	Sorangiineae; Polyangiaceae; *Chondromyces*	Apicularen	Benzolactone enamide	Triggers mitochondrial independent apoptosis, disrupts microtubules, vacuolar-type ATPases inhibition	Anti-bacterial, Cytotoxic	(Jansen *et al.*, 1998; Huss *et al.*, 2005; Kim *et al.*, 2007; Seo *et al.*, 2013; Bhat *et al.*, 2021; Shrivastava and Sharma, 2021; Wang *et al.*, 2024)
Jahnella sp. Strain MSr9139	Sorangiineae; Polyangiaceae; *Jahnella*	Microsclerodermin	Cyclic peptide	NFkB inhibition and apoptosis induction	Anti-fungal	(Hoffmann *et al.*, 2013; Guzmán *et al.*, 2015; Bhat *et al.*, 2021; Shrivastava and Sharma, 2021; Wang *et al.*, 2024)
Polyangium sp. PI 3007	Sorangiineae; Polyangiaceae; *Polyangium*	Thiangazole	Tristhiazolonine	Respiration (Complex I) Inhibition	Cytotoxic	(Kunze *et al.*, 1993; Bhat *et al.*, 2021; Shrivastava and Sharma, 2021; Wang *et al.*, 2024)

Sorangium cellulosum	Sorangiineae; Polyangiaceae; *Sorangium*	Ambruticin	Lactone	Interferes osmoregulation through HOG pathway	Anti-fungal	(Ringel *et al.*, 1977; Knauth and Reichenbach, 2000; Yan *et al.*, 2003; Vetcher *et al.*, 2007; Bhat *et al.*, 2021; Shrivastava and Sharma, 2021; Wang *et al.*, 2024)
Sorangium cellulosum	Sorangiineae; Polyangiaceae; *Sorangium*	Carolacton	Macrolactone	Interferes with cell wall synthesis via PknB signalling and membrane integrity. Interferes with density-dependent signalling pathway	Biofilm formation Inhibition	(Yan *et al.*, 2003; Kunze *et al.*, 2010; Reck *et al.*, 2011; Apel *et al.*, 2013; Sudhakar *et al.*, 2014; Donner *et al.*, 2016; Bhat *et al.*, 2021; Shrivastava and Sharma, 2021; Wang *et al.*, 2024)
Sorangium cellulosum So ce12	Sorangiineae; Polyangiaceae; *Sorangium*	Chivosazol	Macrolactone	Actin Polymerization Inhibition	Anti-fungal, Cytotoxic	(Yan *et al.*, 2003; Diestel *et al.*, 2009; Bhat *et al.*, 2021; Shrivastava and Sharma, 2021; Wang *et al.*, 2024)
Sorangium cellulosum So ce1525	Sorangiineae; Polyangiaceae; *Sorangium*	Chlorotonil	Macrolactone	Hinders all the stages of intraerythrocytic parasite development and stages IV-V gametocytes	Anti-bacterial, Anti-malarial	(Yan *et al.*, 2003; Gerth *et al.*, 2008; Held *et al.*, 2014; Bhat *et al.*, 2021; Shrivastava and Sharma, 2021; Wang *et al.*, 2024)

Sorangium cellulosum So ce12	Sorangiineae; Polyangiaceae; *Sorangium*	Disorazol	Macrodilactone	Microtubule formation Inhibition	Anti-fungal, Cytotoxic	(Jansen *et al.*, 1994; Yan *et al.*, 2003; Elnakady *et al.*, 2004; Carvalho *et al.*, 2005; Hopkins and Wipf, 2009; Tierno *et al.*, 2009; Bhat *et al.*, 2021; Shrivastava and Sharma, 2021; Wang *et al.*, 2024)
Sorangium cellulosum strain So ce90, So ce90/B2 and So ce90/D13	Sorangiineae; Polyangiaceae; *Sorangium*	Epothilone	Macrolactone	Promotes axonal regeneration, Stabilizes microtubules	Anti-fungal, Anti-viral, Cytostatic, promotes axonal regeneration	(Bollag *et al.*, 1995; Muhlradt and Sasse, 1997; Hardt *et al.*, 2001; Yan *et al.*, 2003; Cheng *et al.*, 2008; Reichenbach and Höfle, 2008; Wang *et al.*, 2009; Rivera and Gomez, 2010; Martinez *et al.*, 2013; Lou *et al.*, 2014; Ruschel *et al.*, 2015; Tran and Silver, 2015; Bhat *et al.*, 2021; Shrivastava and Sharma, 2021; Wang *et al.*, 2024)
Sorangium cellulosum strains	Sorangiineae; Polyangiaceae; *Sorangium*	Etnangien	Macrolactone	Bacterial and Viral nucleic acid	Anti-bacterial, Anti-viral	(Yan *et al.*, 2003; Menche *et al.*, 2008; Bhat *et al.*, 2021;

So ce750 and So ce1045				polymerase Inhibition		Shrivastava and Sharma, 2021; Wang *et al.*, 2024)
Sorangium cellulosum So ce701	Sorangiineae; Polyangiaceae; *Sorangium*	Icumazole	Oxazole, polyketide	Targets Respiratory Chain, DNA, RNA and protein synthesis Inhibition	Anti-fungal	(Yan *et al.*, 2003; Barbier *et al.*, 2012; Bhat *et al.*, 2021; Shrivastava and Sharma, 2021; Wang *et al.*, 2024)
Sorangium *cellulosum* So ce701	Sorangiineae; Polyangiaceae; *Sorangium*	Noricumazole	Isochromanone	Aid in stabilization of tetrameric conformation of potassium channel (i.e., KcsA)	Anti-viral, Cation channel blocker	(Yan *et al.*, 2003; Gentzsch *et al.*, 2011; Barbier *et al.*, 2012; Beck *et al.*, 2016; Bhat *et al.*, 2021; Shrivastava and Sharma, 2021; Wang *et al.*, 2024)
Sorangium cellulosum So ce 307	Sorangiineae; Polyangiaceae; *Sorangium*	Jerangolid	Lactone	Alters membrane permeability leading to cell leakage, affects osmoregulation	Anti-fungal	(Gerth *et al.*, 1996; Yan *et al.*, 2003; Bhat *et al.*, 2021; Shrivastava and Sharma, 2021; Wang *et al.*, 2024)
Sorangium cellulosum So ce1485	Sorangiineae; Polyangiaceae; *Sorangium*	Maltepolide	Macrolactone	Spindle Formation Inhibition	Cytotoxic	(Yan *et al.*, 2003; Irschik *et al.*, 2013; Bhat *et al.*, 2021; Shrivastava and Sharma, 2021; Wang *et al.*, 2024)
Sorangium cellulosum So ce880	Sorangiineae; Polyangiaceae; *Sorangium*	Maracin	Vinyl ether	-	Anti-bacterial	(Herrmann *et al.*, 1998; Yan *et al.*, 2003; Bhat *et al.*,

						2021; Shrivastava and Sharma, 2021; Wang *et al.*, 2024)
Sorangium cellulosum So ce1128	Sorangiineae; Polyangiaceae; *Sorangium*	Maracen	Vinyl ether	-	Anti-bacterial	(Herrmann *et al.*, 1998; Yan *et al.*, 2003; Bhat *et al.*, 2021; Shrivastava and Sharma, 2021; Wang *et al.*, 2024)
Sorangium cellulosum So ce38	Sorangiineae; Polyangiaceae; *Sorangium*	Microsclerodermin	Cyclic peptide	Apoptosis induction and NFkB Inhibition	Anti-fungal	(Yan *et al.*, 2003; Hoffmann *et al.*, 2013; Guzmán *et al.*, 2015; Bhat *et al.*, 2021; Wang *et al.*, 2024)
Sorangium cellulosum WXNXJ-C	Sorangiineae; Polyangiaceae; *Sorangium*	Phoxalone	Macrolide	Mitotic arrest in G2/M phase	Cytotoxic	(Yan *et al.*, 2003; Guo *et al.*, 2007; Guo and Tao, 2008; Shrivastava and Sharma, 2021; Wang *et al.*, 2024)
Sorangium cellulosum	Sorangiineae; Polyangiaceae; *Sorangium*	Pellasoren	Polyketide	-	Cytotoxic	(Yan *et al.*, 2003; Jahns *et al.*, 2012; Bhat *et al.*, 2021; Shrivastava and Sharma, 2021; Wang *et al.*, 2024)
Sorangium cellulosum	Sorangiineae; Polyangiaceae; *Sorangium*	Ratjadone	α-Pyrone	CRM1 Inhibitor, Formation of nuclear export complex Inhibition	Anti-fungal, Anti-viral, Cytotoxic	(Schummer *et al.*, 1995; Kalesse *et al.*, 2001; Köster *et al.*, 2003; Yan *et al.*, 2003; Fleta-Soriano

						et al., 2014; Onder *et al.*, 2015; Bhat *et al.*, 2021; Shrivastava and Sharma, 2021; Wang *et al.*, 2024)
Sorangium cellulosum So ce377	Sorangiineae; Polyangiaceae; *Sorangium*	Ripostatin	Macrolactone	Bacterial RNA polymerase Inhibition	Anti-bacterial, Anti-fungal	(O'Neill *et al.*, 2000; Yan *et al.*, 2003; Buurman *et al.*, 2012; Bhat *et al.*, 2021; Shrivastava and Sharma, 2021; Wang *et al.*, 2024)
Sorangium cellulosum KM1003	Sorangiineae; Polyangiaceae; *Sorangium*	Sorangiadenosine	Nucleoside (sesquiterpene adenoside)	-	Anti-bacterial	(Yan *et al.*, 2003; Ahn *et al.*, 2008; Bhat *et al.*, 2021; Shrivastava and Sharma, 2021; Wang *et al.*, 2024)
Sorangium cellulosum So ce12	Sorangiineae; Polyangiaceae; *Sorangium*	Sorangicin	Macrolactone	Bacterial RNA polymerase Inhibition	Anti-bacterial	(Irschik *et al.*, 1987; Yan *et al.*, 2003; Campbell *et al.*, 2005; Bhat *et al.*, 2021; Shrivastava and Sharma, 2021; Wang *et al.*, 2024)
Sorangium cellulosum So ce12	Sorangiineae; Polyangiaceae; *Sorangium*	Sorangiolid	Macrolactone	Disrupts Integrity of Membrane	Anti-bacterial	(Yan *et al.*, 2003; Bhat *et al.*, 2021; Shrivastava and Sharma, 2021; Wang *et al.*, 2024)

Sorangium cellulosum So ce26	Sorangiineae; Polyangiaceae; *Sorangium*	Soraphen	Macrolactone	BC domain Inhibition, Alter virion composition, Inhibition of Fungal acetyl-CoA carboxylase,	Anti-viral, Anti-fungal, Anti-metabolic syndrome, Anti-cancer	(Gerth *et al.,* 1994; Vahlensieck *et al.,* 1994; Gerth *et al.,* 2003; Yan *et al.,* 2003; Shen *et al.,* 2004; Beckers *et al.,* 2007; Schreurs *et al.,* 2009; Jump *et al.,* 2011; Martinez *et al.,* 2013; Berod *et al.,* 2014; Koutsoudakis *et al.,* 2015; Cordonier *et al.,* 2016; Fleta-Soriano *et al.,* 2017; Wang *et al.,* 2024)
Sorangium cellulosum So ce895	Sorangiineae; Polyangiaceae; *Sorangium*	Sorazinone	Pyrazinone	-	Anti-bacterial	(Yan *et al.,* 2003; Jansen *et al.,* 2014; Bhat *et al.,* 2021; Shrivastava and Sharma, 2021; Wang *et al.,* 2024)
Sorangium cellulosum So ce90	Sorangiineae; Polyangiaceae; *Sorangium*	Spirangien	Polyketide, Spiroketal	Modulates IL-8 Expression	Anti-fungal, Anti-viral, Cytotoxic	(Yan *et al.,* 2003; Frank *et al.,* 2007; Reboll *et al.,* 2012; Martinez *et al.,* 2013; Bhat *et al.,* 2021; Shrivastava and Sharma, 2021; Wang *et al.,* 2024)

Sorangium cellulosum	Sorangiineae; Polyangiaceae; *Sorangium*	Spirodienal	Spiroketal	-	Cytotoxic	(Yan *et al.*, 2003; Kwak and Ahn, 2009; Ricca and Rizzacasa, 2021; Wang *et al.*, 2024)
Sorangium cellulosum	Sorangiineae; Polyangiaceae; *Sorangium*	Sulfangolid	Macrolide sulfate ester	-	Antiviral	(Yan *et al.*, 2003; Zander *et al.*, 2012; Martinez *et al.*, 2013; Bhat *et al.*, 2021; Shrivastava and Sharma, 2021; Wang *et al.*, 2024)
Sorangium cellulosum	Sorangiineae; Polyangiaceae; *Sorangium*	Tartrolon	Macrodiolide	Disrupts Integrity of Membrane	Anti-bacterial, Cytotoxic	(Schummer *et al.*, 1994; Irschik *et al.*, 1995; Yan *et al.*, 2003; Bhat *et al.*, 2021; Shrivastava and Sharma, 2021; Wang *et al.*, 2024)
Sorangium cellulosum So ce895	Sorangiineae; Polyangiaceae; *Sorangium*	Thuggacin	Macrolide	Cellular Electron-Transport Chain Inhibition	Anti-bacterial	(Yan *et al.*, 2003; Irschik *et al.*, 2007; Steinmetz *et al.*, 2007; Buntin *et al.*, 2010; Zaburannyi *et al.*, 2016; Bhat *et al.*, 2021; Shrivastava and Sharma, 2021; Wang *et al.*, 2024)
Sandaracinus amylolyticus NOSO-4	Sorangiineae; Sandaracinaceae; *Sandaracinus*	Indiacens	Prenyl indole	-	Anti-bacterial, Anti-fungal	(Steinmetz *et al.*, 2012; Guzmán *et al.*, 2015; Sharma *et*

						al., 2016; Bhat *et al.*, 2021; Shrivastava and Sharma, 2021; Wang *et al.*, 2024)

-: Not Available

According to an analysis of currently available antibiotic compounds, only 1.5% of them exhibit activity exclusively against gram-negative bacteria, while 30% target gram-positive bacteria (Bérdy, 2005; Myers and Clark, 2021). Gram-positive bacteria are more vulnerable to antibacterial drugs, whereas gram-negative bacteria have lipopolysaccharides in their outer membranes that make the cell wall impenetrable (Kakoullis *et al.,* 2021).

Studies on myxobacteria have shown that only a small number of myxobacterial isolates exhibit an inhibitory effect against gram-negative bacteria (Bhat *et al.,* 2021; Saadatpour *et al.,* 2024). In contrast, myxobacteria secrete bioactive chemicals that predominantly exhibit inhibitory effect against gram-positive (G+) bacteria (Schäberle *et al.,* 2014). Conversely, Gaspari and his colleagues (2005) found that over half of the myxobacteria isolated from Israel have been reported to synthesize bioactive compounds that exhibit an inhibitory effect on gram-negative (G-) bacteria (Gaspari *et al.,* 2005). Also, 42% of the myxobacteria isolated from the Slovakian region synthesized bioactive compounds that showed inhibitory effects on G- bacteria.

According to Charousová *et al.,* (2017), myxobacteria holds the potential to address the demand of pharmaceutical industries for bioactive compounds effective against G- pathogens (Charousová *et al.,* 2017). Lately, polyketides have gained significant consideration owing to their capability to synthesize diverse secondary metabolites that are generally synthesized by the enzyme named polyketide synthases (PKSs). Polyketides have diverse biological applications like antibiotics, anti-tumour, and immunosuppressants agents (Bode and Müller, 2008).

2.13. Overview of PKS

Microbes produce various secondary metabolites classified as polyketides, which are synthesized by polyketide synthases (PKSs) and exhibit a wide range of bioactivities and structural diversity (Helfrich and Piel, 2016; Wu *et al.,* 2023). The PKS module typically consists of three functional domains, i.e., the Acyl-transferase domain (AT), the Acyl-carrier protein domain (ACP), and the Ketosynthase domain (KS). The AT domain is involved in activation and binding to a specific substrate (e.g., CoA-activated acyl group). It also aids in transferring substrate to the ACP and KS domains. These domains facilitate the condensation as well as de-carboxylation reactions between the acyl-CoA substrate and the extending polyketide chain. Further chemical diversification of the polyketide chain occurs through the modification of certain domains, such as enoyl reductase (ER), dehydratase (DH), and keto-reductase (KR) (Robbins *et al.,* 2016; Dhanjal *et al.,* 2020), as comprehended in Figure 2.7.

The thioesterase domain (TE) is responsible for terminating the assembly line process. Additionally, there is an alternate mechanism where the trans-AT domain carries out a function comparable to that of the TE. The genes containing AT domains are typically located outside the module, within the gene cluster (Piel, 2010).

The PKSs can be further categorized into three distinct classes based on variations in the arrangement of synthetic modules.

2.13.1. Type I PKSs

This category of PKS is defined by a diverse module distributed across one or more proteins, which sequentially assemble themselves in a linear manner to synthesize the final compound. Furthermore, each module within this category is responsible for executing a single elongation step (Keatinge-Clay, 2012).

2.13.2. Type II PKSs

In this category of PKS, the synthesis involves key structural domains, including ACP, AT, and KS. In spite of the modular organization of these clusters, each gene encodes a catalytic domain with mono-functionality. These modules iteratively synthesize oxidized aromatic compounds (Hertweck *et al.*, 2007).

2.13.3. Type III PKSs

This category of PKS is homo-dimeric enzymes that catalyze the condensation reaction. Unlike type II PKSs, type III PKSs modules directly acts on the Acyl-CoA (substrate) in an iterative manner, as it lacks the ACP domain (substrate binding domain) (Yu *et al.*, 2012).

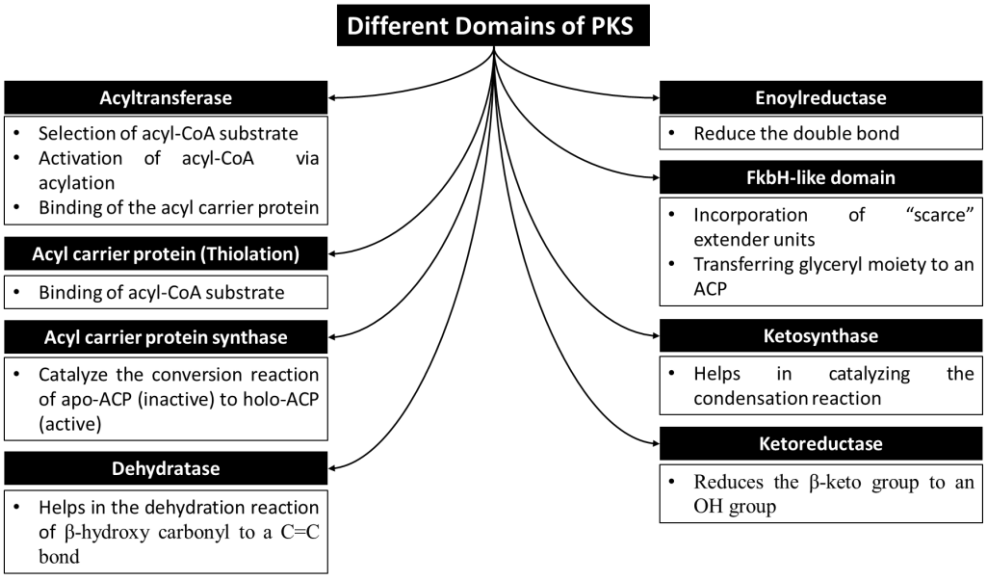


Figure 2.7: Illustration of domains along with function involved in secondary metabolites production by PKS (Dhanjal *et al.*, 2020)

2.14. Myxobacterial PKS

Polyketide synthases (PKSs) derived from myxobacteria are classified as type I PKSs. These enzymes are modular and iterative, capable of generating intricate and diverse polyketide structures (Bode and Müller, 2008). Myxobacteria exhibit a propensity to produce a spectrum of polyketides with novel structures and bioactivities like epothilones, myxovirescins, and myxopyronins by utilizing type I PKSs (Bode and Müller, 2008). The biosynthesis of polyketides by type I PKSs entails the sequential condensation of acyl-CoA units, originating from primary metabolism, facilitated by a series of catalytic domains organized within modules (Neves *et al.,* 2022). Till date, very little literature is available on myxobacteria isolation from Indian habitats (Watve *et al.,* 1999; Bhat *et al.,* 2021). Hence, the present study was designed to isolate myxobacteria from Kashmir Valley and explore their potential for the production of industrially important enzymes and secondary metabolites.

2.15. Purification of enzyme and secondary metabolites

In order to gain a more comprehensive understanding of enzymes and secondary metabolites, it is necessary to carry out the purification process. The main objective of the purification process is to remove the excess water from the cell-free extract. One effective approach involves the concentration of extracts with the help of salts or solvents having high affinity, resulting in the precipitation of bioactive molecules. Additionally, purification methods involve the usage of chromatographic and electrophoretic techniques (Manonmani and Joseph 1993; Su and Lee 2001; Saggu *et al.,* 2023a).

Regarding the concentration of enzymes and secondary metabolites, the culture is first separated from the biomass either via filtration or centrifugation. Then, the supernatant is concentrated through ammonium sulfate salting-out procedures and ultra-filtration (Purohit and Singh, 2009). The salt precipitation extraction procedures involve the utilization of alcohol (methanol or ethanol) or acetone as solvents (Thangam and Rajkumar 2002). Both methanol and ethanol have been found to be effective for enzyme and secondary metabolite purification. Different chromatographic techniques are used to separate and purify substances. Affinity chromatography uses hydroxyapatite as an adsorbent and hydrophobic interaction chromatography operates on different principles to separate and purify substances. Ion exchange chromatography uses cationic exchangers (Gupta *et al.,* 2005; Liu *et al.,* 2020). Additionally, gel filtration employs agarose-based and cross-linked gels that are effective in separating macromolecules based on their size. HPLC enhances the resolving ability of column

techniques, offering both fast separation and high resolution. The purification of enzymes and secondary metabolites can also be achieved using two-phase aqueous systems that involve combinations of dextran and PEG or salts like H_3PO_4, $MgSO_4$, and PEG (Asenjo and Andrews 2012; Yau *et al.,* 2015). Another method involves the utilization of Amberlite® XAD16N resin and processing with concentrated methanol for the extraction of bioactive molecules (Kumar *et al.,* 2017; Albataineh *et al.,* 2021). These purification procedures facilitate the separation and analysis of enzymes and secondary metabolites, thereby providing deep insight into their functions and applications across various scientific fields.

After a comprehensive review of the potential applications as well as the state-of-the-art methods for studying Myxobacteria, there was enough potential to chart out a study hypothesis as well as a problem statement.

Hypothesis of the study

Owing to the potential of myxobacteria, it was hypothesized to isolate them from diverse habitats of the Kashmir Valley for the production of industrially important enzymes and bioactive molecules. The primary hypothesis was to screen for the myxobacteria strains from diverse habitats of Kashmir Valley that have the ability to produce industrially important enzymes and bioactive molecules and purify them. Further, it was hypothesized to clone and express the gene encoding for the enzymes and secondary metabolites. After cloning, sequencing of these genes will provide evidence of the genetic basis underlying their production. Through these hypotheses, the study aims to contribute to the exploration and exploitation of myxobacteria from Indian habitats for industrial and biotechnological applications.

CHAPTER 3

AIMS AND OBJECTIVES

Considering the potential of Myxobacteria to synthesize industrial enzymes and diverse bioactive molecules with unique mechanism. The broad aim of the study was to isolate Myxobacteria from Kashmir Valley and bioprospect it for enzymes and secondary metabolites. The objectives were designed as follows:

1. Isolation and Screening of Myxobacteria from diverse habitats of Kashmir Valley for various industrial enzymes and bioactive molecules.
2. Purification of enzyme and bioactive metabolites from the respective isolates.
3. Cloning and Expression of gene(s) coding for enzyme and for synthesis of bioactive metabolites.
4. Sequencing and analysis of the gene encoding enzyme and bioactive metabolites.

CHAPTER 4

MATERIAL AND METHODS

Chemicals

Molecular reagents: dNTP mix, Lambda DNA (uncut), Taq DNA Polymerase, Proteinase K, RNase were procured from Merck Specialties Pvt. Ltd, Bangalore.

Salts and media:

Acetic acid, Acrylamide, Agarose, Ammonium persulphate, Boric Acid, Bovine Serum Albumin, Calcium Chloride Dihydrate, Carboxymethyl cellulose (CMC), Casein, Casitone, Chloroform, Cobalt Dichloride, Coomassie brilliant blue G-250, Coomassie brilliant blue R 250, Copper Sulfate, Dimethyl sulfoxide, Dipotassium Phosphate, Dithiothreitol, EDTA, Ethidium bromide, Ferric Chloride/Iron Trichloride, Glycerol, Isopropyl-1-thio-β-D-galactopyranoside, Luria Bertani, Lysozyme, Magnesium Chloride, Magnesium Sulfate, Manganese Chloride Tetrahydrate, Manganese Sulfate Heptahydrate, Methanol, N,N'-methylene-bis-acrylamide, Phenol: Chloroform: Isoamyl alcohol (25:24:1), Phosphoric Acid, PMSF, Polyethylene Glycol, Potassium dihydrogen phosphate, Potassium Iodide, Potassium Nitrate, SDS, Skim Milk Powder, Sodium chloride, Sodium Hydroxide, Sodium Molybdate Dihydrate, Starch, TEMED, Trichloroacetic acid, Tris base and Zinc Chloride were procured from HiMedia Laboratories Pvt. Limited, Bombay and Central Drug House (P) Ltd., Delhi.

4.1. Collection of soil samples

The soil samples were collected from two different wetlands of Kashmir Valley at high altitude (1584 m) namely Hokersar (34°06' N latitude, 74°05' E longitude) and Mirgund (33°58' N latitude, 74°45' E longitude) Wetland. For physio-chemical analysis, the soil samples were sealed in zip-lock bags and stored at 2-4°C till the completion of analysis. For microbiological analysis, the soil samples were air-dried and stored in sterile zip lock bags at ambient temperature not exceeding 25°C.

4.2. Analysis of physico-chemical properties of different soil samples

4.2.1. Moisture content

The soil moisture content was determined by drying one gram of soil for 30 mins at 103°C and the moisture content (%) was determined using the following formula (Rayment and Higginson, 1992):

$$Moisture\ Content = \frac{(Initial\ Weight - Dry\ Weight)}{Initial\ Weight} \times 100$$

4.2.2. pH

The soil pH was determined via dilution of the sample in water (i.e., dissolving of 1gm of soil sample in 9 mL of distilled water) and its measurement with pH meter (Hanna Instruments, Italy) and pH strip (Weligama *et al.*, 2022).

4.3. Isolation and determination of characteristic features of myxobacteria

Different methods have been reported for the isolation of myxobacteria from soil, among which dung baiting method has been extensively used in the laboratory (Gaspari *et al.*, 2005). Different methods of isolation of myxobacteria have been appended in Table 4.1.

Table 4.1: Overview of the methods used for the isolation of myxobacteria from soil (Gaspari *et al.*, 2005)**.**

Method	**Media Used**	**Soil Treatment**	**Incubation Conditions**	**Observation**
E. coli Baiting	WCX agar	Soil Moistened with cycloheximide	32°C for 1-4 weeks	Fruiting Bodies Formation and Swarming Pattern
Dung Pellets Baiting Method	CY Media	Soil Moistened with cycloheximide	25-32°C for 1-3 weeks	Fruiting Bodies Formation on Dung Pellets
Filter Paper Method	ST21CX Agaar	Clump of Soil treated with cycloheximide	32°C for 1-4 weeks	Swarming Pattern and Fruiting Bodies Formation on Filter Paper
Moist Chamber Method	bark saturated with cycloheximide, Whatman filter paper, Sterilized petri plate	Clump of Soil treated with cycloheximide	32°C for 1-4 weeks	Fruiting Bodies Formation on the Bark

4.3.1. Dung pellets baiting method

The dung pellets baiting method involves the preparation of Casitone media (Composition: tryptone [0.5% (w/v)], peptone [0.1% (w/v)], and 2% (w/v) agar), supplemented with cycloheximide (25 µg/mL). The prepared casitone media was sterilized by autoclaving (121^{o}C/15 psi, held for 20 minutes), cooled to under 50^{o}C. Cycloheximide was then added to attain the final concentration of 25µg/mL, and poured into petri plates. Once solidified, 2 grams of soil was laid as a mount on the medium and dried-and-autoclaved pellets of goat-dung were embedded in such a way that half pellet remain exposed. The prepared plates were then kept in incubator at 28°C for the period of 2 weeks and the goat dung pellet were examined for presence of white patches (Gaspari *et al.*, 2005).

4.3.2. Fruiting bodies formation and swarming pattern

For fruiting bodies, the white patches that migrated on the goat-dung pellets were visualized under stereomicroscope (Radical, India) (40X magnification). Additionally, these white patches were inoculated in test tubes with 10 mL of Casitone broth supplemented with 10 µL of cycloheximide (25 mg/mL) with incubation at 28°C for 1 week at 180 rpm. Post-incubation, the tubes were examined for a whitish-ring on the wall of test tubes. The single-line of this culture was streaked onto a casitone agar plate and incubated for a period of 1 week at 28°C. Following the incubation period, the plates were assessed for swarming pattern as well as fruiting bodies under the stereomicroscope at 4X magnification (Thakur *et al.*, 2017; Saadatpour *et al.*, 2024).

4.3.3. Gram staining

For preliminary identification of myxobacteria, gram staining was performed as per a previously reported protocol (Coico, 2006). Briefly, a few drops of sterile distilled water and the colony was mixed properly to make a smear on a microscopic glass slide. The smear was then heat-fixed on a Bunsen burner 3-4 times. Next, the smear was stained with crystal violet for 1 min, followed by gentle washing with sterile distilled water. After crystal violet, the slides were stained with gram's iodine for 1 min, followed by washing with a drop of 95% ethyl alcohol. The slides were again washed with sterile distilled water and counter-stained with safranin for 30 secs. The slides were then washed gently with distilled water, dried and visualized under the microscope (Olympus (India) Pvt. Ltd., India) at 40X and 100X magnification with immersion oil (Coico, 2006).

4.4. Isolation of genomic DNA from myxobacterial isolates

The cultures were grown in Casitone broth at 30°C for 36-48 hours. Subsequently, 10 mL of the overnight-grown cultures was centrifuged in a 2 mL microcentrifuge tube, by repeated centrifugation (REMI, India) and centrifugation at 6000 rpm for 10 mins. The pelleted culture was then re-suspended in 1000 µL of TE buffer through repeated pipetting. Following resuspension, 50 µL of 10% SDS, 10 µL of proteinase K (20 mg/mL), and 10 µL of RNase A were added, mixed thoroughly, and incubated horizontally in a shaking incubator at 37°C for 15 mins. Followed by 65°C in a water bath for an additional 15 mins. Next, the tubes were centrifuged at 10,000 rpm for 10 mins, and the supernatant was transferred into a fresh tube. To the supernatant, equal volume of Phenol: Chloroform: Isoamyl alcohol (25:24:1) solution was added, and mixed by repeated inversions. Subsequently, the tubes were centrifuged at 10,000 rpm for 10 mins at 4°C. The aqueous phase was carefully transferred to a new tube, 1/10th volume of 7.5M ammonium acetate was added and mixed. After mixing, 0.7 mL of chilled isopropanol was added. The DNA was precipitated by gently inverting the tubes until the spools were visible. The tubes were then subjected to centrifugation at 12000 rpm for 20 mins at 4°C. Following centrifugation, the supernatant was discarded. The resulting DNA-containing pellets were washed with 70% ethanol. After centrifuging at 12000 rpm for 10 mins, the supernatant was again discarded, and the DNA pellets were air-dried. Finally, the DNA pellets were dissolved in 50 µL of TE buffer (Green and Sambrook, 2017).

4.5. Analysis of DNA by agarose gel electrophoresis

Agarose gel electrophoresis is a highly efficient approach for separating nucleic acid molecules based on their charge, size, and conformation under the influence of an external electric field. This technique, known for its sensitivity, speed, and high resolution, allows the precise resolution of closely-sized DNA fragments with excellent reproducibility. Additionally, it facilitates the estimation of DNA fragment lengths by running a molecular weight marker alongside the DNA fragments of interest. The fundamental principle governing the separation of DNA on an agarose gel is electrophoretic mobility, influenced not only by the molecular weight of DNA but also by its conformation (single or double-stranded), agarose gel concentration, and DNA base composition.

To conduct agarose gel electrophoresis, essential equipment and consumables include a gel casting tray, gel running tank, electrophoretic power supply, electrophoresis buffer (1X TAE buffer), sample loading buffer, and a UV illuminator for analysis. The detection of DNA is

achieved by incorporating ethidium bromide (an intercalator) into the gel. Ethidium bromide (*aka* fluorescent dye) binds to DNA, producing orange-colored fluorescence when exposed to UV light. Thus, when observed under a UV transilluminator (GeneI, India), DNA appears as distinct orange bands. Notably, agarose gel electrophoresis offers a semi-quantitative analysis of DNA content in a given sample.

The procedure for analysis of DNA involved the preparation of 0.5% agarose gel containing 2µL of ethidium bromide dye [10mg/mL]. Approximately, 5µL of the isolated genomic DNA samples were loaded on the gel and gel was subjected to electrophoresis at 60 volts. Uncut Lambda DNA (48,502 bp) was also run as a reference high molecular weight DNA. The gel was observed under a UV transilluminator.

4.6. Spectrophotometric analysis of genomic DNA

The purity of genomic DNA samples extracted by modified Green and Sambrook (2017) was assessed by spectrophotometric analysis based on the ratio of absorbance at 260 and 280 nm (for protein contamination) (Wilfinger *et al.,* 1997; Alemán-Duarte *et al.,* 2023). The absorbance of DNA was assessed to determine the concentration of DNA within the sample. For checking the absorbance, the dilution of 1:200 i.e., 15µL of Genomic DNA in the 2985 µL of 1X TE Buffer was prepared. The absorbance of the diluted DNA sample was recorded at 260 nm and 280 nm. The ratio of absorbance of 260/280 was calculated and concentration of the pure dsDNA was determined (Barbas *et al.,* 2007) by the following formula. One absorbance unit considered as equivalent to 50µg/mL of pure double stranded DNA.

$$Concentration\ (\mu g/mL) = Absorbance\ at\ 260\ nm\ \times 50\ \mu g/mL \times dilution\ factor$$

4.7. PCR amplification of genomic DNA with 16S primers

The amplification of the isolated genomic DNA was carried out with universal 16S primers 27F (5'- AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'- GGTTACCTTGTTACGAC TT-3') (Kumar *et al.,* 2017) following the PCR reaction composition outlined in Table 4.2. Genomic DNA of samples (Hok1, Hok2, Mir1 and Mir2) were utilized as the templates for the PCR amplification.

Table 4.2: PCR reaction mixture for the amplification of 16s rRNA region from isolated genomic DNA

Components	**Hok1**	**Hok2**	**Mir1**	**Mir2**
	Volume (µL)	**Volume (µL)**	**Volume (µL)**	**Volume (µL)**
Water (Molecular Grade)	7.0	7.0	7.0	7.0
Taq Polymerase Buffer (10X Buffer)	2.5	2.5	2.5	2.5
10µM Forward Primer (27F)	1.0	1.0	1.0	1.0
10µM Reverse Primer (1492R)	1.0	1.0	1.0	1.0
Template DNA (diluted)	5.0	5.0	5.0	5.0
dNTP mix (2 mM)	2.5	2.5	2.5	2.5
Taq Polymerase (5 U/10 µL)	1.0	1.0	1.0	1.0
Total	**20**	**20**	**20**	**20**

After the preparation of reaction mixture, the thermal cycler conditions were set as follows:

95°C – 2 mins

95°C – 30 secs

50°C – 50 secs

72°C – 1 min 45 secs

30 cycles

72°C – 8 mins

Hold at 4°C.

The amplified products were visualized on 1.5% (w/v) agarose gel along with 1kb DNA ladder.

4.8. Sequencing of 16S ribosomal RNA gene of the isolates

For molecular identification of the isolates, the PCR amplicons were sent for sequencing to Biologia Research India Pvt. Ltd, Karnal. The obtained sequencing results were further

subjected to analysis via the BLASTn tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to assess sequence similarity. The phylogenetic tree was constructed using MEGA X software.

Molecular Evolutionary Genetics Analysis X (MEGA X) is an extensively used software for the construction of phylogenetic trees and the analysis of molecular evolutionary relationships among biological sequences, particularly nucleotide and amino acid sequences (Kumar *et al.*, 2018). During pre-processing stages, contig sequence obtained after sequencing are compared to a known database using a comparison tool like BLAST. The sequencing showing similarity shows the highest identity score. The 15 sequences that are showing the highest identity score are retrieved and subjected to MEGA X software for construction of phylogenetic tree as illustrated in Figure 4.1.

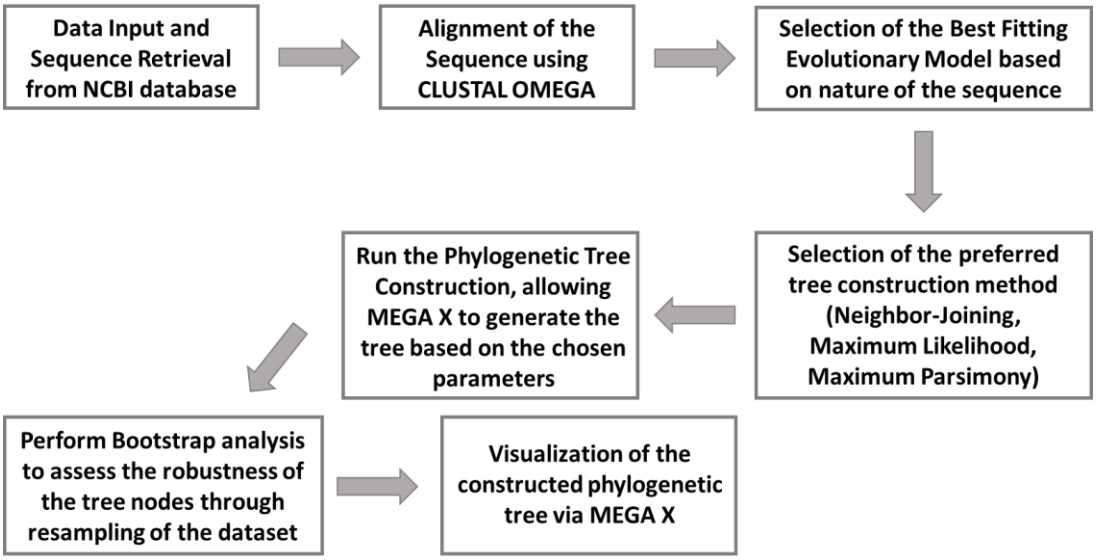


Figure 4.1: Overview of steps involved in the phylogenetic tree construction using MEGA X

4.9. Screening of the isolates for the production of hydrolases and bioactive molecules

4.9.1. Primary screening of the isolates for enzyme production

The three isolates of myxobacteria i.e., Hok1, Mir1 and Mir2 were cultured in casitone broth and plated on the substrate (-agar) plates for the screening of amylase, cellulase and protease activity by employing standard plate assays. The plates were incubated for 24-48 hours at 37°C.

(a) Amylase activity: The isolates were tested on the starch agar plates containing 1% (w/v) starch and were incubated for 48-72 hours at 30°C. The isolates having the potential to synthesize amylase show a zone of hydrolysis on the plates revealed by staining with gram's iodine solution (Kumar *et al.*, 2016).

(b) Cellulase Activity: The isolates were tested for cellulolytic activity on the CMC-agar plates containing 1% (w/v) CMC and were incubated for 48-72 hours at 30°C. The isolates having the potential to synthesize cellulase show a zone of hydrolysis on the plate revealed by staining with 0.1% congo red and destaining with 1M NaCl solution (Bai *et al.,* 2012).

(c) Protease Activity: The isolates were tested on the skimmed milk agar plates containing 1.5% (w/v) skimmed milk and were incubated for 48-72 hours at 30°C. The isolates having the potential to synthesize protease show a zone of hydrolysis on the skimmed milk agar Plate (Tennalli *et al.,* 2022).

4.9.2. Growth of culture, preparation and storage of crude extract

The isolates showing the positive results in primary screening were streaked on casitone agar plates supplemented with cycloheximide (25 mg/mL) and incubated at 30°C for 5-6 days (Gaspari *et al.,* 2005). A loopful of fresh culture from the plates was inoculated in a 25mL conical flask containing 10 mL casitone broth supplemented with cycloheximide (25 mg/mL) and incubated for 5-6 days with 180 rpm at 30°C to prepare a seed culture. Next, 1% of the seed culture was used to inoculate a 500 mL conical flask containing 200mL Casitone Broth supplemented with 0.1% CMC (w/v) for cellulase production and 0.1% (w/v) skimmed milk for protease production, and incubated for 5-6 days at 30°C. After incubation, cells were separated by centrifugation at 10000 rpm for 10 mins. After that, supernatant was collected in sterile 50 mL centrifuge tubes and stored at 4°C for further use. The pellets were subjected to lysis in the lysis buffer (50mM Tris HCl (pH 7.5), 5mM EDTA (pH 8.0) and 0.5% Triton-X 100). After the addition of lysis buffer, an equal amount of 0.5 mm glass beads was added. Then, mixtures were repeatedly vortexed for 10 secs and kept on ice for 20 secs, for a total of 30 mins. Next, the mixtures were centrifuged at 10000 rpm for 10 mins at 4°C. After centrifugation, the supernatant was collected in 15 mL centrifuge tubes, and stored as cell free extract (CFE) for further analysis (Kaur *et al.,* 2020).

4.9.3. Secondary screening for detecting the localization of enzymes

After primary screening of the enzyme, secondary screening was carried out for the two enzymes i.e., protease and cellulase. As both enzymes showed the positive results for isolate Hok1, Mir1 and Mir2. The supernatant and CFE were evaluated for enzyme localization via

standard agar diffusion method and were visualized for zone of clearance (Sitoke *et al.,* 2017; Kaur *et al.,* 2020).

4.9.3.1. Assay for testing cellulase activity

1% (w/v) CMC agar plates were prepared by dissolving 1g of CMC in 100 mL hot distilled water containing 2% agar. This mixture was thoroughly mixed and poured on sterile plastic petri plates and allowed to solidify. On solidifying, 10 mm holes were bored on the solidified media. Then, 100 µL of CFE and 200 µL supernatant of each sample was loaded to the respective wells in the 1% (w/v) CMC plates. The plates were then incubated at 37°C overnight to 24 hours. After incubation, the plates were flooded with 0.1% congo red dye for 20 mins on a rocking shaker. After that, destaining was done with 1M NaCl solution until the zone of clearance appeared (Bai *et al.,* 2012).

4.9.3.2. Assay for testing protease activity

1.5% (w/v) skimmed milk agar plates were prepared by dissolving 2g of Agar in 95 mL of hot distilled water and boiled till it gets completely dissolved. After that 5 mL of 15% skimmed milk heated till dissolution was added. This mixture was thoroughly mixed and 20 mL of the media was poured in sterile plastic petri plates and allowed to solidify. On solidifying, 10 mm holes were bored on the solidified media. Then, 100 µL of CFE and 200 µL supernatant of each sample was loaded to the respective wells in the1.5% (w/v) skimmed milk plates. The plates were then incubated at 37°C overnight to 24-48 hours and was visualized for clear zone (Tennalli *et al.,* 2022).

4.10. Screening of the isolates (Hok1, Mir1 and Mir2) for antimicrobial activity

The cultures of Hok1, Mir1 and Mir2 were grown in 50 mL of casitone broth containing cycloheximide (25 mg/mL) at 30°C for 3-4 days. The grown cultures were centrifuged at 6000 RPM for 10 mins. The supernatant was transferred to new tubes. The pellets obtained were subjected to 2 mL of lysis buffer for the preparation of CFE as mentioned in section 4.9.2. Next, the antimicrobial activity of isolates was assessed on the test microbial strains i.e., Gram-positive *Bacillus cereus* MTCC 8714, Gram-negative *Escherichia coli* MTCC 1679, *and* Gram-negative *Pseudomonas aeruginosa* MTCC 2582. with the supernatant and CFE from different myxobacterial isolates via agar well diffusion method. Moreover, the plate was visualized for zone of inhibition to check the antimicrobial activity (Thakur *et al.,* 2017).

4.11. Extraction of antimicrobial compounds by organic extraction protocol from Mir2

The culture was prepared by inoculating the culture of Mir2 in 100 mL casitone broth supplemented with cycloheximide (25 mg/mL) and 0.01% (w/v) L-arabinose followed by the incubation at 180 rpm for 5-6 days at 30°C. The extraction of metabolites was done using modified protocol of Rajan and Kannabiran (2014). For which, 10 mL of methanol was added to the grown culture and homogenized in a homogenizer for 30 mins. The mixture was extracted twice by adding equal volume of ethyl acetate. The culture extract was concentrated to dry powder using rotavapor. The residue was dissolved in dimethyl sulfoxide (DMSO) (Rajan and Kannabiran, 2014)

4.12. Partial purification of antimicrobial compounds from Mir2

The obtained ethyl acetate extract dissolved in DMSO containing the antimicrobial agent was run on the TLC plates. Chloroform: Methanol (9:1) was used as the solvent system. Azithromycin and vancomycin drug (2mg/mL) were used as reference antibiotics. For the assessment, the compound from isolate (acting as control) and reference drug were spotted using the fused capillaries on the marked line on TLC plates. The spot of the samples was visualized under the UV chamber (at short wavelength of 254 nm) and after the exposure iodine chamber.

4.13. Identification of gene(s) coding for hydrolases enzymes

4.13.1. Amplification of cellulase gene from the isolate

The isolated genomic DNAs of isolate Hok1, Mir1 and Mir2 served as the templates for the amplification of cellulase genes. For gene amplification, the set of degenerate cellulase primers were used (Table 4.3). The degenerate primers were designed following the procedure stated by Pandey *et al.*, (2014). Fifteen sequences of the bacterial cellulases were retrieved from NCBI database i.e., GenBank. The retrieved nucleotide sequences of these genes were aligned with the help of T-Coffee alignment tool (http://www.ebi.ac.uk/Tools/msa/tcoffee/). The alignment of the sequence was observed for variation in the first 10-15 nucleotide base pair for manually preparing the forward degenerate primer. Whereas, last 10-15 nucleotide base pair was observed and reversed complementary sequence was used for manually preparing the reverse degenerate primer (Pandey *et al.*, 2014).

Table 4.3: Summary of sets of degenerate primers for the amplification of cellulase gene from the isolates

SET NO	Sequence Type	Sequence
SET I	FW	5'- TACATGCGCACCCGCAGCGCCGCGACCTGA -3'
	RV	5'- GGWCARGCCRTRMCCGAACGSRWASAKCGGSKT -3'
SET II	FW	5'- ACTTTTTGCTGAAATGGG -3'
	RV	5'- GCRAANCCYAGHTARACGGT -3'
SET III	FW	5'- ACCMGITAYTGGGAYTGYTG -3'
	RV	5'- AAGRYICCNAVICCNCCICCNGG -3'

FW: Forward, RV: Reverse

Further, 20 µL of reaction mixture was prepared containing 10 µL of 1X of the 2X Master Mix (Takara Bio, USA), 0.6 µL of DMSO, 1 µL of each 0.5 µM of 10 µM forward and reverse primer, 2 µL of DNA template and 5.4 µL of Milli Q Water. After the preparation of reaction mixtures, the thermal cycler conditions were set as follow:

95°C – 1 min

95°C – 10 secs

64°C, 66°C, 68°C for set I

44°C, 51°C, 58°C for set II — 35 secs

53°C, 60°C, 66°C for set III

72°C – 1 min

35 cycles

72°C – 5 mins

Hold at 4°C.

The amplified products were visualized on 1.5 % (w/v) agarose gel along with 1 kb DNA ladder.

4.13.2. Amplification of protease gene from the isolate

The isolated genomic DNAs of isolate Hok1, Mir1 and Mir2 served as template for the amplification of protease genes. For the amplification, the set of degenerate protease primers were designed (Table 4.4). The degenerate protease primers were designed as per the protocol stated in section 4.13.1.

Table 4.4: List of primers for the amplification of protease genes from myxobacterial isolates

SET NO	Sequence Type	Sequence
SET IV	FW	5'- ATGCAGACAATGACTCACACCCGCC -3'
	RV	5'- GGGCTTGCCSAGGYTCGTCCAGG -3'
SET V	FW	5'- ATGAARCGADKCCARYTCGCRGGTYT -3'
	RV	5'- CTACTGGABKYTCCASACCRC -3'
SET VI	FW	5'- ATGTCGGAGTCGGTCGAGCTGAGG -3'
	RV	5'- CTACCGCYGCCATTCCGCGGGC -3'

FW: Forward, RV: Reverse

The PCR reaction mixtures for amplification of protease genes were as mentioned in section 4.13.1. After the preparation of reaction mixtures, the thermal cycler conditions were set as follow:

95°C – 1 min

95°C – 10 secs

59.1°C, 62°C, 65°C for set IV

50°C, 53°C, 56°C for set V — 35 secs — 35 cycles

60°C, 62°C, 65°C for set VI

72°C – 1 min

72°C – 5 mins

Hold at 4°C.

The amplified products were visualized on a 1.5% (w/v) agarose gel along with 1 kb DNA ladder. After visualization, the amplicons from set V at 53°C and 56°C and from set VI at 62°C and 65°C were selected for scale up through multiple reactions for further use. After scale-up, the amplified products were visualized on 1.5% (w/v) agarose gel along with 1 kb DNA ladder. On visualization, the amplicon from primer set V at 56°C was selected and sent to Biologia Research India Pvt. Ltd, Karnal for purification and Sequencing.

4.14. Analysis of the sequence of protease amplicon

The sequence homology analysis was conducted using the BLASTx program (https://blast.ncbi.nlm.nih.gov/Blast.cgi) from NCBI for similarity assessment with protein database using a translated nucleotide.

4.15. Identification of polyketide synthase (PKS) gene from the myxobacterial isolates

The genomic DNA of myxobacterial isolates Hok1, Mir1 and Mir2 were used as a template for amplification process. For the amplification, the set of degenerate Polyketide synthase (PKS) primers were used (Table 4.5). Wherein, primer sets VII and VII were taken from the published literature (Komaki *et al.,* 2008) and the primer set IX was designed as per the protocol stated in section 4.13.1.

Table 4.5: List of primers for the amplification of PKS genes from myxobacterial isolates

SET NO	Sequence Type	Sequence
SET VII	FW	5'- CCSCAGSAGCGCSTSTTSCTSGA- 3'
	RV	5'- GTSCCSGTSCCGTGSGTSTCSA- 3'
SET VIII	FW	5'- CCSCAGSAGCGCSTSCTSCTSGA- 3'
	RV	5'- GTSCCSGTSCCGTGSGCCTCSA- 3'
SET IX	FW	5'- TSGCSTGCTTCGAYGCSATC- 3'
	RV	5'- TCGCCBAAGCCGCCNAAGGT- 3'

FW: Forward, RV: Reverse

The PCR reaction mixtures for amplification of protease genes were prepared as mentioned in section 4.13.1. After preparation of reaction mixtures, the thermal cycler conditions were set as follows:

95°C – 1 min

95°C – 10 secs

57°C, 61.5°C, 65°C for set VII, VIII, IX – 35 secs

72°C – 1 min

35 cycles

72°C – 5 mins

Hold at 4°C.

The amplified products of each sample for each set of primers were visualized on a 1.5% (w/v) agarose gel along with 1 kb DNA ladder. After visualization, the amplicon from primer set IX at 61.5°C was selected for scale up through multiple reactions for further use. The amplicon product obtained after the scale up was sent to Biologia Research India Pvt. Ltd, Karnal for purification and sequencing (Sanger sequencing, and Applied Biosystems™ 3500 genetic analyzer).

4.16. Analysis of the sequence for PKS amplicon

The sequence homology analysis was carried out using the BLASTx program (https://blast.ncbi.nlm.nih.gov/Blast.cgi) of NCBI for similarity assessment with protein database using a translated nucleotide.

4.17. Cloning of protease gene and PKS gene from Mir2

4.17.1. Cloning of protease gene

The cloning of protease gene was done by the Gateway cloning approach (Reece-Hoyes and Walhout 2018), as per the kit protocol (https://assets.thermofisher.com/TFS-Assets/LSG/manuals/pentr_dtopo_man.pdf).

4.17.1.1. Vector

pENTR-D-TOPO vector (entry vector) was procured from Invitrogen, Life Technologies, USA and directly used for cloning

4.17.1.2. Preparation of insert

For gateway cloning, the 4 base pair sequence (CACC) was added to 5′ end of the forward primer set V (Table 4.6) for directional cloning. The gene of interest "Pro" was amplified with new set of primer for the preparation of insert

Table 4.6: Primer set for the amplification of protease gene (Pro) for preparation of insert

SET NO	Sequence Type	Sequence
Modified SET V	FW	5'- **CACC**ATGAARCGADKCCARYTCGCRGGTYT -3'
	RV	5'- CTACTGGABKYTCCASACCRC -3'

FW: Forward, RV: Reverse

4.17.1.3. Ligation and transformation

The ligation rection for Pro (protease gene) in pENTR-D-Topo vector was set as mentioned in Table 4.7. The mixture was gently mixed and incubated at room temperature for 5 mins and then kept on ice. The transformation was done in *E. coli* DH5α competent cell and kept on ice for 30 mins. After the incubation on ice, the tube containing the cells and plasmid was given a heat shock at 42°C for 90 secs. Then, 1 mL of LB broth was added to the tube and incubated in shaking incubator at 37°C for 1 hour. After incubation, 50 µL of bacterial culture was spread on the LB agar plate containing kanamycin (100 µg/ml).

Table 4.7: Ligation of gene of interest (Pro) in pENTR-D-Topo gateway entry vector

Component	**Volume for reaction**
Salt solution	1 µL
Entry Vector (pENTR-D-Topo)	1 µL
PCR product	3 µL
Water (Molecular Grade)	1 µL
Total volume	6 µL

4.17.2. Colony PCR for screening of transformants

On observing the growth of colonies on the plate, random single-cell colonies were picked and resuspended in molecular grade water for selection of clones through colony PCR method. To perform the colony PCR, gene specific primers (Table 4.6) were used and the reaction was prepared as mentioned in the Table 4.8. After the preparation of reaction mixtures, the thermal cycler conditions were set as mentioned in Table 4.9. Colony PCR products for cloning of Pro (protease gene) in pENTR-D-Topo were analyzed on 1.5% (w/v) agarose gel and amplicons were visualized under UV transilluminator.

Table 4.8: PCR reaction composition for the colony PCR screening of protease gene (Pro)

Component	**Final Concentration**	**Reaction Volume**
2X Master Mix	1X	10 µL
10 µM Forward Primer	0.5 µM	1 µL
10 µM Reverse Primer	0.5 µM	1 µL
Template DNA	-	2 µL
Milli Q Water	-	6 µL
Total Volume	-	20 µL

Table 4.9: Thermal cycler conditions for the amplification of protease gene (Pro) for the colony PCR screening

Temperature (°C)	Time (min: sec)	Cycles
94	01:00	x1
98	00:10	x35
56 (modified SET V)	00:35	
72	01:00	
72	5:00	x1
4	∞	

4.17.3. Plasmid isolation

Clones selected from colony PCR screening were inoculated for overnight culture in LB broth supplemented with Kanamycin (100 µg/ml) and plasmid was isolated following the protocol provided with FavorPrep^{TM} Plasmid Extraction Mini Kit (https://www.favorgen.com/data/manual/files/1703561598024940124.pdf). Initially, 2-3 mL of grown culture of bacteria were transferred to a micro-centrifuge tube and centrifuged at 12000 rpm for 1 min for harvesting the cells. To the pellet 200 µL of FAPD1 Buffer, encompassing RNase A was added and resuspended by pipetting. After that, 200 µL of FAD2 Buffer was added and tube was gently inverted 8-10 times. This mixture was then incubated at room temperature for 2-5 mins to lyse the cells. After incubation, the lysate was neutralized by addition of 300 µL of FAPD3 Buffer and mixed by inverting 8-10 times. After mixing, the lysate was subjected to centrifugation at 12000 rpm for 5 mins and supernatant was carefully transferred to FAPD column. The column was then centrifuged at 11000 rpm for 30 secs, the resultant flow-through was then discarded and the FAPD column was repositioned for subsequent washing step. The washing steps involved the sequential addition and removal of 400 µl of WP Buffer and 700 µl of Wash Buffer, both followed by centrifugation at 11000 rpm for 30 secs for effective removal of impurities. The FAPD column was then subjected to full-speed centrifugation for 3 mins to dry. The dried column was transferred to a new microcentrifuge tube. Then, plasmid DNA elution was achieved by addition of 50-100 µl of Elution Buffer or ddH_2O to the membrane center, standing for 1 min. The elution process was finalized by centrifuging the column at 18,000 rpm for 1 min. The eluted plasmid DNA was then sent for Sanger Sequencing to Biologia Research India Pvt. Ltd, Karnal.

4.17.4. LR transformation

The expression of clone, was done by using pEZY19 gateway expression vector. LR reaction was set between the pENTR-D-Topo clone and pEZY19 empty vector in presence of LR enzyme mix. Reaction was set as mentioned in Table 4.10. The mixture was gently mixed and incubated at room temperature for 5 mins and then kept on ice. Ligated mixture was transformed in *E. coli* BL21 (DE3) cells following the same procedure of transformation as mentioned in section 4.17.1.3. Further, positive colonies were selected on LB agar plate supplemented with ampicillin (100 µg/ml).

Table 4.10: LR transformation mix for ligation of gene of interest (Pro) in pEZY19 destination vector

Component	**Reaction Volume (µL)**
pENTR-D-Topo clone	1 (100 ng)
Destination vector (pEZY19)	1 (100 ng)
LR enzyme mix	1
Water (Molecular Grade)	1
Total Volume	4

4.17.5. Colony PCR

Colony PCR-based screening of random clone was performed using gene-specific primers (Table 4.6). Reaction mix was prepared as mentioned in Table 4.8. The thermal cycling conditions were used as mentioned in Table 4.9. On amplification, the PCR amplified Pro gene in the pEZY19 expression vector was confirmed by running the PCR product on a 1.5% (w/v) agarose gel followed by visualization on UV transilluminator.

4.17.6. Cloning of PKS gene

The cloning of PKS gene was done by the Gateway cloning approach in accordance to the procedure mentioned in section 4.17.1.

4.17.6.1. Preparation of insert

For gateway cloning, the 4 base pair sequence (CACC) was added to 5′ end of the forward primer set IX (Table 4.11) for directional cloning. The gene of interest “PKS” was amplified with the new set of primer for the preparation of insert.

Table 4.11: Primer set for the amplification of PKS gene for preparation of insert

SET NO	Sequence Type	Sequence
Modified SET IX	FW	5'- **CACC**TSGCSTGCTTCGAYGCSATC- 3'
	RV	5'- TCGCCBAAGCCGCCNAAGGT- 3'

FW: Forward, RV: Reverse

4.17.6.2. Ligation and transformation

The ligation reaction for PKS gene in pENTR-D-Topo vector was performed as per the protocol mentioned in section 4.17.1.3.

4.17.7. Colony PCR for screening of transformants

On observing the growth of colonies on the plate, single-cell colonies were picked randomly from the plate. Then randomly picked colony was resuspended in molecular grade water for selection of positive colonies through colony PCR method. To perform the colony PCR, gene-specific primers were used (Table 4.11) and the reaction was prepared as mentioned in the Table 4.8. After the preparation of reaction mixtures, the following thermal cycler conditions were set as mentioned in the Table 4.12. Colony PCR product for cloning of PKS gene in the pENTR-D-Topo were analyzed on 1.5% (w/v) agarose gel and amplicons were visualized under UV transilluminator.

Table 4.12: Thermal cycler conditions for the amplification of PKS gene for the colony PCR screening

Temperature (°C)	Time (min: sec)	Cycles
94	01:00	x1
98	00:10	x35
61.5 (modified set IX)	00:35	
72	01:00	
72	5:00	x1
4	∞	

4.17.8. Plasmid isolation

Colonies positive in colony PCR screening, were inoculated for overnight culture in LB broth supplemented with Kanamycin (100 µg/ml) and plasmid was isolated following the protocol

as mentioned in section 4.17.3. The obtained plasmid was then sent for Sanger Sequencing to Biologia Research India Pvt. Ltd, Karnal.

4.17.9. LR transformation

The LR Transformation was done in accordance to the procedure mentioned in section 4.17.4.

4.17.10. Colony PCR

Colony PCR-based screening of random clones was performed using gene-specific primers (Table 4.11). Reaction mix was prepared as mentioned in Table 4.8. The thermal cycling conditions were used as mentioned in Table 4.12. On amplification, the PCR amplified PKS gene in the pEZY19 expression vector was confirmed by running the PCR product on a 1.5% (w/v) agarose gel followed by visualization on UV transilluminator.

4.18. Primary screening of the clone (Mir2*Pro*) for protease enzyme

The primary screening of the clone (Mir2*Pro*) for protease enzyme was done as mentioned in section 4.9.1.

4.19. Secondary screening of the clone (Mir2*Pro*) for protease enzyme

The crude extract from the clone (Mir2*Pro*) was obtained by culturing the clone on Luria agar plates supplemented with ampicillin and grown at 37°C for 18-24 hours. A loopful of the freshly-grown culture was used for inoculating 10 mL of LB broth in a 25mL conical flask and incubated at 37°C for overnight at 180 rpm, for preparing the seed culture. Next, 1% of the seed culture was used to inoculate a 500 mL conical flask containing Luria Broth and incubated at 37°C till OD reached 0.5. Next, IPTG (Isopropyl-1-thio-β-D-galactopyranoside) was added to a final concentration of 1mM for inducing the protease expression and culture was incubated at 18°C for 20 hours. After incubation, the cells were harvested by centrifugation at 10,000 rpm for 10 mins. After centrifugation, supernatant was collected and stored at 4°C for further use. The pellet was further subjected to cell lysis according to procedure mentioned in section 4.9.2. to prepare the CFE.

The secondary screening of the Mir2*Pro* was further assessed with the supernatant and CFE according to the section 4.9.3.

4.20. Protein expression and purification of protease enzyme

The *E. coli* BL21 (DE3) expression strain carrying the pEZY19+Mir2*Pro* gene (Mir2*Pro*) plasmid is used for protein expression. For the primary culture, 3 mL of culture was prepared and incubated overnight at 37°C in LB broth. Subsequently, a secondary culture was initiated using a 1% inoculum from the primary culture and grown at 37°C. Later, the secondary culture was assessed to check if the culture has attained the optical density (OD) of 0.5. On attaining 0.5 OD, induction was performed by adding 1mM IPTG to a final concentration. The induction process was conducted at 18°C for 20 hours. After induction, bacterial culture was harvested through centrifugation at 6000 rpm at 4°C for 10 mins. The resulting cells were then resuspended in a protein isolation buffer containing 50 mM Tris-Cl (pH 7.5), 10 mM $MgCl_2$, 100 mM KCl, 5% glycerol, and 0.5% NP-40. The resuspended cells were supplemented with 100X Protease Inhibitor Cocktail (PIC) to achieve a final concentration of 1X. Additional supplements, including 100mM phenylmethylsulfonyl fluoride (PMSF) at a final concentration of 1mM and 1M dithiothreitol (DTT) at a final concentration of 1mM, were also added. The bacterial suspension was then vortexed for 5 mins.

For cell disruption, the suspension was subjected to sonication with 5 cycles (30 secs ON and 60 secs OFF) at 35% power. Following sonication, the suspension was centrifuged at 10,000 rpm for 50 mins at 4°C, and the soluble fraction was separated. The expressed protein was then purified using Ni-NTA beads. These beads (100 µL per protein sample) were washed and equilibrated with protein isolation buffer at 1200 rpm at 4°C. The equilibrated beads were added to the soluble fraction of proteins and incubated in an end-to-end rotor for 2 hours at 4°C. After incubation, the soluble fraction was removed from the beads as the flow-through, and the beads were washed with protein isolation buffer. A gradient of imidazole (10 and 20 mM) was used to eliminate non-specifically bound proteins, and the purified protein was eluted in 50-200 mM imidazole (Sambrook and Russell, 2006). The eluted protein underwent analysis and quantification using SDS-PAGE.

For SDS-PAGE, the resolving gel was prepared by mixing deionized water (DDW), 10% (w/v) SDS, Tris-HCl buffer (pH 8.8), and TEMED to prepare the 40 mL separating gel (12 % gel concentration). This mixture was allowed to deaerate for 10 mins. After deaeration, 10% (w/v) ammonium persulphate was added and this resulting solution was immediately poured to initiate gel polymerization. Whereas, for the stacking gel, a spacer gel with a gel concentration of 5% was prepared using a stock gel solution containing 1M Tris-HCl buffer (pH 6.8), DDW, 10% (w/v) SDS, 10% (w/v) ammonium persulphate, and TEMED. This spacer gel was layered over the polymerized separating gel, and a comb was immediately inserted between the plates

for polymerization to take place. Prior to loading, the protein samples were denatured by heating for 15 mins at 100°C in SDS containing-protein loading dye. Subsequently, the gel slab was placed into the electrophoresis apparatus, and the samples were loaded into their respective wells. Electrophoresis was conducted at 100 volts until the front indicator dye (Bromophenol blue) reached the bottom. The process was stopped, and the gel slab was removed from the electrophoresis apparatus. Then, the gel between the glass plates was taken out and subjected to staining with Coomassie Brilliant Blue R-250 for one hour. Later, destaining by methanol, water and acetic acid solution in the ratio of 45:45:10 (v/v%) was continuously repeated until the gel became transparent, revealing distinct blue bands (Sambrook and Russell, 2006). Moreover, the amount of sample loading was determined by Bradford Method.

4.21. Biochemical characterization of the protease (Mir2*Pro*)

The purified enzyme was characterized for optimum temperature, optimum pH, effect of metal ions and detergents. For these experiments, 2.5% of BSA was used as the substrate. The tyrosine release was measured at 660 nm using the standard protease assay. All experiments were conducted in triplicates.

4.21.1. Protease assay

The method used by Kunitz (1947) was modified and used for protease assay. The tyrosine gradient curve was plotted by determining the absorbance of free tyrosine that reacts with Folin's reagent in alkaline condition. Dilutions of pure tyrosine were prepared from 1.1mM tyrosine stock solution. To each dilution containing gradient tyrosine and phosphate buffer (pH 7.5), 0.5 mL of 10% TCA was added and mixed. After which, 1 mL NaOH and 2 mL of diluted folin's reagent (containing 1 part of FC reagent and 2 Part of DDW) was added. After that, mixture was incubated at room temperature for 10 mins. The absorbance was measure at 660 nm and graph was plotted between absorbance and concentration of tyrosine (Cupp-Enyard, 2008).

4.21.2. Standard curve of BSA

1 mg/mL BSA stock solution was prepared and different concentration of the protein were prepared. To the mixture, 1 mL of Bradford reagent was added and thoroughly mixed. The tubes were kept at room temperature for 5 mins. The absorbance was measured at 595 nm and graph was plotted between absorbance and concentration of BSA (Ernst and Zor, 2010).

4.21.3. Effect of temperature on protease enzyme activity

The effect of temperature on the enzyme activity was determined spectrophotometrically by incubating the purified enzyme synthesized by Mir2*Pro* with 2.5% BSA at different temperature ranging from 10-100°C for 30 minutes (Sitoke *et al.,* 2017).

4.21.4. Effect of pH on protease enzyme activity

The effect of pH on the enzyme activity was determined by estimating the tyrosine release by incubating the purified enzyme synthesized by Mir2*Pro* with 2.5% BSA in buffer of pH values ranging from 3-12 (Sitoke *et al.,* 2017).

4.21.5. Effect of metal ions on protease enzyme activity

The enzyme synthesized by Mir2*Pro* was incubated with 2.5% BSA in the presence of different divalent metal ions (Ca^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Mn^{2+}, Mg^{2+}, Zn^{2+}) at 2mM and 5mM. The residual activity was determined via tyrosine estimation assay (Sitoke *et al.,* 2017).

4.21.6. Effect of detergents on protease enzyme activity

The effect of detergents on the enzyme activity of purified enzyme synthesized by Mir2*Pro* with 2.5% BSA was determined under standard assay conditions in the presence of SDS (2% and 5%), Tween 20 (2% and 5%), and Triton X-100 (2% and 5%) (Sitoke *et al.,* 2017).

4.22. Analysis of antibacterial activity of clone containing the PKS gene

The clone (Mir2*PKS*) containing PKS gene was grown on LB agar plates containing ampicillin (100 µg/mL) for 1 days at 37°C. After that, the culture clone was grown in 50 mL of LB broth at 37°C for 24 hours. The culture of clone containing PKS gene was centrifuged at 6000 rpm for 10 mins. The supernatant obtained was transferred to new tubes. The pellets obtained was subjected to 2 mL of lysis buffer following the protocol mentioned in section 4.9.2.

Further, the antimicrobial activity of Mir2*PKS* was assessed on the test microbial strains i.e., Gram-positive *Bacillus cereus* MTCC 8714, Gram-negative *Escherichia coli* MTCC 1679, *and* Gram-negative *Pseudomonas aeruginosa* MTCC 2582. The supernatant and CFE obtained from Mir2*PKS* was assessed via agar well diffusion method and the culture plates were visualized for zone of clearance to check the antimicrobial activity (Thakur *et al.,* 2017).

4.23. Induction and molecular weight determination of the PKS

The *E. coli* BL21 (DE3) expression strain carrying the pEZY19+Mir2*PKS* gene (Mir2*PKS*) plasmid was used for studying the protein expression. For the primary culture, 3 mL of culture was prepared and incubated overnight at 37°C in LB media. Subsequently, a secondary culture was initiated using a 1% inoculum from the primary culture and grown at 37°C. Later, the secondary culture was assessed to check if the culture has attained the optical density (OD) of 0.5. On attaining 0.5 OD, induction was performed by adding 1mM IPTG to a final concentration. The induction process was conducted at 18°C for 20 hours. After induction, bacterial cultures were harvested through centrifugation at 6000 rpm at 4°C for 10 mins. The resulting cells were then resuspended in a protein isolation buffer containing 50 mM Tris-Cl (pH 7.5), 10 mM $MgCl_2$, 100 mM KCl, 5% glycerol, and 0.5% NP-40. The resuspended cells were supplemented with 100X PIC to achieve a final concentration of 1X. Additional supplements, including 100mM PMSF at a final concentration of 1mM and 1M DTT at a final concentration of 1mM were also added. The bacterial suspension was then vortexed for 5 mins. The expression of uninduced and induced protein was determined by SDS-PAGE as mentioned in section 4.20.

4.24. Extraction of antimicrobial compounds by organic extraction protocol from clone (Mir2*PKS*)

The clone (Mir2*PKS*) was culture in LB broth containing ampicillin (100 µg/mL) for 24 hours at 37°C. For the extraction of antimicrobial compounds from clone (Mir2*PKS*), the protocol mentioned in section 4.11 was used.

4.25. Partial purification of antimicrobial compounds from clone (Mir2*PKS*)

For the partial purification of antimicrobial compounds from the clone (Mir2*PKS*), the protocol mentioned in section 4.12. was used.

4.26. Computational analysis of cloned PKS gene sequence for the lack of antimicrobial activity

4.26.1. *Sequence retrieval*

Mir2*PKS* clone for recombinant protein *E. coli* didn't show antibacterial activity. To study the probable reasons, sequence analysis of the PKST1 (polyketide synthase type I) components from the *E. coli* were studied. The sequence of PKST1, acyl carrier protein, and acyltransferase for *Myxococcus* and *E. coli* were retrieved from the NCBI protein database. The sequences of the proteins are enlisted in Annexure 2.

4.26.2. Protein-protein interaction

To study whether the *Myxococcus* PKST1 is able to interact with the *E. coli* proteins of PKS complex, the STRING database (https://string-db.org/) was used. The amino acid sequences of the *Myxococcus* PKS protein and *E. coli* acyltransferase protein were submitted in the STRING database in multiple proteins section and the auto-detect was selected in the organism section. After sequence submission, a window appeared to select the organism, and *E. coli* was selected. As the recombinant protein expression was performed in the *E. coli* BL21 strain. STRING window showed whether the submitted proteins were interacting with each other in *E. coli*.

4.26.3. Alignment study

All the protein sequences for the components of PKS extracted from NCBI were used for alignment study with CLUSTAL Omega (https://www.ebi.ac.uk/jdispatcher/msa/clustalo). The amino acid sequences were pasted in FASTA format in CLUSTAL Omega and protein dataset was selected. The sequences were submitted, and the alignment files were downloaded.

CHAPTER 5

RESULTS AND DISCUSSION

5.1. Collection of soil sample

The soil samples were collected from different regions of Kashmir Valley at high altitudes (1584 m) namely Hokersar and Mirgund Wetland as shown in Figure 5.1. The soil samples were collected in sterile autoclave bags/zip lock bags using autoclaved digging tools to avoid any experimental contamination.

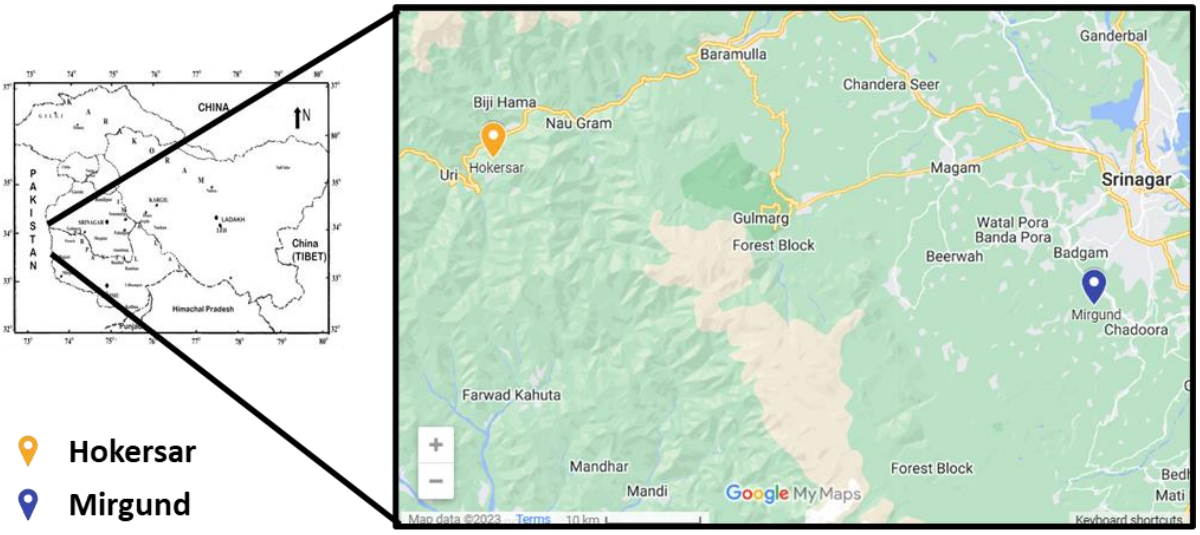


Figure 5.1: Geo-location map for sample sites used in the study

5.2. Analysis of physico-chemical properties of different soil samples

The moisture content of the Hokersar and Mirgund soil sample was 33.8 and 27.7% respectively. pH of the Hokersar and Mirgund soil sample was found to be 8 and 7 respectively. The pH of soil sediments from Hokersar wetland has also been reported to be within pH range of 7-8, which aligns with our findings (Bano *et al.,* 2022). Whereas, it is the first report to show the soil pH of Mirgund wetland. The recorded pH value aligned with the published literature, confirming that most of myxobacterial species isolated from soil flourish within a pH range of 6.5-8.5 (Shimkets *et al.,* 2006; Mohr, 2018; Saadatpour *et al.,* 2024). According to the study conducted by Hook (1997), ten different species of myxobacteria named *Archangium gephyra, Myxococcus fulvus*, *Polyangium cellulosum*, *Myxococcus disciformis*, *Myxococcus coralloides*, *Myxococcus virescens*, *Myxococcus stipitatus*, *Myxococcus xanthus*, *Polyangium sorediatum*, and *Melittangium lichenicola*, isolated from alkaline bog waters and adjoining soil that had the pH between 6.0 to 8.7 (Hook, 1977). Dawid (2000) reported about the global

distribution of myxobacteria in soil, according to which myxobacterial species are found in both alkaline (pH 8.0–9.2) and acidic soil (pH 3.7) (Dawid, 2000).

5.3. Isolation and determination of characteristic features of myxobacteria

Four pure isolates of myxobacteria from Hokersar and Mirgund soil (two from each site) were isolated by using the dung-baiting method (Gaspari *et al.,* 2005; Zhang *et al.,* 2013). After an incubation of 2-weeks, white colour patches were observed on the pellets of goat dung (Figure 5.2).

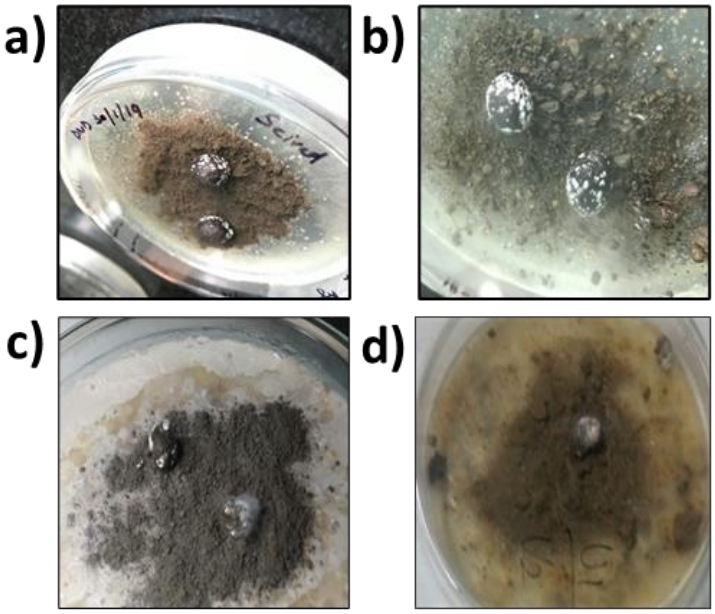


Figure 5.2: White patches on the goat dung pellet. a) Hok1, b) Hok2, c) Mir1 and d) Mir2

The patches observed on the dung-pellet were picked and inoculated in casitone broth. The inoculated test tubes after the incubation of 1 week showed the formation of yellow-whitish ring on the walls of glass tubes. Using this approach, 4 isolates of Myxobacteria were obtained i.e., Hok1, Hok2, Mir1 and Mir2 respectively (Figure 5.3).

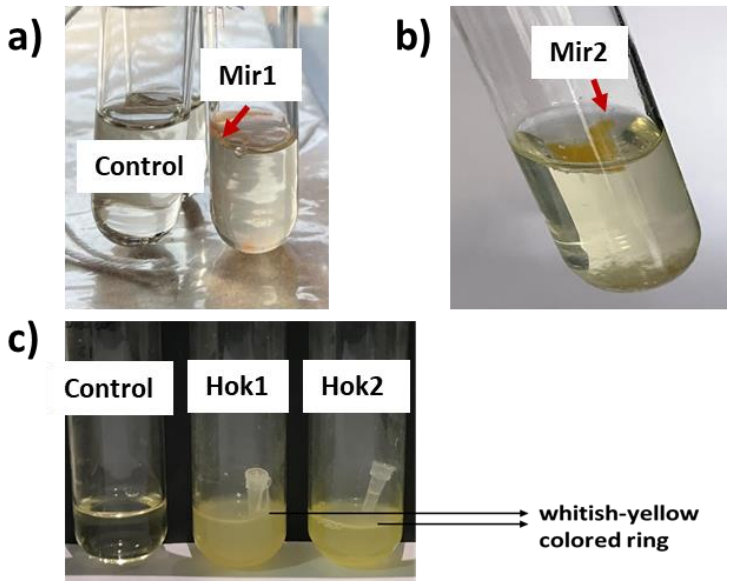


Figure 5.3: Ring formation on the wall of test tubes. a) Control and Mir1; b) Mir2 and c) Control, Hok1 and Hok2

The white patches on goat dung pellets were also observed under a stereomicroscope to observe the presence of fruiting bodies and swarming growth pattern. All isolates showed the characteristic myxobacterial features, *viz* fruiting bodies formation and swarming pattern. These findings preliminarily confirmed that the isolates belonged to the myxobacterial group, as shown in Figure 5.4 and 5.5. The formation of fruiting bodies and growth in swarming pattern displayed the similarity of growing pattern and morphological characteristics as stated by previously published literature on myxobacteria isolated from different geo-locations (Hook, 1977; Dawid, 2000; Zhang *et al.,* 2013; Mohr *et al.,* 2018a; Saggu *et al.,* 2023b).

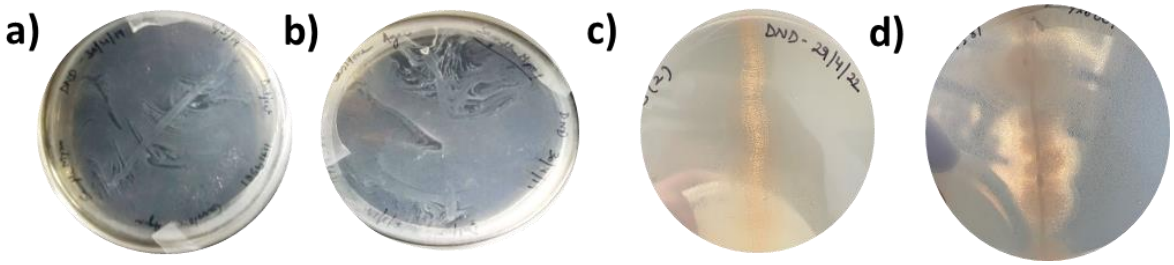


Figure 5.4: Swarming morphology of isolates. a) Hok1, b) Hok2, c) Mir1, and d) Mir2

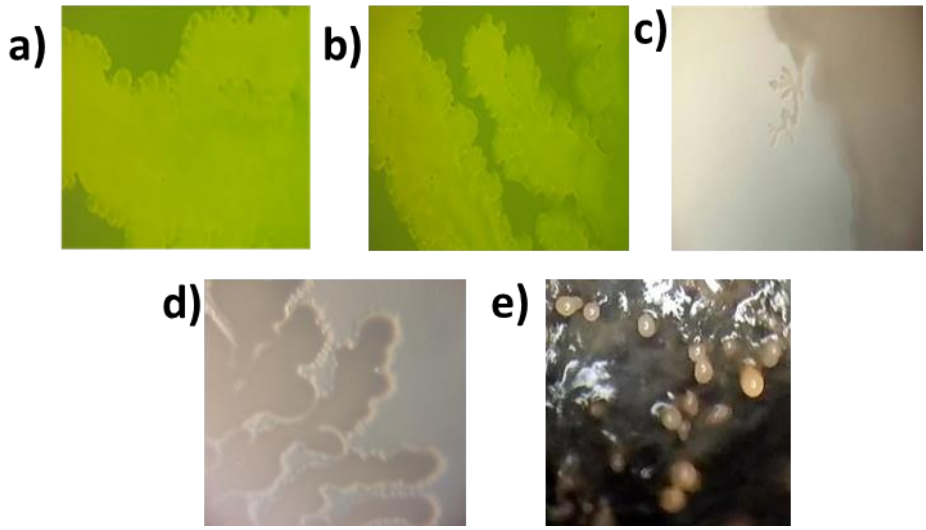


Figure 5.5: Fruiting bodies formation. a) Hok1, b) Hok2, c) Mir1, and d) Mir2 observed under stereomicroscope at 40X and e) Migrated fruiting bodies of Mir2 on the goat dung pellet

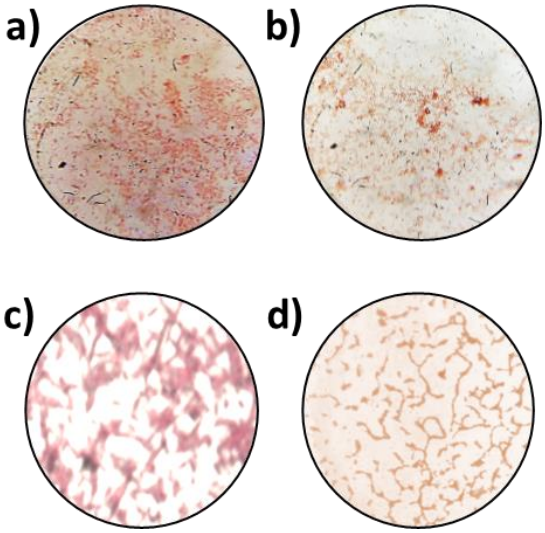


Figure 5.6: Gram staining of the isolates. a) Hok1, b) Hok2, c) Mir1, and d) Mir2

The pure isolates were also studied for gram staining for confirming them to be Gram-negative (Figure 5.6).

5.4. Isolation of genomic DNA from myxobacterial isolates and its analysis by agarose gel electrophoresis

In the current study, the isolation of DNA by the standard protocol (Green and Sambrook, 2017) yielded high molecular weight (HMW) genomic DNA. Good quality of genomic DNA was obtained from all four samples, which is essential for the cloning experiments. As described in

section 4.5, the total genomic DNA isolated from all the four isolates was analyzed on 0.5% (w/v) agarose gel electrophoresis (Figure 5.7).

Figure 5.7: 0.5 % Agarose gel electrophoresis of genomic DNA. Lane 1: Mir1, Lane 2: Mir2, Lane 3: Hok1 Lane 4: Hok2 and Lane 5: Uncut lambda DNA

5.5. Spectrophotometric analysis of genomic DNA

The isolated high molecular weight DNA was checked for yield and purity based on the A_{260} value and absorbance ratios at A_{260}/A_{280} (DNA/ protein) spectrophotometrically. The purity of extracted DNA with respect to A_{260}/A_{280}, where high ratio was an indicative of the purity with respect to protein contamination (Purohit and Singh, 2009). The DNA preparation was considered for molecular biology techniques and applications, as the DNA obtained fulfilled the yield and purity criteria, spectrophotometrically. The DNA concentration was calculated using the formula (Table 5.1):

dsDNA concentration = 50 µg/mL × OD_{260} × dilution factor (200);

Table 5.1: Spectrophotometric analysis of genomic DNA from different isolates

Sample	OD at 260	OD at 280	Purity	DNA concentration (µg/mL)
Hok1	0.136	0.097	1.402	1360
Hok2	0.089	0.062	1.435	890
Mir1	0.107	0.074	1.446	1070

Mir2	0.203	0.136	1.493	2030

5.6. PCR amplification of genomic DNA with 16S primers

16S r-RNA gene was amplified from genomic DNA of Hok1, Hok2, Mir1 and Mir2. PCR conditions were standardized based on the Tm of the 16S primers [27F & 1492R] (Kumar *et al.,* 2017). This experiment also served as a test for the suitability of the quality of DNA for cloning. All samples showed a single amplicon at 1.5 kb (Figure 5.8). Further, after amplification the amplicons were sent for purification and sequencing to Biologia Research India Pvt. Ltd, Karnal for identification of isolates.

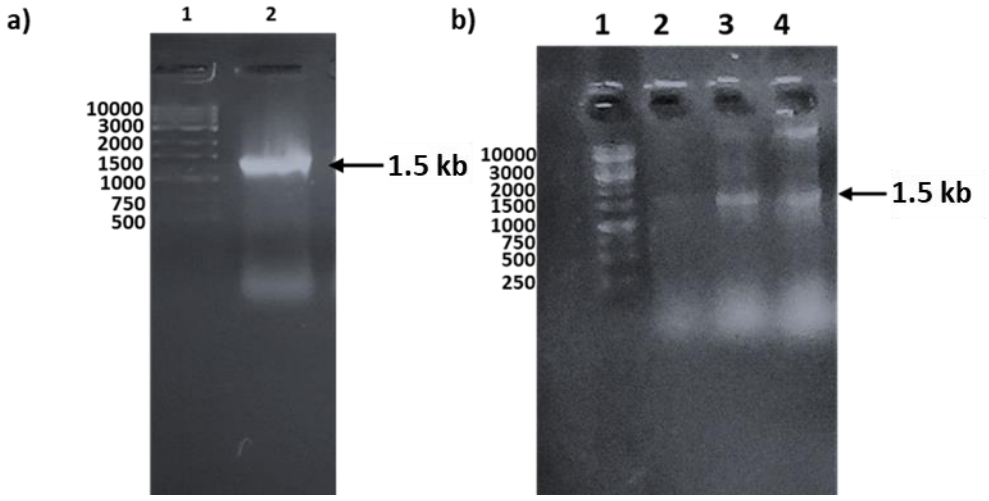


Figure 5.8: 1.5 % Agarose gel showing PCR amplified product of 16S rRNA gene from the isolate. a) Lane 1: 1 kb ladder and Lane 2: Hok1. b) Lane 1: 1 kb Ladder, Lane 2: Hok2, Lane 3: Mir1 and Lane 4: Mir2

5.7. Sequencing of 16S ribosomal RNA gene of the isolates

The sequencing results obtained were subjected to analysis through BLASTn tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi), as mentioned in Annexure 1. The BLASTn results showed the similarity with Uncultured *Anaeromyxobacter* sp. (86.5%), *Pyxidicoccus fallax* (99.93%) and *Myxococcus* sp. (100%) for Hok1, Mir1 and Mir2 respectively as shown in Figure 5.9. Out of the four isolates, Hok2 showed 100% similarity to *Cystobacillus kochi*, which was not identified as Myxobacteria and hence was not considered for any further experiments. This is the first report to show the presence of myxobacterial strains in the soils of Hokersar and Mirgund wetlands.

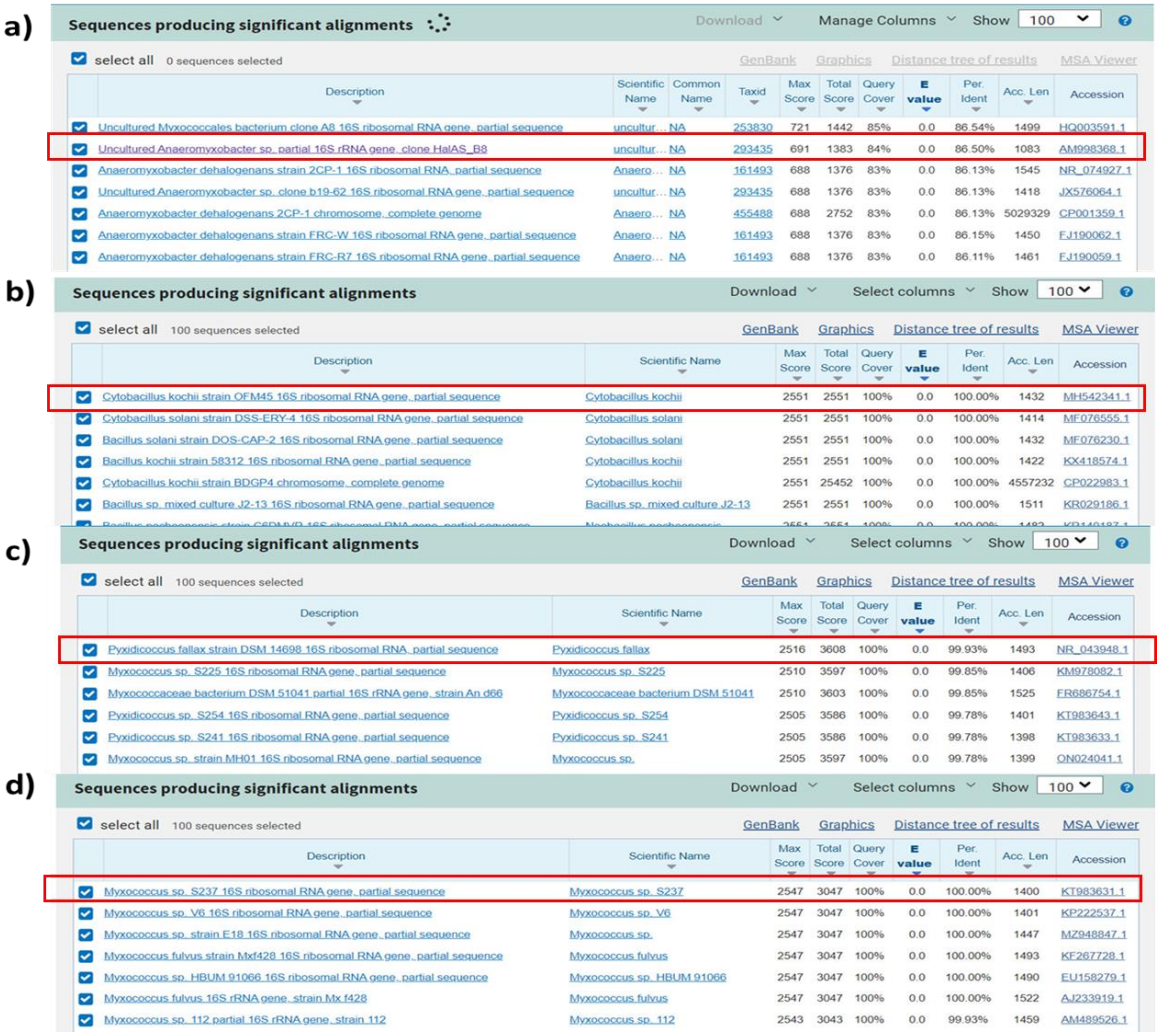
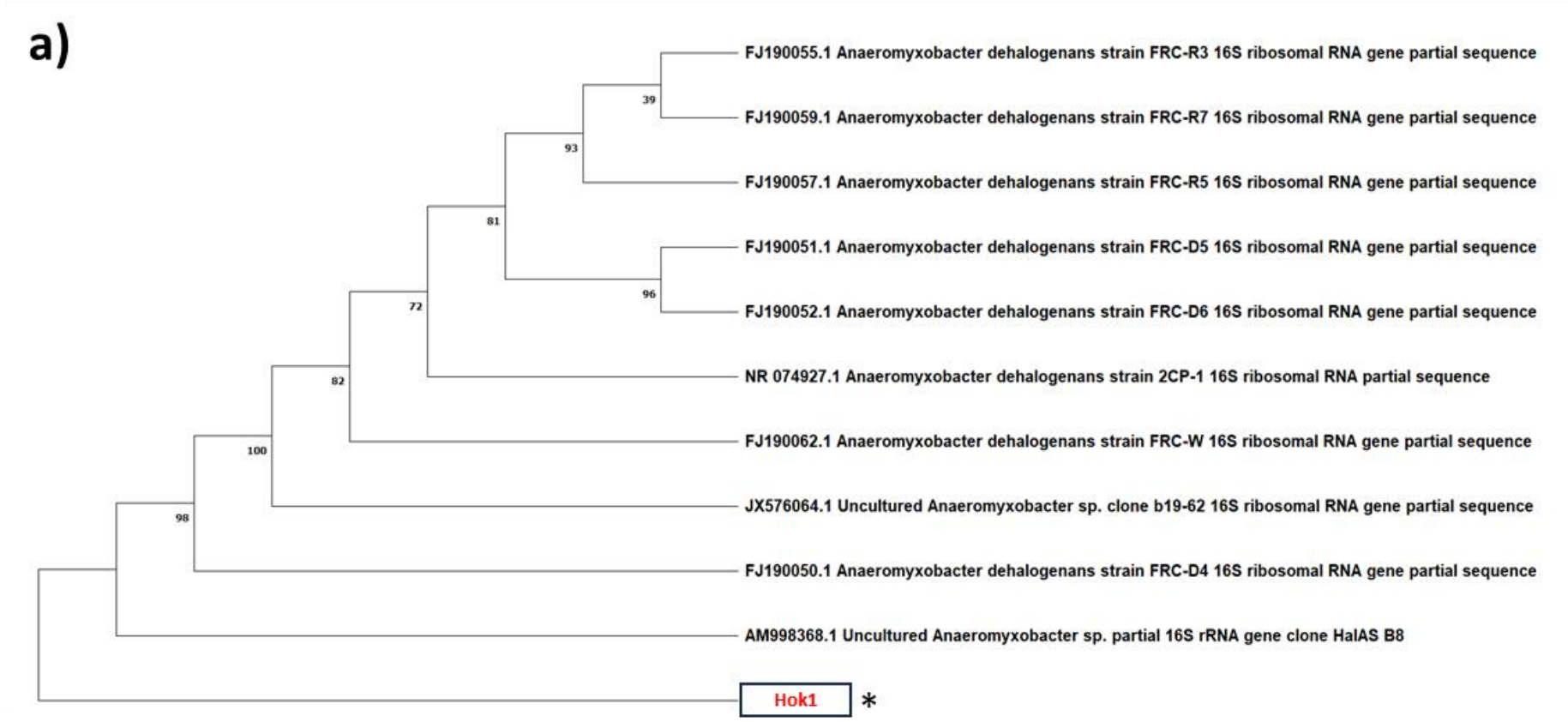
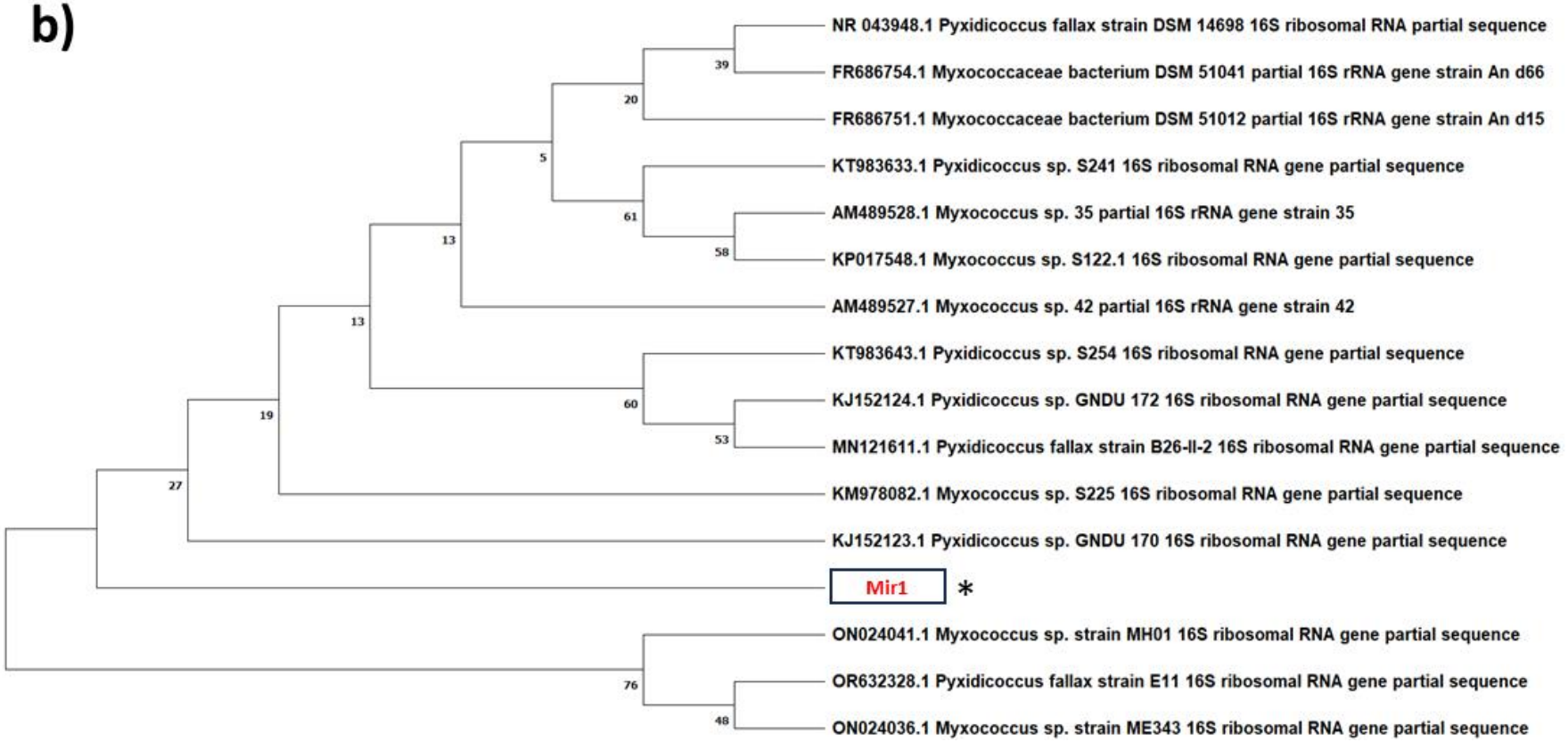


Figure 5.9: BLASTn based molecular identification of myxobacterial isolates a) Hok1, b) Hok2, c) Mir1 and d) Mir2

The phylogenetic tree was constructed using the MEGA X tool for (a) Hok1, (b) Mir1 and (c) Mir2 as illustrated in Figure 5.10. MEGA X is a highly used software for the construction of phylogenetic trees and is used to conduct comparative analysis of molecular sequences to discover functional and adaptive genome differences among different species (Kumar *et al.,* 2018).


a)
FJ190055.1 Anaeromyxobacter dehalogenans strain FRC-R3 16S ribosomal RNA gene partial sequence
FJ190059.1 Anaeromyxobacter dehalogenans strain FRC-R7 16S ribosomal RNA gene partial sequence
FJ190057.1 Anaeromyxobacter dehalogenans strain FRC-R5 16S ribosomal RNA gene partial sequence
FJ190051.1 Anaeromyxobacter dehalogenans strain FRC-D5 16S ribosomal RNA gene partial sequence
FJ190052.1 Anaeromyxobacter dehalogenans strain FRC-D6 16S ribosomal RNA gene partial sequence
NR 074927.1 Anaeromyxobacter dehalogenans strain 2CP-1 16S ribosomal RNA partial sequence
FJ190062.1 Anaeromyxobacter dehalogenans strain FRC-W 16S ribosomal RNA gene partial sequence
JX576064.1 Uncultured Anaeromyxobacter sp. clone b19-62 16S ribosomal RNA gene partial sequence
FJ190050.1 Anaeromyxobacter dehalogenans strain FRC-D4 16S ribosomal RNA gene partial sequence
AM998368.1 Uncultured Anaeromyxobacter sp. partial 16S rRNA gene clone HalAS B8
Hok1
*
39
93
81
96
72
82
100
98


b)
NR 043948.1 Pyxidicoccus fallax strain DSM 14698 16S ribosomal RNA partial sequence
FR686754.1 Myxococcaceae bacterium DSM 51041 partial 16S rRNA gene strain An d66
FR686751.1 Myxococcaceae bacterium DSM 51012 partial 16S rRNA gene strain An d15
KT983633.1 Pyxidicoccus sp. S241 16S ribosomal RNA gene partial sequence
AM489528.1 Myxococcus sp. 35 partial 16S rRNA gene strain 35
KP017548.1 Myxococcus sp. S122.1 16S ribosomal RNA gene partial sequence
AM489527.1 Myxococcus sp. 42 partial 16S rRNA gene strain 42
KT983643.1 Pyxidicoccus sp. S254 16S ribosomal RNA gene partial sequence
KJ152124.1 Pyxidicoccus sp. GNDU 172 16S ribosomal RNA gene partial sequence
MN121611.1 Pyxidicoccus fallax strain B26-II-2 16S ribosomal RNA gene partial sequence
KM978082.1 Myxococcus sp. S225 16S ribosomal RNA gene partial sequence
KJ152123.1 Pyxidicoccus sp. GNDU 170 16S ribosomal RNA gene partial sequence
Mir1
*
ON024041.1 Myxococcus sp. strain MH01 16S ribosomal RNA gene partial sequence
OR632328.1 Pyxidicoccus fallax strain E11 16S ribosomal RNA gene partial sequence
ON024036.1 Myxococcus sp. strain ME343 16S ribosomal RNA gene partial sequence
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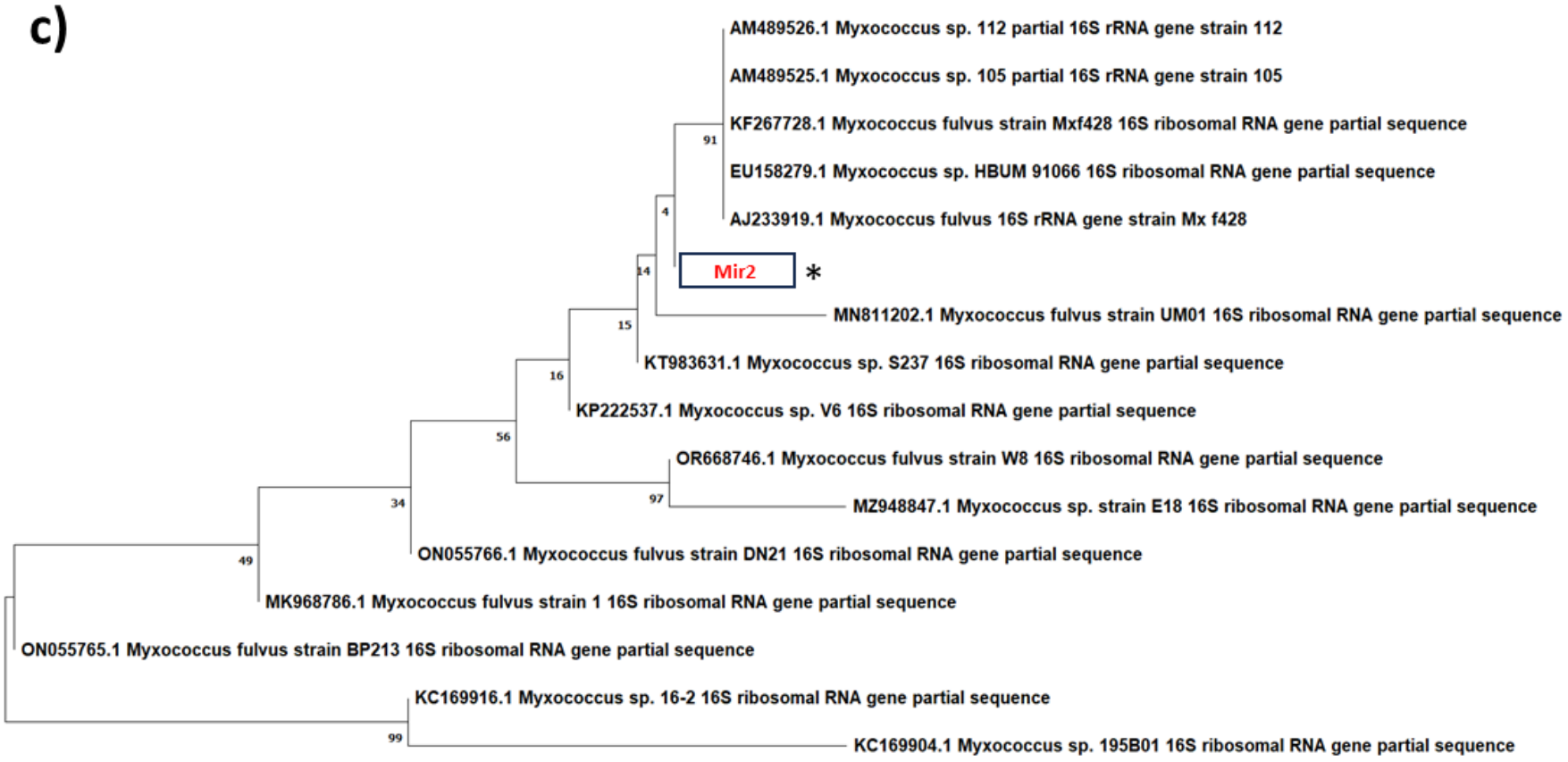


Figure 5.10: Phylogenetic tree inferred by the neighbor-joining method of a) Hok1, b) Mir1, and c) Mir2 based on 16S rRNA gene sequence, which have been aligned with their closely related species

5.8. Screening of the isolates for the production of hydrolases and bioactive molecules

5.8.1. *Primary screening of isolates for enzyme production*

The primary screening of the isolates for the production of hydrolase enzymes (amylases, cellulases and proteases) involved the formation of zone of hydrolysis. For this, isolates streaked as straight single line on the casitone media supplemented with starch (1%) for amylase (incubated at 30°C for 72 hours) (Kumar *et al.,* 2016), CMC (1%) for cellulase (incubated at 30°C for 72 hours) (Bai *et al.,* 2012) and skimmed milk (1.5%) for protease (incubated at 30°C for 24-72 hours) (Tennalli *et al.,* 2022).

Amylase Activity: No zone of hydrolysis was observed around the marked area of the growth of isolates Hok1, Mir1 and Mir2 on the plates after iodine staining (Figure 5.11). A zone of hydrolysis indicates enzymatic breakdown of starch in the media (Kumar *et al.,* 2016). This confirmed that none of the isolated strains of myxobacteria were producing amylase enzyme.

Cellulase Activity: Zone of hydrolysis was observed around the marked area of the growth of isolates Hok1, Mir1 and Mir2 on the plates after congo red staining followed by NaCl destaining. The presence of a hydrolysis zone indicates the degradation of cellulose from the media (Bai *et al.,* 2012) (Figure 5.11). Other available reports on myxobacteria have also confirmed the production of cellulolytic enzymes by myxobacterial genera (Hou *et al.,* 2006; Zhou *et al.,* 2020).

Protease Activity: Zone of hydrolysis was observed on the skimmed milk plates that were single streaked with isolates i.e., Hok1, Mir1 and Mir2. The result of the protease enzyme showed similarity with the results reported for alkaline protease synthesized by *Bacillus cereus* (Tennalli *et al.,* 2022). The presence of a zone of hydrolysis indicated the degradation of casein from the media (Figure 5.11).

The primary screening experiments with the isolates were performed in triplicates and repeated at least once in order to establish the reproducibility of the results. The primary screenings gave an indication that all the three isolates i.e., Hok1, Mir1 and Mir2 had the potential for synthesizing cellulase and protease enzymes.

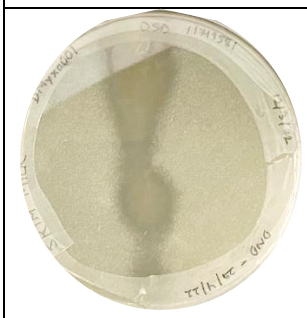
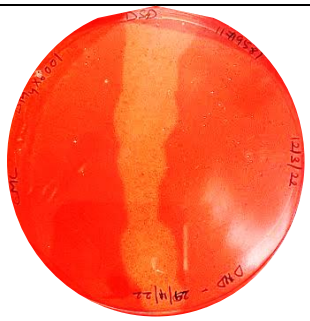
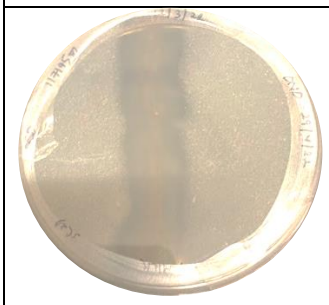
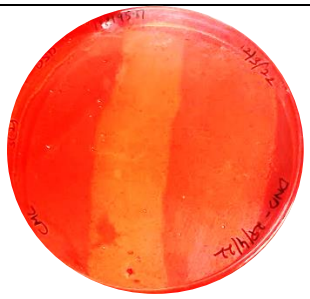
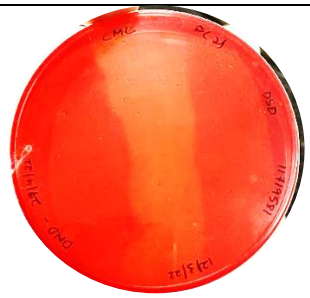
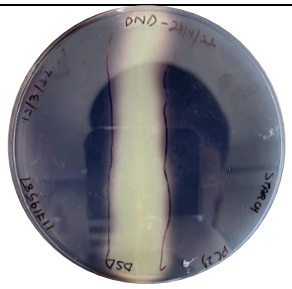
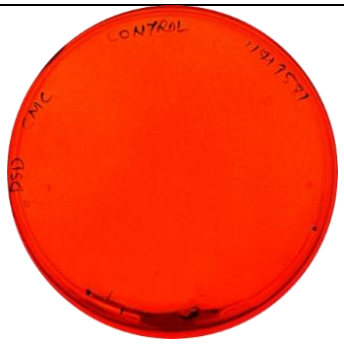
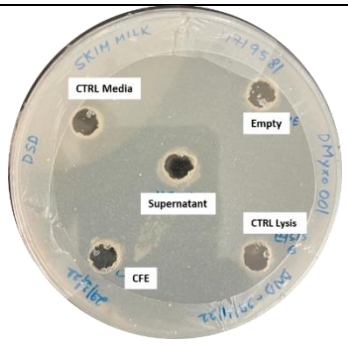
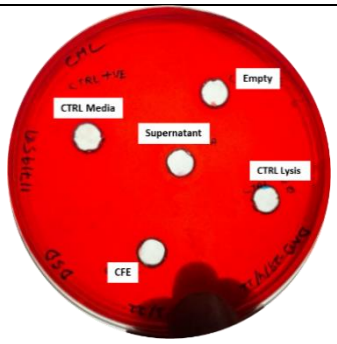
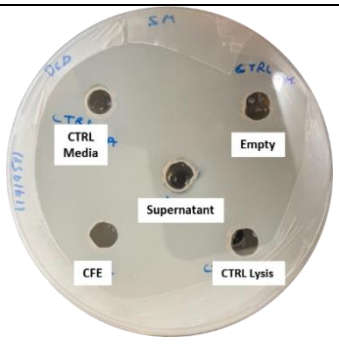
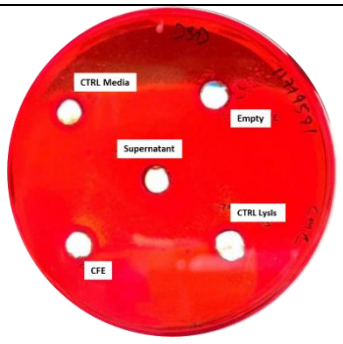
Strain	a) Skimmed Milk (Protease)	b) CMC (Cellulase)	c) Starch (Amylase)
Hok1	**Positive**	**Positive**	**Negative**
			
Mir1	**Positive**	**Positive**	**Negative**
			
Mir2	**Positive**	**Positive**	**Negative**
			

Figure 5.11: Primary screening of Hok1, Mir1 and Mir2 isolates for different hydrolases enzymes on a) skimmed Milk (protease), b) CMC (cellulase) and c) starch (amylase)

5.8.2. *Secondary screening for detecting the localization of enzyme*

The secondary screening of isolates for enzymes (cellulase and protease) was performed to confirm the localization of enzyme (extracellular or intracellular) via agar-diffusion method (Sitoke *et al.,* 2017; Kaur *et al.,* 2020). For both cellulase and protease, five wells were punctured on the casitone media supplemented with CMC (1%) for cellulase and skimmed milk (1.5%) for protease (Figure 5.12). On evaluating the media plates for enzyme localization, out of three isolates, i.e., Hok1, Mir1, and Mir2, only the isolate Mir2 showed positive results for both cellulolytic and proteolytic activity. Thus, it was used for further analysis.

The result of localization of our study revealed that the majority of the activity of both the enzymes in Mir2 was localized in the extracellular fraction (Figure 5.12). The result corroborated with the result obtained for cellulase-producing bacteria adapted to cold temperature which was isolated from boniyar region of Kashmir (Kaur *et al.,* 2020). On the other hand, the result of protease localization in our study revealed to be extracellular which aligned with the result obtained by Sharma *et al.,* that reported the alkaline protease enzyme synthesized by *Pyxidicoccus* sp. 252 to be extracellular in nature (Sharma *et al.,* 2021). Whereas, no activity by Hok1 and Mir1 may be due to membrane-bound nature of the protein (Louime *et al.,* 2006; Dalbey *et al.,* 2012).

Strain	**a) Skimmed Milk (Protease)**	**a) CMC (Cellulase)**
Control		
Hok1		
Mir1		

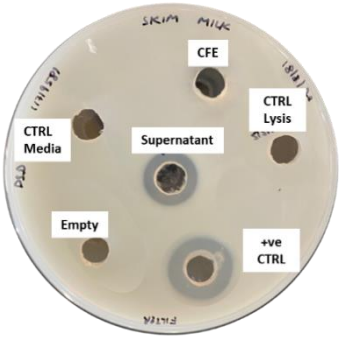
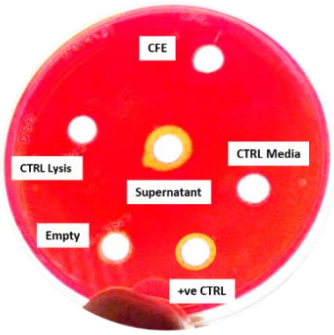
Mir2		
Diameter of Zone of Inhibition	**Supernatant = 1.7 cm**	**Supernatant – 1.5 cm**

Figure 5.12: Screening of localization of enzyme of isolates (Hok1, Mir1, and Mir2) for different hydrolases enzyme a) Skimmed milk (protease) and b) CMC (cellulase)

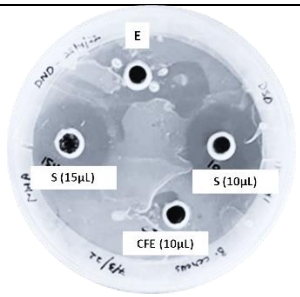
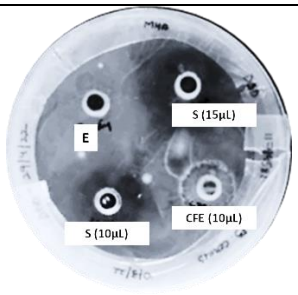
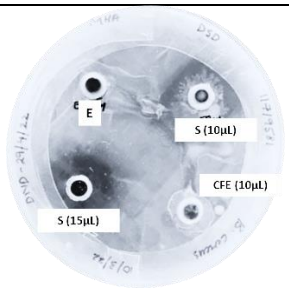
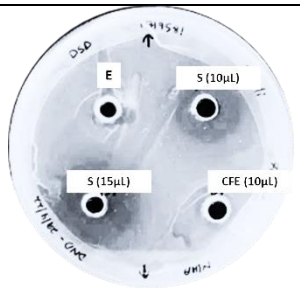
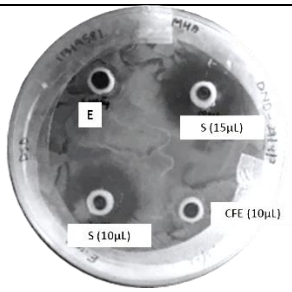
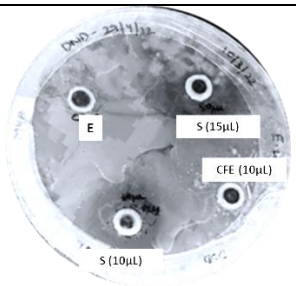
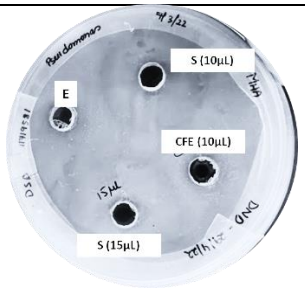
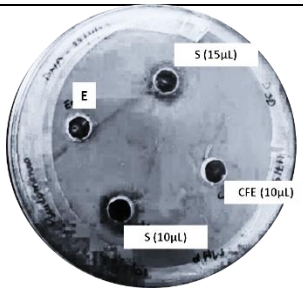
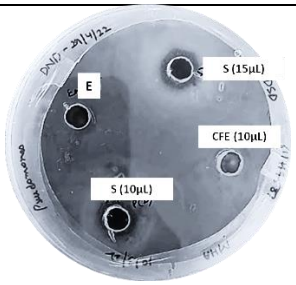
5.9. Screening of the isolates (Hok1, Mir1 and Mir2) for antimicrobial activity

Myxobacteria are previously known for their ability to synthesize bioactive molecules that exhibit antibacterial activity. In the current study, the antimicrobial potential of three isolates, i.e., Hok1, Mir1 and Mir2 were evaluated against the three test organisms *viz Bacillus cereus* MTCC 8714*, Escherichia coli* MTCC 1679 and *Pseudomonas aeruginosa* MTCC 2582*,* by the agar diffusion method. All isolates showed the zone of inhibition (ZOI) against the test organisms (Table 5.2 and Figure 5.13). The result obtained corroborated with the previously published findings on the antimicrobial activity of the myxobacterial isolate (Myxo010) against the test organism *viz Bacillus cereus, Escherichia coli* and *Pseudomonas sp*. (Thakur *et al.,* 2017). Another study, reported about the antibacterial potential of *Myxococcus xanthus* against *Escherichia coli* (Gaspari *et al.,* 2005; Xiao *et al.,* 2011). Different reports have also been comprehended about the antibacterial potential of myxobacterial species against various eubacteria (Norén and Raper, 1962).

Table 5.2: Antimicrobial activity of different isolates against test strains

Test Strains	**Hok1**		**Mir1**		**Mir2**	
	Supernatant	CFE	Supernatant	CFE	Supernatant	CFE
Bacillus cereus MTCC 8714	+	-	+	-	+	-
Escherichia coli MTCC 1679	+	-	+	-	+	-

Pseudomonas aeruginosa MTCC 2582	+	-	+	-	+	-

Strain Name	**Hok1**	**Mir1**	**Mir2**
Bacillus cereus MTCC 8714			
	Positive	Positive	Positive
Escherichia coli MTCC 1679			
	Positive	Positive	Positive
Pseudomonas aeruginosa MTCC 2582			
	Positive	Positive	Positive

*S: Supernatant; E: Empty; CFE: Cell Free Extract

Figure 5.13: Antibacterial assessment of supernatant and cell free extract of myxobacterial strain, i.e., Hok1, Mir1, and Mir2 against *Bacillus cereus* MTCC 8714, *Escherichia coli* MTCC 1679, and *Pseudomonas aeruginosa* MTCC 2582

5.10. Identification of the gene(s) coding for hydrolases enzymes

5.10.1. *Amplification of cellulase gene from the isolate*

The genomic DNAs of Hok1, Mir1, and Mir2 were screened for the presence of cellulase gene fragments using three sets of degenerate primers at different annealing temperatures (as

mentioned in section 4.13.1). The amplified product of each sample was subjected to 1.5% (w/v) agarose gel electrophoresis. No positive result was obtained for cellulase gene amplification, which could be due to an unknown sequence of the cellulase gene, which could not be bound by the degenerate primers. The probable reason could be a variation in gene sequence present within the isolate on which the degenerate primers have been used (Busk and Lange, 2013) (Figure 5.14, 5.15, 5.16).

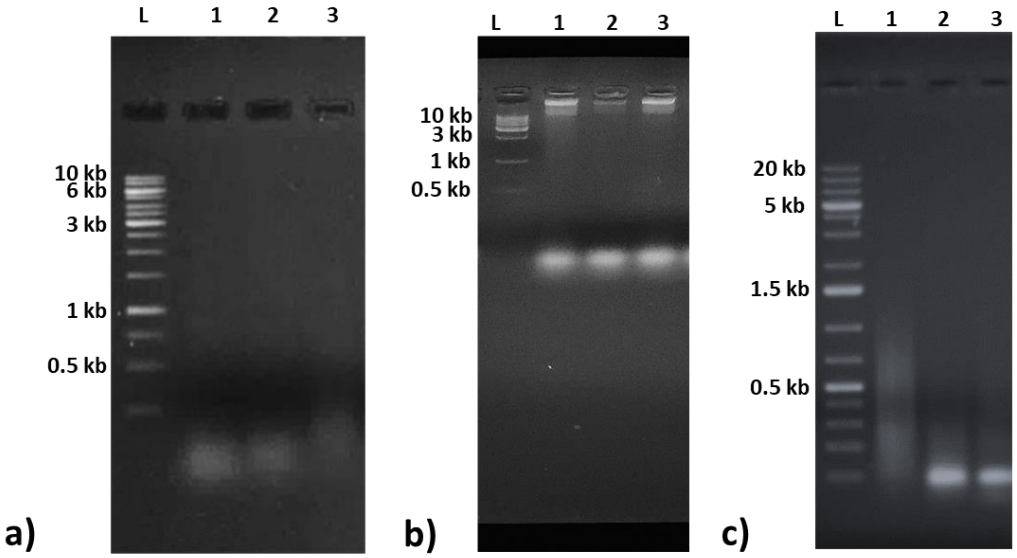


Figure 5.14: 1.5% Agarose gel showing amplified gradient PCR product of cellulase gene for Hok1. a) With primer set I. Lane L: 1 kb ladder, Lane 1-3: Product of primer set I at 64°C, 66°C, 68°C, respectively, b) With primer set II. Lane L: 1 kb ladder, Lane 1-3: Product of primer set II at 44°C, 51°C, 58°C respectively, c) With primer set III. Lane L: 1 kb ladder, Lane 1-3: Product of primer set III at 53°C, 60°C, 66°C, respectively

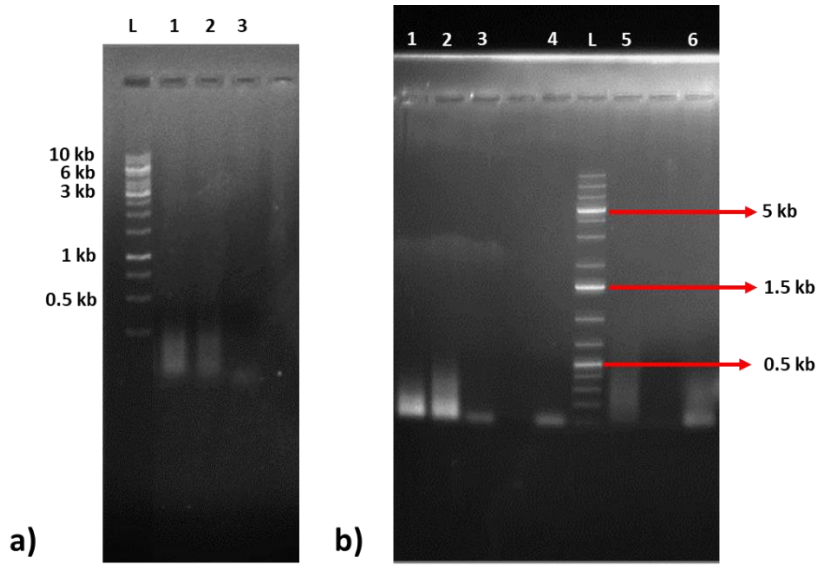


Figure 5.15: 1.5% Agarose gel showing amplified gradient PCR product of cellulase gene for Mir1 a) With primer set I. Lane L: 1 kb ladder, Lane 1-3: Product of primer set I at 64°C, 66°C, 68°C,

respectively, b) With primer set II and III. Lane L: 1 kb ladder, Lane 1-3: Product of primer set II at 44°C, 51°C, 58°C respectively and Lane 4-6: Product of primer set III at 53°C, 60°C, 66°C, respectively

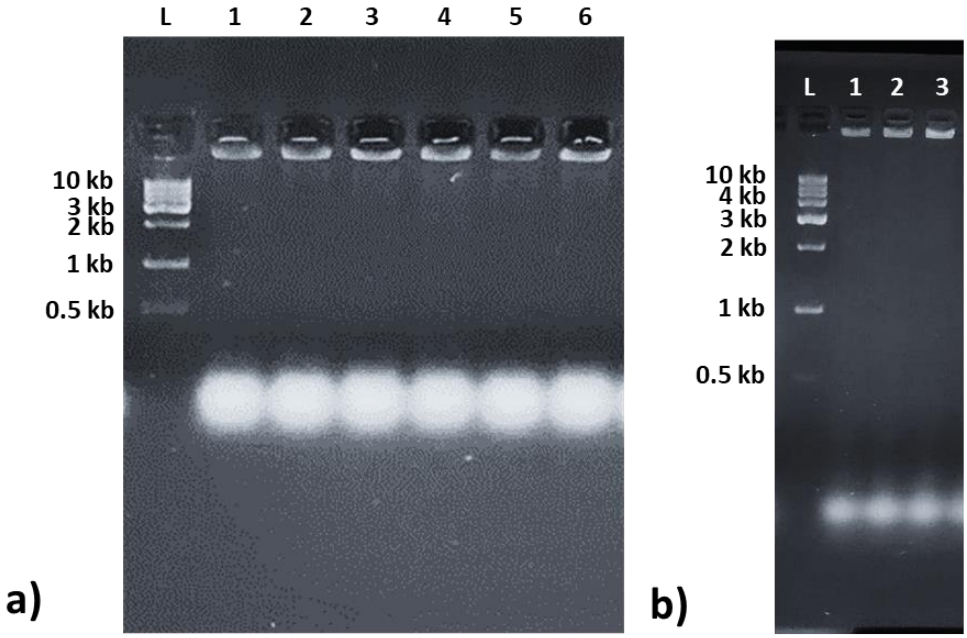


Figure 5.16: 1.5% Agarose gel showing amplified gradient PCR product of cellulase gene for Mir2. a) With primer set I and II. Lane L: 1 kb ladder, Lane 1-3: Product of primer set I at 64°C, 66°C, 68°C, respectively, and Lane 4-6: Product of primer set II at 44°C, 51°C, 58°C respectively; b) With primer set III. Lane L: 1 kb ladder, Lane 1-3: Product of primer set III at 53°C, 60°C, 66°C, respectively

5.10.2. *Amplification of protease gene from the isolate*

The genomic DNAs of Hok1, Mir1, and Mir2 were screened for the presence of protease gene fragments using three sets of degenerate primers at different annealing temperatures (as mentioned in section 4.13.2). The amplified product of each sample was subjected to 1.5% (w/v) agarose gel electrophoresis (Figure 5.17, 5.18, 5.19)**.** The primer set V at 53°C and 56°C and primer set VI at 62°C and 65°C showed positive results by showing the amplified product of ~2 kb on 1.5% (w/v) agarose gel for Mir2 (Figure 5.18, 5.19).

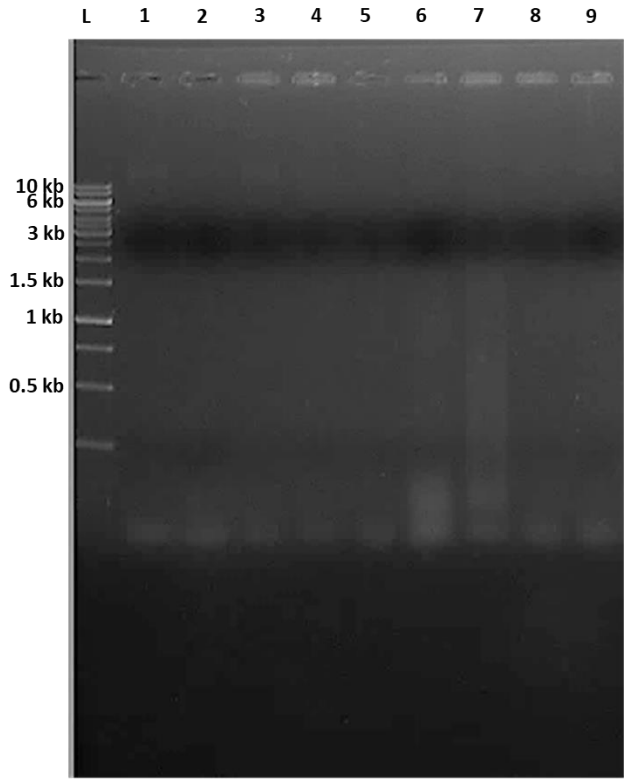


Figure 5.17: 1.5% Agarose gel showing amplified gradient PCR product of protease (Pro) gene for Hok1 with primer set IV, V and VI. Lane L: 1 kb ladder, Lane 1-3: Product of primer set IV at 59.1°C, 62°C, 65°C respectively; Lane 4-6: Product of primer set V at 50°C, 53°C, 56°C, respectively and Lane 7-9: Product of primer set VI at 60°C, 62°C, 65°C, respectively

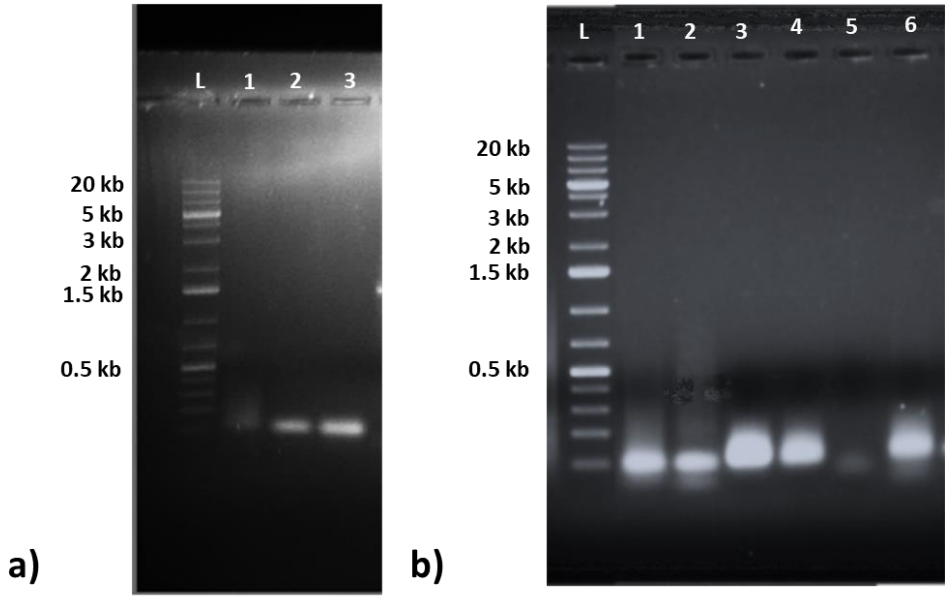


Figure 5.18: 1.5% Agarose gel showing amplified gradient PCR product of protease (Pro) gene for Mir1 a) With primer set IV. Lane L: 1 kb ladder, Lane 1-3: Product of primer set IV at 59.1°C, 62°C, 65°C respectively, b) With primer set V and VI. Lane L: 1 kb ladder, Lane 1-3: Product of primer set V at 50°C, 53°C, 56°C, respectively and Lane 4-6: Product of primer set VI at 60°C, 62°C, 65°C, respectively

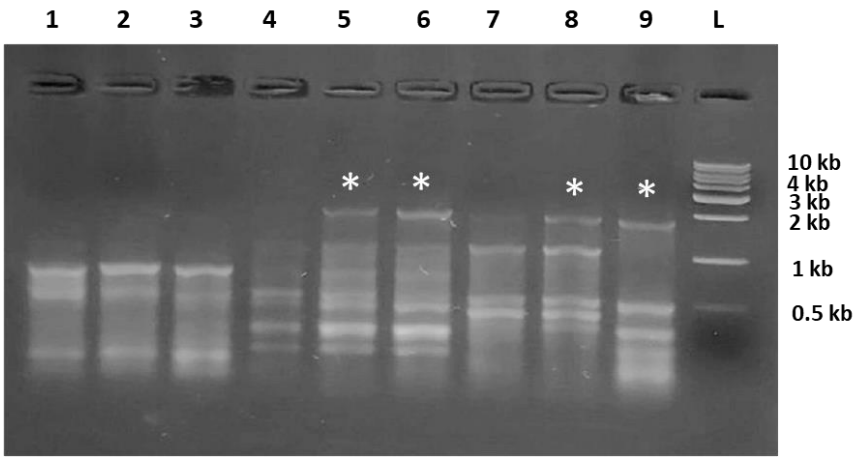


Figure 5.19: 1.5% Agarose gel showing amplified gradient PCR product of protease (Pro) gene for Mir2 with primer set IV, V and VI. Lane 1-3: product of primer set IV at 59.1°C, 62°C, 65°C respectively; Lane 4-6: product of primer set V at 50°C, 53°C, 56°C, respectively and Lane 7-9: product of primer set VI at 60°C, 62°C, 65°C, respectively and Lane L: 1 kb ladder

Further, the scale-up PCR was performed using the same set of primers and PCR conditions (as mentioned in section 4.13.1) for Mir2. The amplified product of scale-up was subjected to 1.5% (w/v) agarose gel electrophoresis, which showed an effective result at 56°C for primer set V and 65°C for primer set VI (Figure 5.20). The amplicon (~2 kb) of primer set V at 56°C was sent for sequencing to Biologia Research India Pvt. Ltd., Karnal, and was subjected for further analysis.

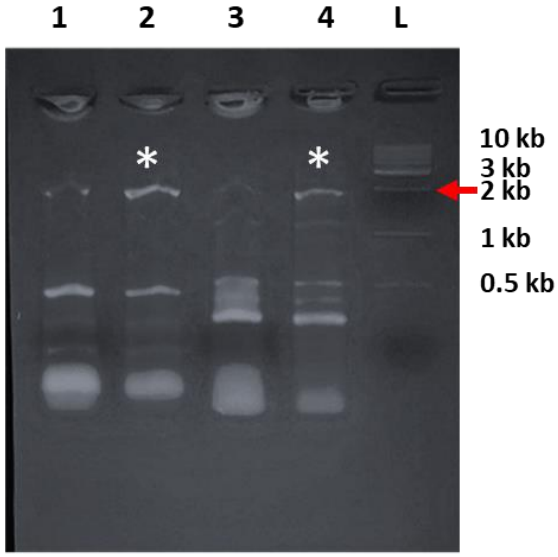


Figure 5.20: 1.5% Agarose gel showing scale up PCR product of protease "Pro" gene for Mir2. Lane 1-2: Product of primer set V at 53°C and 56°C respectively; Lane 3-4: Product of primer set VI at 62°C and 65°C respectively and Lane L: 1 kb ladder

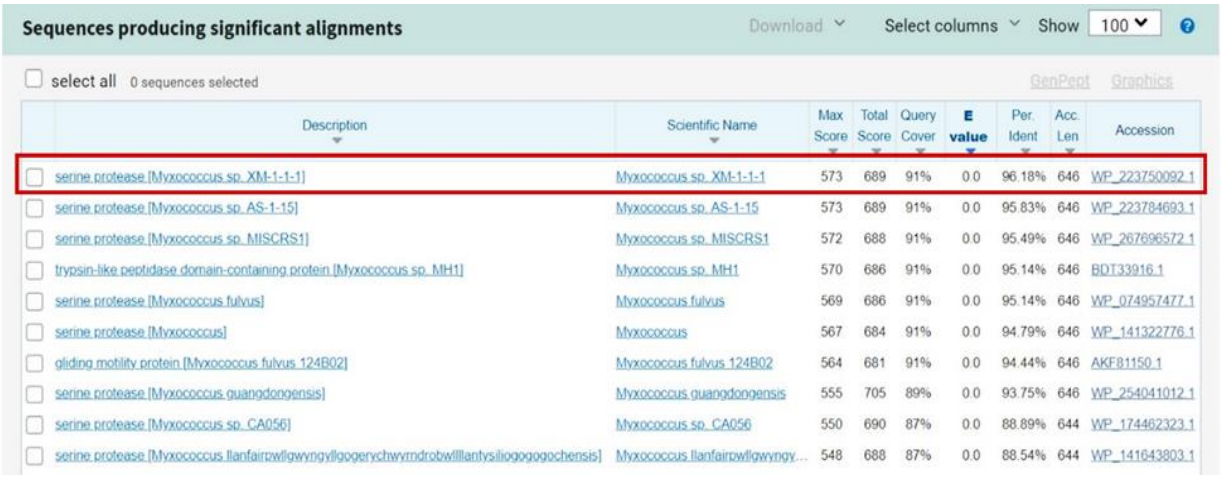
5.11. Analysis of the sequence of protease amplicon

The sequence of 1233 bp was provided by Biologia Research India Pvt. Ltd., Karnal, for the protease gene amplicon (~2 kb). Further, the sequencing result obtained was subjected to analysis through the BLASTx tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) as follow:

>Mir2*Pro*

CCGATTGTAGGCGCTGTCTCAGCGCGCCGGCGGCGCTGGCCTCGGCGCCCGTGTG
CGAGGCGGCCGTGTCGCCCGCCTCCCAGCGCGCCACGAAGGTGGGCGAAGACGT
GCACCGGCGTTTCGAGTCGGCCCACCCCTACGCCACCACGGAGCTGCGCGCTTCG
AGCGGTCCGGTCCACACCGATGTCATCACCCACCCGGGGGCCGCGTACATCGCCG
CGCACTTCGAGCGGCTGGAGCTCGAGGAGGGCGACTTCGTCGTGGTGCGCGCCC
CGGACGGCTCTCGCTCCAAGCGCTATGACAACGGCCACCCGGGCGCGCGCGACG
GCTTCTGGGCCCTGCACATCCCCGGTGACACGGCCATCATCGAGCTGCACAGCGC
CGACCACCCCGGCCGTCGGGGCATCCTGAACCAGCACGGCTACAGCATCGACCGG
TTCGCGCGCGGCTACACCAACGAGGAGATGGGCTTCACGTCCGAGCTGAACAAG
GCCGTGTGTGGCGCGGACGACTCGCGCTGGGCGCCCTGCTACGCCGCCAGCGAC
CCCACCCTCTACGGCCGCGCCCGCCCCGTGGCGCGCCTGCTCATCGGTGGCTCCA
GCGCGTGCACCGGTTGGCTCGTGGGCAGCCAGGGCCACGTCATGACCAACCAGC
ACTGCATCGCCACCGCGAGCGACGCGCAGAACACCGACTTCGAGTTCATGGCGG
AGGGCGCCAGCTGCGCCACGAGCTGCGGGAGCTGGTTCGGCTGCCCCGGCCACG
TCATCTCCGGCGGCACCCTGGTGCAGGCGGACGCGCCCCGCGACTACGCGCTGGT
GCGGCTGGCCGTGAACCCCACCGCCAGCTTCGGCTACCTCCAGCTTCGCGACTCG
GGCGCGGTGGTGAACGAGCGCATCTACGTCCCGCAGCACCCCGCCGGGCACGGG
AAAGAAGATCGCCGTCGCCTCCTCGGACTCCACCGACGCGTCCGGCTTCGCGGAG
GTCTACAGCCTCGACGAGCCGTCCTGCCAGTCCGGCGGCCCCAACGACGTGGGCT
ACTTCGCGGACACGCAGGGCGGCTCCTCGGGCTCCCCCGTGATTGGCCACGGGG
ACCACCTGGGTGGTGTCGCTGCACCACTGGGCCAACTGCCCCAACCGGGGGGTG
GCCCATCCAGGGCGTCATCAGCCACCTGGGGCGCCAACCTGCCCACGTGGTGCCC
TGGCCCGCCGGCGGGTGGCCCAACCCCAAAGGGA

Further, BLASTx results showed a 96.18 % similarity of the amplified gene with serine protease (*Myxococcus* sp. XM-1-1-1) with accession number WP_223750092 (Figure 5.21).



Sequences producing significant alignments

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select all 0 sequences selected

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Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
serine protease [Myxococcus sp. XM-1-1-1]	Myxococcus sp. XM-1-1-1	573	689	91%	0.0	96.18%	646	WP_223750092.1
serine protease [Myxococcus sp. AS-1-15]	Myxococcus sp. AS-1-15	573	689	91%	0.0	95.83%	646	WP_223784693.1
serine protease [Myxococcus sp. MISCRS1]	Myxococcus sp. MISCRS1	572	688	91%	0.0	95.49%	646	WP_267696572.1
trypsin-like peptidase domain-containing protein [Myxococcus sp. MH1]	Myxococcus sp. MH1	570	686	91%	0.0	95.14%	646	BDT33916.1
serine protease [Myxococcus fulvus]	Myxococcus fulvus	569	686	91%	0.0	95.14%	646	WP_074957477.1
serine protease [Myxococcus]	Myxococcus	567	684	91%	0.0	94.79%	646	WP_141322776.1
gliding motility protein [Myxococcus fulvus 124B02]	Myxococcus fulvus 124B02	564	681	91%	0.0	94.44%	646	AKF81150.1
serine protease [Myxococcus guangdongensis]	Myxococcus guangdongensis	555	705	89%	0.0	93.75%	646	WP_254041012.1
serine protease [Myxococcus sp. CA056]	Myxococcus sp. CA056	550	690	87%	0.0	88.89%	644	WP_174462323.1
serine protease [Myxococcus llanfairpwllgwyngyllgogerychwyrndrobwllllantysiliogogogochensis]	Myxococcus llanfairpwllgwyngy...	548	688	87%	0.0	88.54%	644	WP_141643803.1

Figure 5.21: Mir2*Pro* showing 96.18% similarity with serine protease upon BLASTx on NCBI database

5.12: Identification of polyketide synthase (PKS) gene from the myxobacterial isolates

Metabolites obtained from microbes have long been known to be the primary source of important therapeutic drugs. Polyketides are one of the leading class of molecules that open new avenues for novel drug therapy (Musiol-Kroll and Wohlleben 2018). In general, polyketides are a diverse family of natural products that are synthesized by plants, bacteria, and fungi. This family is known to synthesize different antibiotics such as erythromycin, tetracycline, tylosin, rifamycin, and rapamycin and anti-cancerous compounds like doxorubicin and mithramycin (Wu et al. 2023).

The genomic DNA of Hok1, Mir1, and Mir2 were screened for the presence of PKS gene fragments using three sets of degenerative primers at different annealing temperatures (as mentioned in section 4.15). The amplified product of each sample was subjected to 1.5% (w/v) agarose gel electrophoresis (Figure 5.22, 5.23, 5.24)**.** The primer set IX at 61.5°C for Mir2 showed a positive result by showing the amplified product of ~600 bp on 1.5% (w/v) agarose gel.

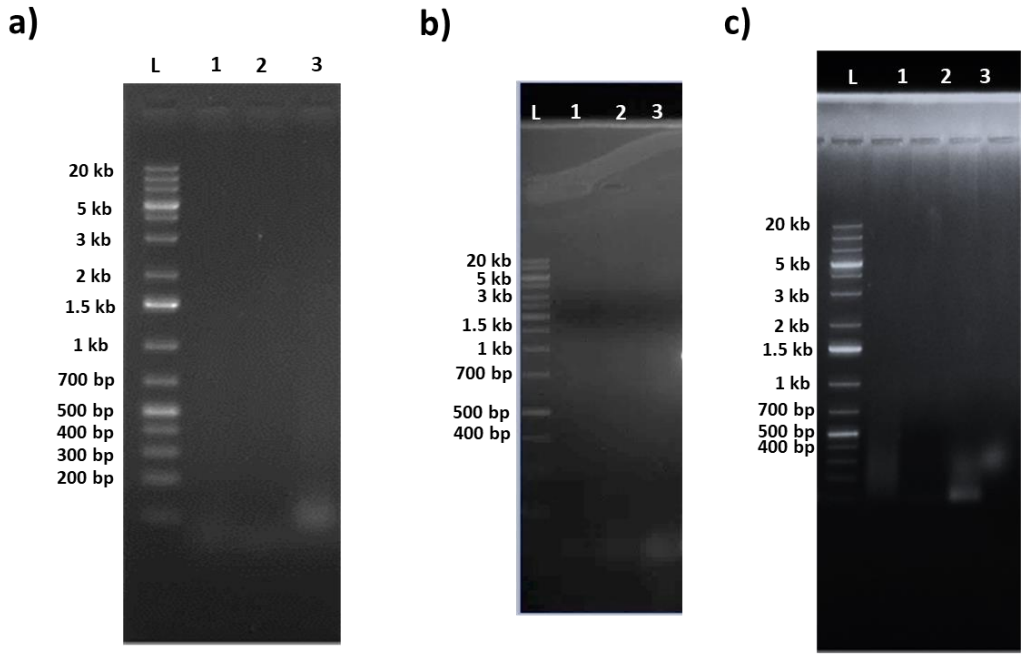


Figure 5.22: 1.5% Agarose gel showing amplified gradient PCR product of PKS gene for Hok1. a) with primer set VII. Lane L: 1 kb Ladder, and Lane 1-3: product of primer set VII at 57°C, 61.5°C and 65°C respectively; b) with primer set VIII. Lane L: 1 kb Ladder and Lane 1-3: product of primer set VIII at 57°C, 61.5°C, 65°C respectively; c) with primer set IX. Lane L: 1 kb ladder and Lane 1-3: product of primer set IX at 57°C, 61.5°C, 65°C respectively

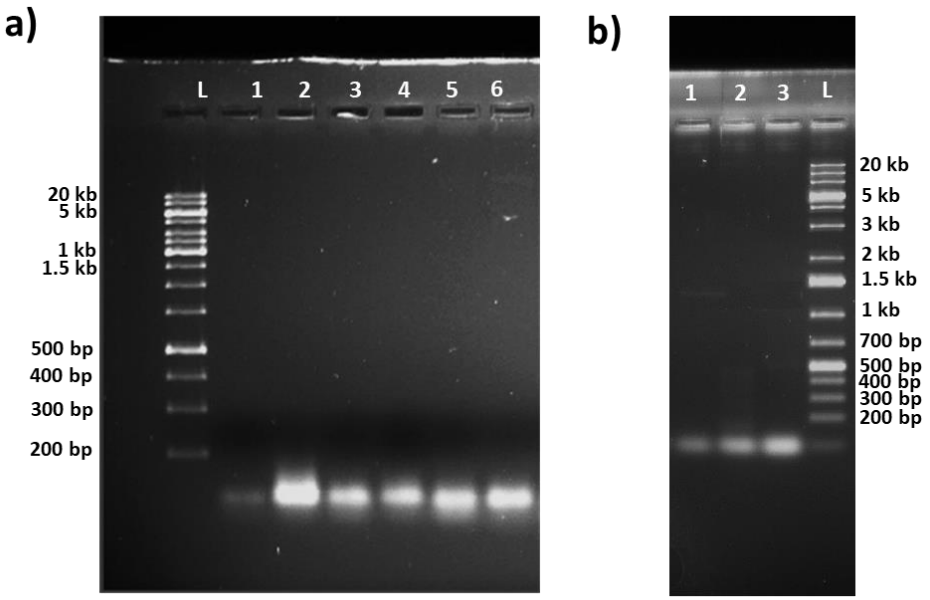


Figure 5.23: 1.5% Agarose gel showing amplified gradient PCR product of PKS gene for Mir1. a) With primer set VII and VIII. Lane L: 1 kb Ladder, Lane 1-3: Product of primer set VII at 57°C, 61.5°C and 65°C respectively and Lane 4-6: Product of primer set VIII at 57°C, 61.5°C, 65°C respectively; e) With primer set IX. Lane 1-3: Product of primer set IX at 57°C, 61.5°C, 65°C respectively and Lane L: 1 kb Ladder

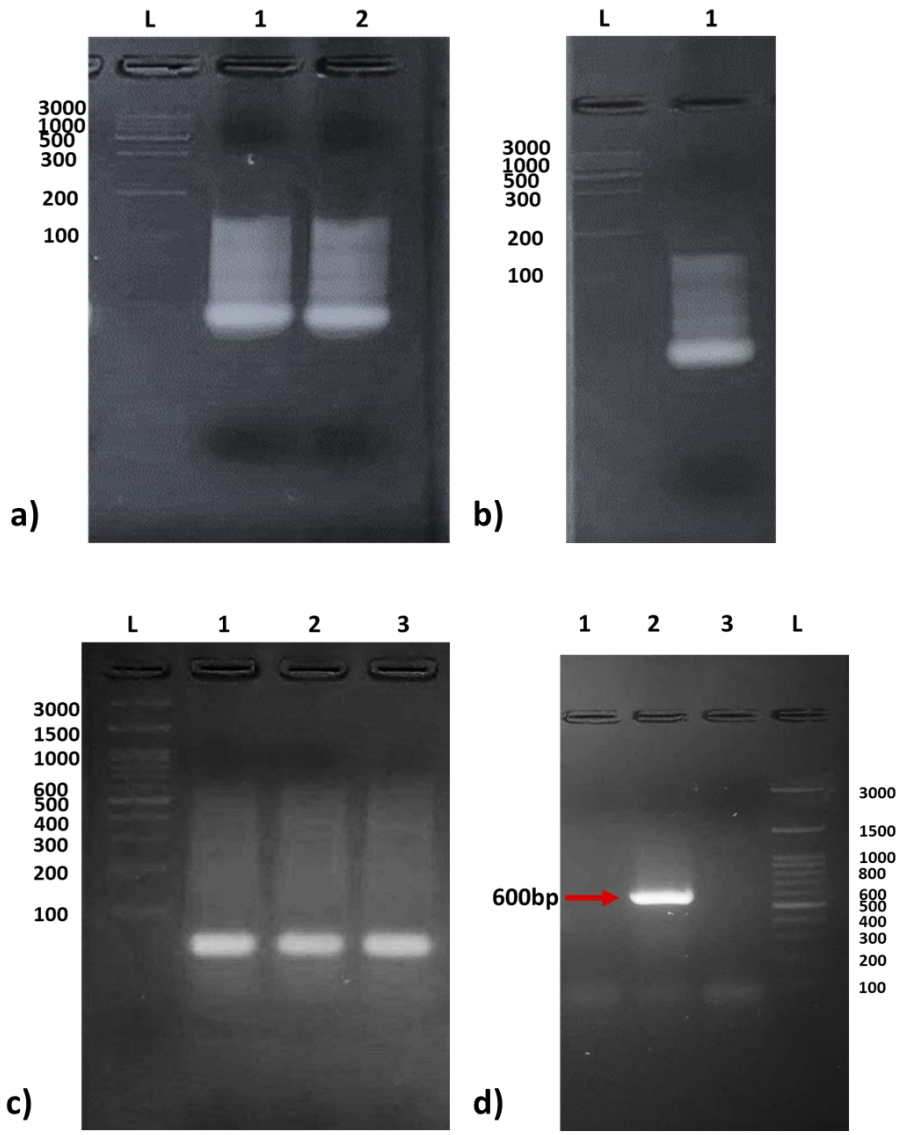


Figure 5.24: 1.5% Agarose gel showing amplified gradient PCR product of PKS gene of Mir2 by gradient PCR a) With primer set VII. Lane L: 100 bp Ladder, and Lane 1-2: Product of primer set VII at 57°C, 61.5°C respectively; b) With primer set VII. Lane L: 100 bp Ladder and Lane 1: Product of primer VII at 65°C; c) With primer set VIII. Lane L: 100 bp Ladder and Lane 1-3: Product of primer set VIII at 57°C, 61.5°C, 65°C respectively; d) With primer set IX. Lane 1-3: Product of primer set IX at 57°C, 61.5°C, 65°C respectively and Lane L: 100 bp Ladder

Further, the scale-up PCR was performed using the same set of primers and PCR conditions (as mentioned in section 4.15) for Mir2. The amplified product of scale-up was subjected to 1.5% (w/v) agarose gel electrophoresis (Figure 5.25). The amplicon (~600 bp) by primer set IX at 61.5°C was sent for sequencing to Biologia Research India Pvt. Ltd., Karnal and was subjected for further analysis.

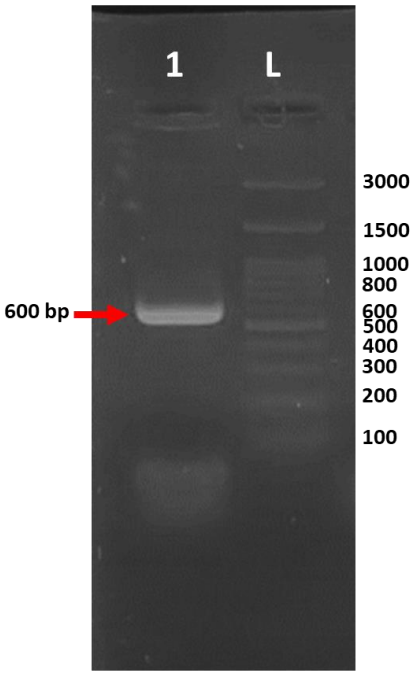


Figure 5.25: 1.5% Agarose gel electrophoresis showing the scale up PCR product of PKS gene for Mir2 with primer set IX. Lane 1: Product of primer set IX at 61.5°C and Lane L: 100 bp ladder

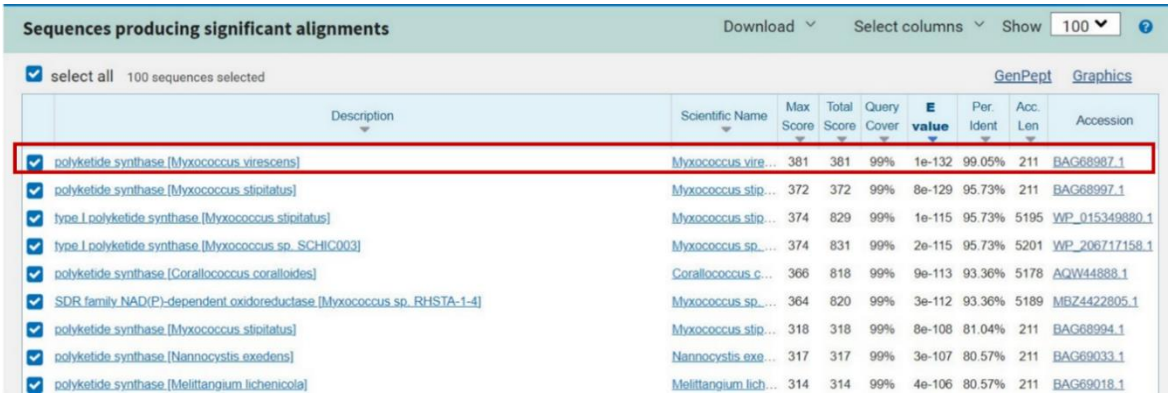
5.13. Analysis of the sequence for PKS amplicon

The sequence of 634 bp was provided by Biologia Research India Pvt. Ltd., Karnal for the PKS gene amplicon (~600 bp). Further, the sequencing result obtained was subjected to analysis through the BLASTx tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) as follow:

>Mir2*PKS*

GTTGCTCGAGGTCGCCTGGGAGGCGCTCGAGGACGCCGCGGTGGACGCGGAGAA
GCTCGCGGGGAGCCGGGGGGGCGTGTTCATCGGCGCCTGCAACGACGACTACCA
CCTCATGCAGCTCGAGCGCCCGGAGTCCGGCGATGCCTTCAGCATCCCGGGCCTG
GCGATGAGTGTGCTGTCGGCACGGCTCTCGTACACCCTCAACTTGCAGGGGCCGA
GCCTCGTGGTGGACACGGCGTGCTCGTCCTCGCTGGTGTCCGTCCACCTGGCCTG
CCAGAGCTTGCGGGCGCGTGAGTGCAACGTGGCGCTCGCCGGTGGCGTCAACCT
CATCCTCTCGCCGCTCTCGCTGGTGCTGGTGTCGAAGCTTCAAGCCCTCGCGCCG
GATGGGCGCTGCAAGGCCTTTGACGCGGCGGCGAATGGCTTCTCGCGCGGCGAG
GGCTGTGGCGTGCTCGTGCTCAAGCGCCTCCCCGACGCCCAGGCGGATGGAGAC
CGCATCCTCGCCCTCATCCGTGGCTCGGCGAGCAATCAGGATGGCAAGTCGACCG
GGTTGACGGCGCCCAACGTCCTCTCGCAGCAGGCGCTCATCCAGCAGGCGTTGGA
GAACGCCCGGCTCCAGCCCGCGCAGGTGACGTAC

Further, BLASTx results showed the 99.05 % similarity of amplified gene with polyketide synthase (*Myxococcus virescens*) with accession number BAG68987 (Figure 5.26).



Sequences producing significant alignments Download Select columns Show 100

select all 100 sequences selected GenPept Graphics

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
polyketide synthase [Myxococcus virescens]	Myxococcus vire...	381	381	99%	1e-132	99.05%	211	BAG68987.1
polyketide synthase [Myxococcus stipitatus]	Myxococcus stip...	372	372	99%	8e-129	95.73%	211	BAG68997.1
type I polyketide synthase [Myxococcus stipitatus]	Myxococcus stip...	374	829	99%	1e-115	95.73%	5195	WP_015349880.1
type I polyketide synthase [Myxococcus sp. SCHIC003]	Myxococcus sp....	374	831	99%	2e-115	95.73%	5201	WP_206717158.1
polyketide synthase [Corallococcus coralloides]	Corallococcus c...	366	818	99%	9e-113	93.36%	5178	AQW44888.1
SDR family NAD(P)-dependent oxidoreductase [Myxococcus sp. RHSTA-1-4]	Myxococcus sp....	364	820	99%	3e-112	93.36%	5189	MBZ4422805.1
polyketide synthase [Myxococcus stipitatus]	Myxococcus stip...	318	318	99%	8e-108	81.04%	211	BAG68994.1
polyketide synthase [Nannocystis exedens]	Nannocystis exe...	317	317	99%	3e-107	80.57%	211	BAG69033.1
polyketide synthase [Melittangium lichenicola]	Melittangium lich...	314	314	99%	4e-106	80.57%	211	BAG69018.1

Figure 5.26: Mir2*PKS* showing 99.05% similarity with polyketide synthase upon BLASTx on NCBI database

5.14. Cloning of protease and PKS gene from Mir2

5.14.1 *Cloning of protease gene*

The cloning of protease gene (Pro) was done by gateway cloning (The Gateway® Technology), using pENTR™ Directional TOPO® Cloning Kit (Invitrogen, Life Technologie, USA). It is a universal cloning method that involves site-specific recombination capability of bacteriophage lambda (Landy, 2015). It offers highly efficient and rapid means of transferring gene of interest into different vector systems. The steps involved in gateway cloning were as following:

1. Vector for cloning
2. Preparation of insert of cloning
3. Ligation and Transformation

5.14.1.1. *Vector*

pENTR-D-TOPO vector was used for cloning present in the kit. The vector includes attL1 and attL2 sites to facilitate site-specific recombination of insert with the vector. The vector pENTR-D-TOPO contains an overhang sequence (GTGG) at 3' end for directional cloning. The vector has a size of 2580 bp (Figure 5.27), which is suitable to clone gene fragment ranging between 1-3 kb (Xu and Li, 2008).

Figure 5.27: Vector map of pENTR-D-TOPO (https://www.genomics-online.com/vector-backbone/44/pentr-d-topo/**)**

5.14.1.2. *Preparation of insert*

Gene fragment (~2 kb) was amplified using the modified set V forward primer containing CACC at 5' end and the set V reverse primer. The sequence CACC was added to facilitate directional cloning.

5.14.1.3. *Ligation and transformation*

Purified insert in vector were ligated in the molar excess ratio 2:1 (https://assets.thermofisher.com/TFS-Assets/LSG/manuals/pentr_dtopo_man.pdf). Ligation was carried out according to the kit protocol, and then the ligated mixture was transformed into chemically competent *E. coli* DH5α cells. The transformed mixture was serially diluted and plated onto an LB agar plate containing Kanamycin (100 mg/mL). Overnight incubation of the plates at 37°C produced white colonies (Figure 5.28a), demonstrating efficient transformation.

5.14.2. *Colony PCR of the transformants*

Colony PCR was carried out with the transformants to confirm the presence of insert into the plasmid/host cell (Xu and Li, 2008), using gene-specific primers. The amplicons (~2 kb) were checked on 1.5% (w/v) agarose gel electrophoresis (Figure 5.28c).

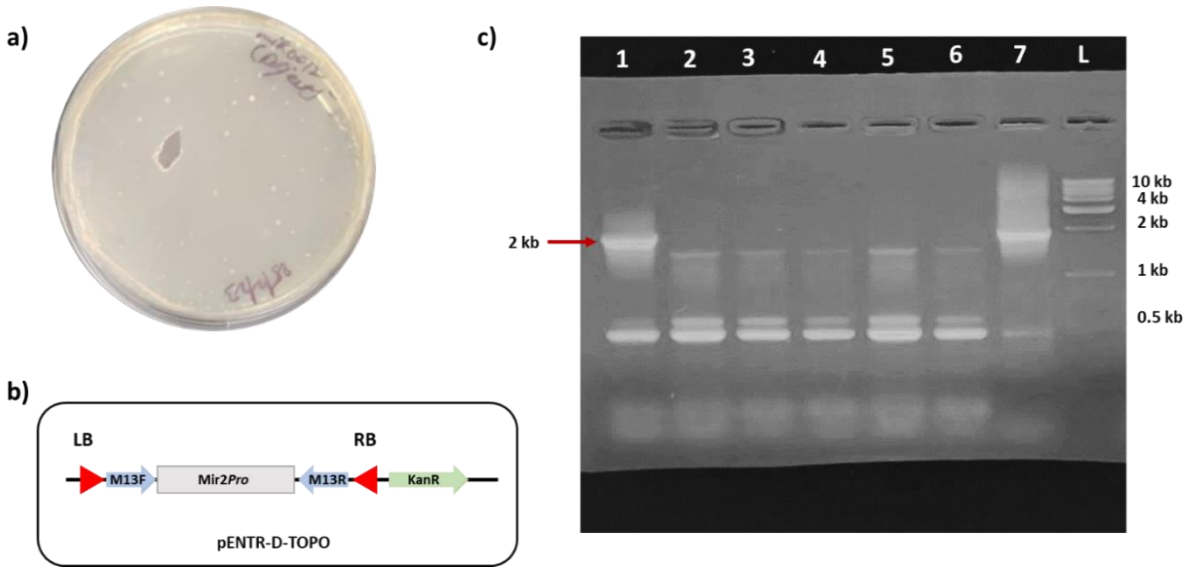


Figure 5.28: Cloning of Mir2Pro gene in pENTR-D-TOPO (entry vector). a) Transformed *E. coli* DH5α containing Mir2*Pro* gene in entry vector; b) Physical map of pENTR-D-TOPO (entry vector); c) 1.5% (w/v) agarose gel of cPCR of Mir2*Pro* gene in entry vector. Lane 1-7: cPCR of randomly selected transformants, Lane L: 1 kb DNA ladder

5.14.3. *Plasmid isolation*

The recombinants showing the presence of insert by colony PCR were further processed for plasmid isolation, as mentioned in the section. 4.17.3. and confirmed by sequencing.

5.14.5. *LR transformation*

The expression of the protease clone was studied by using the pEZY19 expression vector (7934 bp) (Figure 5.29), also known as the destination vector (Invitrogen, Life Technologies, USA). pEZY19 is designed to carry the recombinant (entry clone) for gateway LR reaction to generate an expression clone (showing the expression of the gene) in competent *E. coli* BL21 (DE3) cells (Guo *et al.,* 2008). The transformed mixture was serially diluted and plated onto an LB agar plate containing ampicillin (100 mg/mL). Overnight incubation of the plates at 37°C produced white colonies (Figure 5.30b), demonstrating efficient transformation.

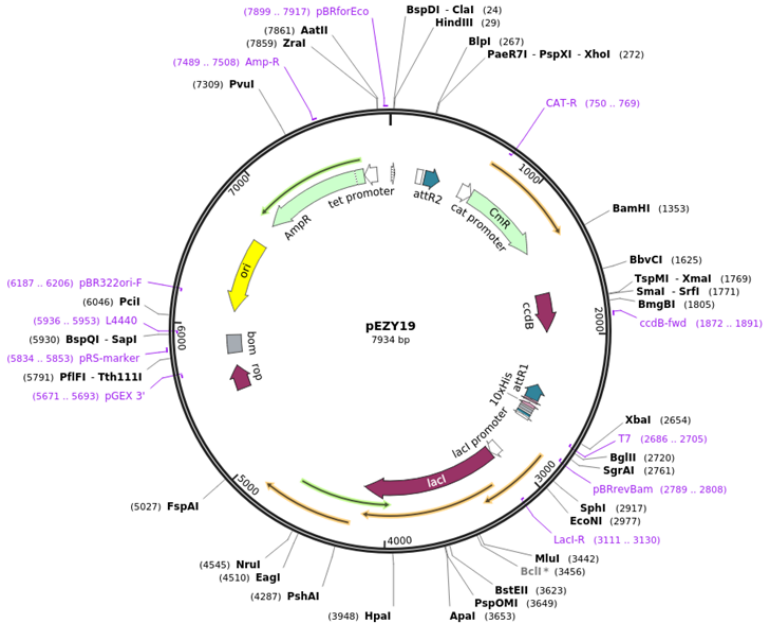


Figure: 5.29: Vector map of pEZY19 (https://www.addgene.org/18668/**)**

5.14.6. *Colony PCR*

Colony PCR was carried out to confirm the presence of gene of interest in the clone (Guo *et al.,* 2008), using gene-specific primers. The amplicons (~2 kb) were checked on 1.5% (w/v) agarose gel electrophoresis (Figure 5.30c).

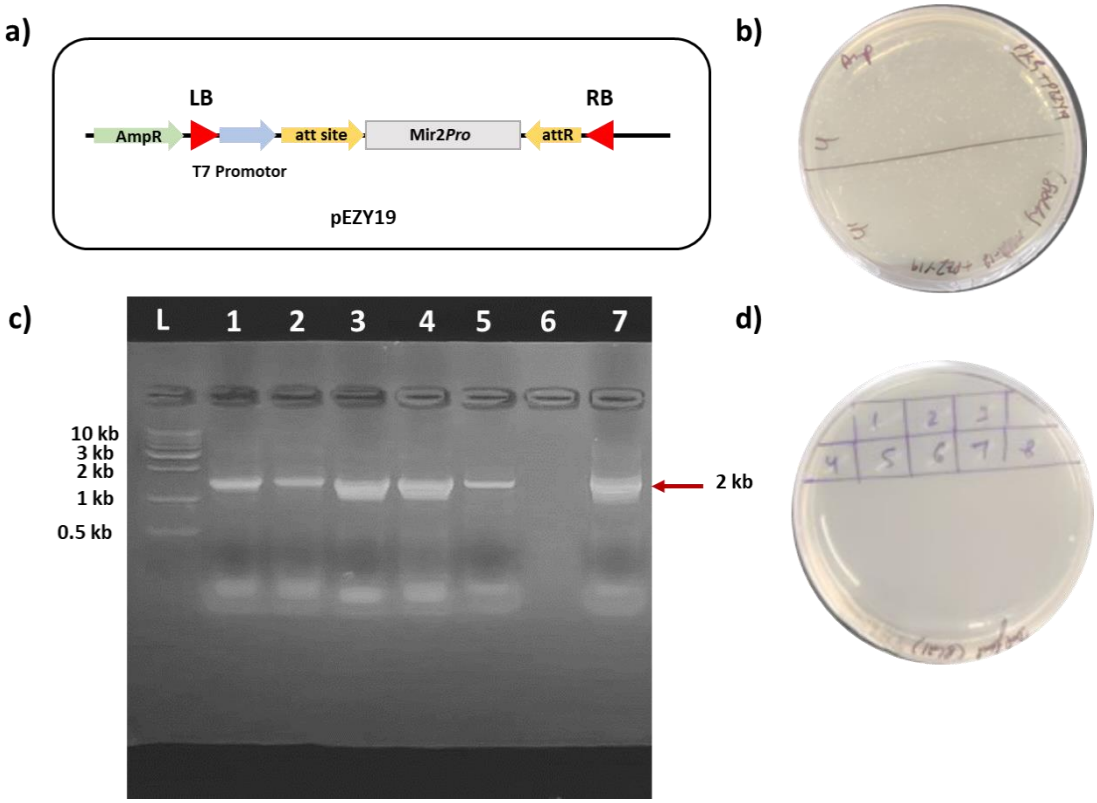


Figure 5.30: Cloning of Mir2*Pro* gene in pEZY19 (expression vector). a) Physical map of pEZY19 (expression vector); b) Transformed *E. coli* DH5α containing Mir2*Pro* gene in expression vector; c) 1.5%

(w/v) agarose gel of cPCR of Mir2*Pro* gene in expression vector. Lane L: 1 kb DNA ladder, Lane 1-7: cPCR of randomly selected transformants; d) Positive colonies of transformed *E. coli* BL21(DE3) containing Mir2*Pro* gene in expression vector

5.14.7. *Cloning of PKS gene*

Cloning of PKS gene (~600 bp) was done in accordance to section 5.14.1. using the modified set IX forward primer containing CACC at 5' end and the set IX reverse primer. The sequence CACC was added to facilitate directional cloning.

5.14.8. *Colony PCR of the transformants*

Colony PCR was carried out with the transformants to confirm the presence of the gene in the clone, using gene-specific primers. The amplicons (~600 bp) were checked on 1.5% (w/v) agarose gel electrophoresis (Figure 5.31c) (Xu and Li, 2008).

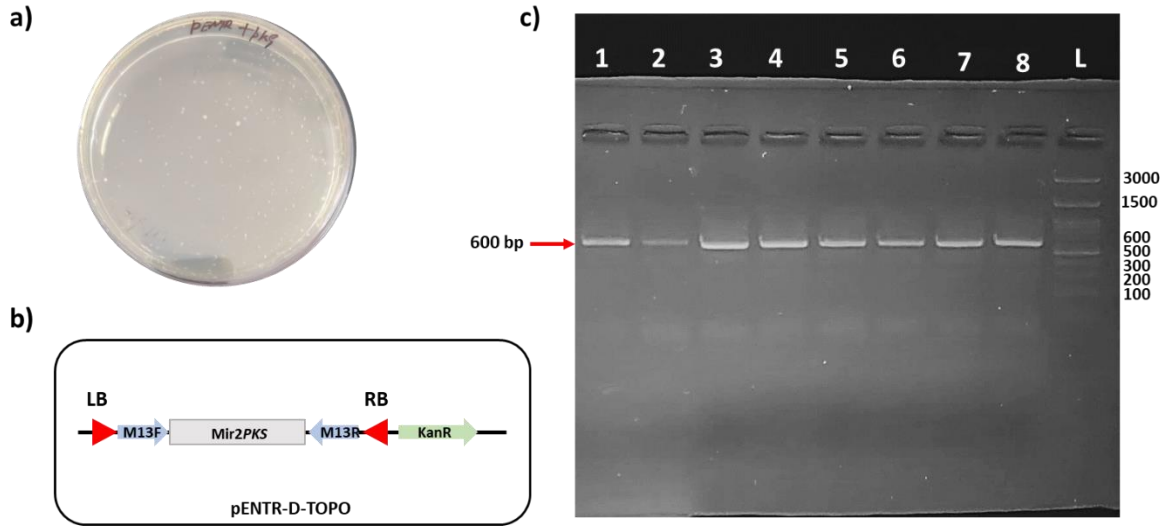


Figure 5.31: Cloning of Mir2PKS gene in pENTR-D-TOPO (entry vector). a) Transformed *E. coli* DH5α containing Mir2PKS gene in entry vector; b) Physical map of pENTR-D-TOPO (entry vector); c) 1.5% (w/v) agarose gel of cPCR of Mir2PKS gene in entry vector. Lane 1-8: cPCR of randomly selected transformants, Lane L: 100 bp DNA ladder

5.14.9. *Plasmid isolation*

The recombinants showing the presence of insert by colony PCR were further processed for plasmid isolation, as mentioned in the section. 4.17.3. and confirmed by sequencing.

5.14.10. *LR transformation*

The expression of the PKS gene was studied by using the pEZY19 expression vector (7934 bp), also known as the destination vector (Invitrogen, Life Technologies, USA). pEZY19 is

designed to carry the recombinant (entry clone) for gateway LR reaction to generate an expression clone (showing the expression of the gene) in competent *E. coli* BL21 (DE3) cells. The transformed mixture was serially diluted and plated onto an LB agar plate containing ampicillin (100 mg/mL). Overnight incubation of the plates at 37°C produced white colonies demonstrating efficient transformation (Figure 5.32b)

5.14.11. *Colony PCR*

Colony PCR was carried out to confirm the presence of the gene of interest in the clone, using gene-specific primers. The amplicons (~600 bp) were checked on 1.5% (w/v) agarose gel electrophoresis (Figure 5.32c).

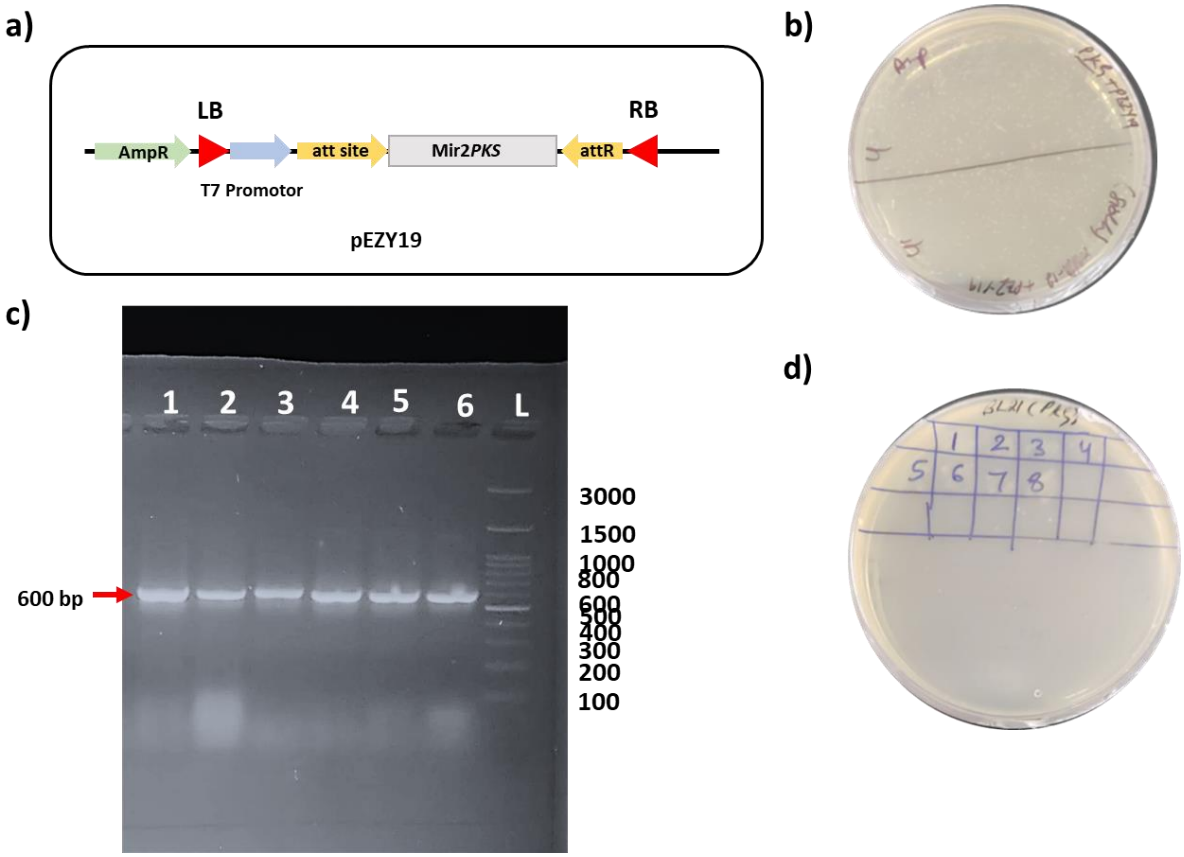


Figure 5.32: Cloning of Mir2*PKS* gene in pEZY19 (expression vector). a) Physical map of pEZY19 (expression vector); b) Transformed *E. coli* DH5α containing Mir2*PKS* gene in expression vector; c) 1.5% (w/v) agarose gel of cPCR of Mir2*PKS* gene in expression vector. Lane 1-6: cPCR of randomly selected transformants, Lane L: 100 bp DNA ladder; d) Positive colonies of transformed *E. coli* BL21(DE3) containing Mir2*PKS* gene in expression vector

5.15. Primary screening of the clone (Mir2*Pro*) for protease enzyme

Primary screening of clone (Mir2*Pro*) protease enzyme was performed to test the efficacy of the enzyme. To perform this, a 1% skimmed milk agar plate was prepared, and the enzyme production capability of the recombinant clone was assessed. The clone harbouring the protease gene was streaked as a single line on the 1% skim milk agar and incubated at 37°C for 12 hours. After the incubation period, a zone of clearance (hydrolysis) developed on both sides of the streaked line, as shown in Figure 5.33. The result of the recombinant protease enzyme in the experiment showed a similarity with the results reported for alkaline protease synthesized by *Bacillus cereus* (Tennalli *et al.,* 2022). Moreover, it also showed the similarity of results for alkaline protease synthesized by *Bacillus mycoides* isolated from Industrial soil (Sitoke *et al.,* 2017). The result of our study also aligns with the result obtained for *Archangium* sp. UTMC 450 (soil myxobacterium) has been reported to produce thermostable alkaline serine protease (Rouhizohrab and Mohammadipanah, 2021).

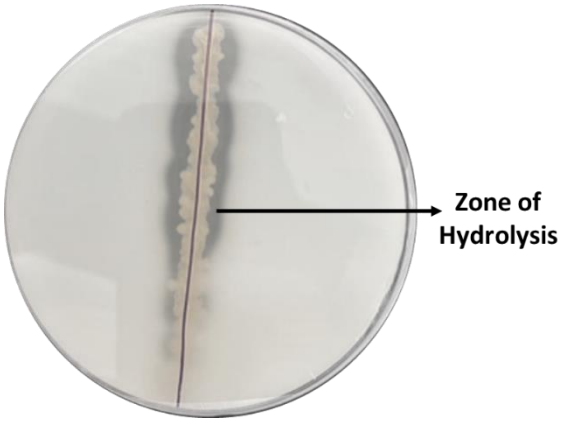


Figure 5.33: Zone of hydrolysis by Mir2*Pro* clone on skimmed milk plate

5.16. Secondary screening of the clone (Mir2*Pro*) for protease enzyme

Secondary screening of recombinant protease enzyme was performed to test the localization of the expressed protein. To perform the secondary screening of the protease activity of the recombinant enzyme, the cells harbouring the protease gene were subjected to cell lysis to generate the cell free extract (CFE). The supernatant and cell free extract were used for protease activity assessment using the standard agar diffusion assay (Figure 5.34). The CFE, supernatant, and proteinase K showed 0.9, 1.1-, and 1.4-cm zones of hydrolysis on the skimmed milk plate, respectively. These results confer that the enzyme is intracellular as well as extracellular, though the diameter of the zone of clearance for supernatant is more prominent, indicating the enzyme to be extracellular. The results obtained were contrasting with those

reported by Sitoke *et al.,* (2017), which reported the alkaline protease enzyme to be intracellular (Sitoke *et al.,* 2017). Moreover, the result of our study aligns with the result obtained for *Pyxidicoccus* sp. S252, which has been reported to produce extracellular alkaline protease (Sharma *et al.,* 2021).

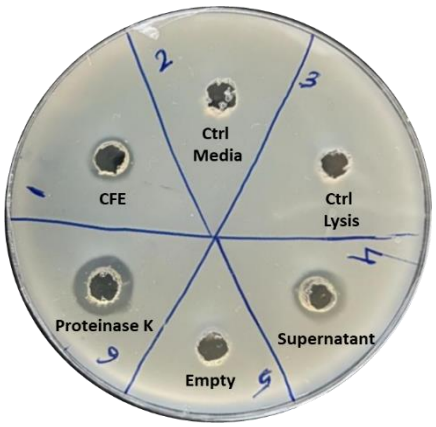


Figure 5.34: Determination of localization of enzyme synthesized by Mir2*Pro* clone

5.17. Protein expression and purification of protease enzyme

At commercial level, protease enzymes are one of the most exploited ones. Characterization and purification of various alkaline proteases have already been reported (Deng *et al.,* 2010; Hammami *et al.,* 2017; Sharma *et al.,* 2021). Still, exploration for new enzymes with unique features that meet industrial demand is a continuous endeavor. Usually, crude protease enzymes are extensively used in the detergent industries. However, the purification of protease is highly important to gain insight into the structural and functional relationship of enzymes during different hydrolytic and synthetic reactions (Song *et al.,* 2023). Expression and purification of protease enzyme from the myxobacterial isolates is a step in this direction. To express the protease enzyme, gene was cloned in pEZY19 vector followed by transformation in the BL21 (DE3) expression strain of *E. coli*. A colony of the clone was inoculated and primary culture was prepared, followed by secondary culture and IPTG based induction of the His-tagged protease enzyme as mentioned in section 4.20. Induced and uninduced bacterial cells were harvested and subjected to lysis followed by SDS-PAGE. Induced cells showed the expression of protease enzyme of molecular weight (~63 kDa) as shown in the Figure 5.35. Uninduced cells did not show the expression of the gene. Induced cells were subjected to lysis through sonication as mentioned in the section 4.20 and affinity chromatography with Ni-NTA Sepharose was performed. Bound beads were checked on a denaturing 10% SDS-PAGE, stained with CBB dye. Stained gel showed the expression of the protease gene in the induced

culture as revealed by a band at the same molecular weight (~63 kDa) (Figure 5.36). Further, eluted protein via varied imidazole concentration is illustrated in Figure 5.37.

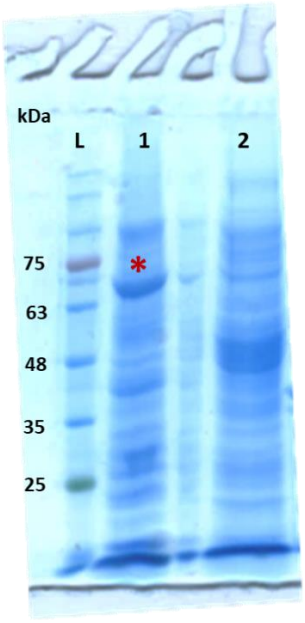


Figure 5.35: SDS PAGE (10%) showing IPTG induced and uninduced protein expression of the protease enzyme. Lane L: Protein marker, Lane 1: IPTG-induced protein expression (50 µg/mL) and Lane 2: Uninduced protein

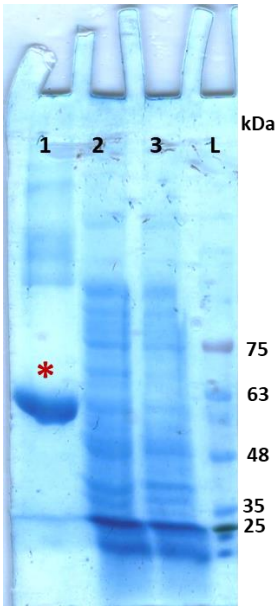


Figure 5.36: SDS PAGE (10%) showing Ni-NTA column bound protein and its remaining flowthrough. Lane 1: Eluted bead binded protein, Lane 2-3: Eluted flowthrough 1 and 2 respectively; Lane L: Protein marker

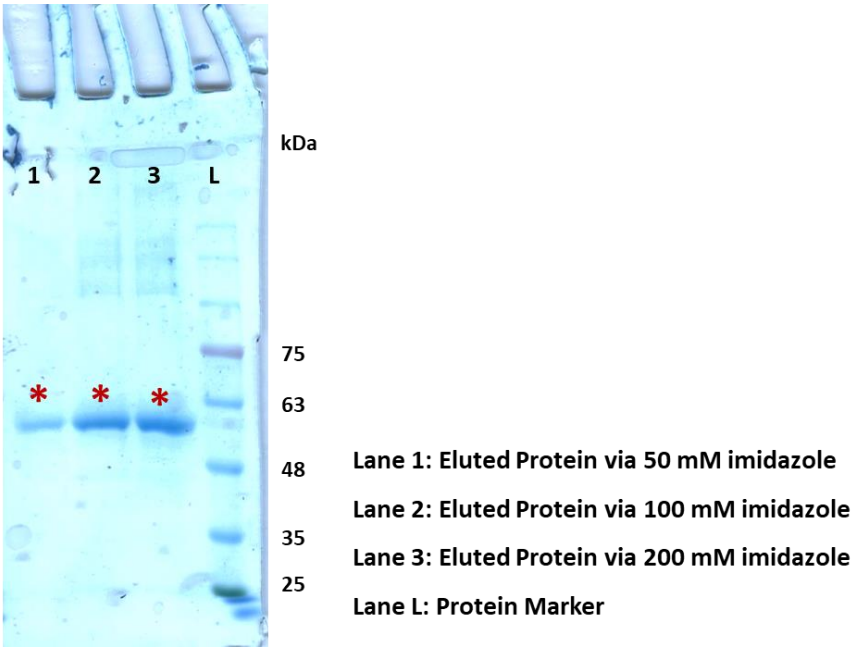


Figure 5.37: SDS PAGE (10%) under reducing conditions for eluting His-tagged protein (Mir2Pro) via varied imidazole concentration

5.18. Biochemical characterization of the protease (Mir2*Pro*)

5.18.1. *Protease assay*

Determination of tyrosine release is the standard procedure for determining the protease activity. The action of protease enzyme on casein acting as substrate, leads to the liberation of amino acid tyrosine along with other peptide fragments and amino acids. Folin's reagent reacts with free tyrosine and produce blue colour chromophore, which can be measure as an absorbance by spectrophotometer at 660nm. The more liberation of tyrosine from casein, the higher the intensity of chromophores and stronger the activity of protease. The standard curve generated serve as the reference and help in ascertaining the amount of tyrosine release by the protease enzyme. This quantification is expressed in terms of enzymatic Units, representing the micromoles of tyrosine equivalents liberated from casein per minute, thereby providing a quantitative assessment of protease activity (Cupp-Enyard, 2008). Figure 5.38 represents the tyrosine standard curve.

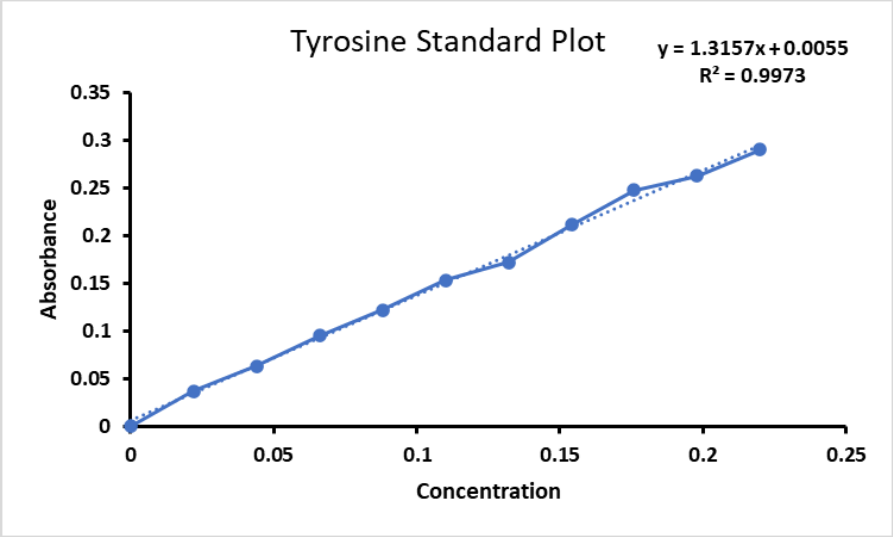


Figure 5.38: Scatter plot of tyrosine standard curve

5.18.2. *Standard curve of BSA*

The bradford protein assay is extensively used method for quantification of total protein concentration in solutions. This assay is based on the principle of absorbance variation, which take place due to the proportional binding of proteins to Coomassie Blue G250 dye. The ionic and hydrophobic interaction of the protein stabilize the anionic form of the dye, leading to visible change in colour, which is measured by spectrophotometer at 595 nm. The linear range of this assay is within range of 5-25 µg/mL. Thus, high amount of protein, more is the binding of the dye (Figure 5.39.). This approach allows the determination of protein concentration of unknown sample by comparing its absorbance to series of the protein's standard with known concentration (Ernst and Zor, 2010).

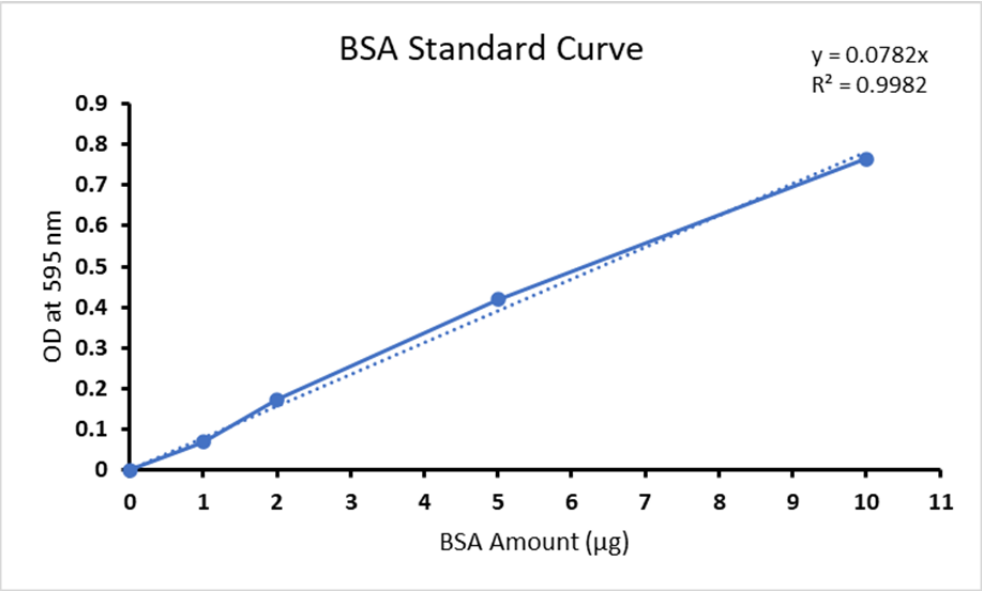


Figure 5.39: Scatter plot of BSA standard curve

5.18.3. *Effect of temperature on protease enzyme activity*

The optimum temperature for protease enzyme (Mir2*Pro*) was 40°C, exhibiting a maximum activity (i.e., 3.656 µM Tyrosine per mL enzyme per hour) at this temperature. However, there was decrease in the enzyme activity after 40°C (Figure 5.40). Gerth and Miller (2005) reported about the significant morphogenesis of myxobacteria occurring in the 42–48°C temperature range (Gerth and Müller, 2005). Study conducted by Iizuka *et al.,* (2006) reported about the myxobacteria isolation from hot springs having the temperature range of 45-49°C (Iizuka *et al.,* 2006). Mohr et al (2018) also reported about the isolation of *Nannocystis konarekensis* sp. from the desert soil of Iran, which confirmed about their optimum growth at 30°C (Mohr *et al.,* 2018a). These studies aligned with our findings, hence providing the evidence about the mesophilic nature of myxobacteria.

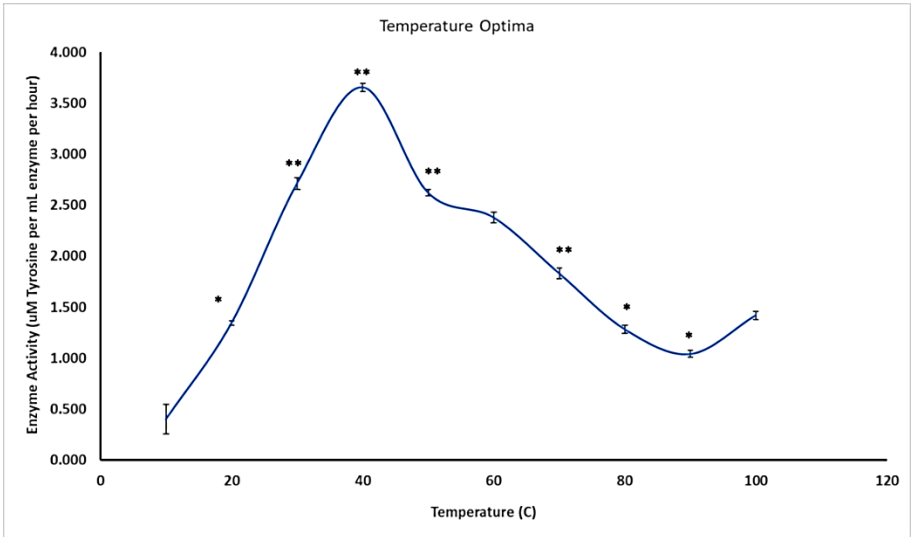


Figure 5.40: Graph depicting optimum temperature of Mir2*Pro* clone. '*' represent p<0.05 and '' represent p<0.01**

5.18.4. *Effect of pH on protease enzyme activity*

The purified enzyme (Mir2*Pro*) showed maximum activity at pH 7.0 (i.e., 4.031 µM Tyrosine per mL enzyme per hour) (Figure 5.41). This result aligned with the isolation of *Archangium, Myxococcus* and *Sorangium* sp. from the alkaline pH bogs (Hook, 1977). Additionally, previously published literature has also provided evidence about the inhabitation of myxobacteria within the range of 6.5-8.5 i.e., within the neutral and slightly alkaline pH range (Mohr, 2018).

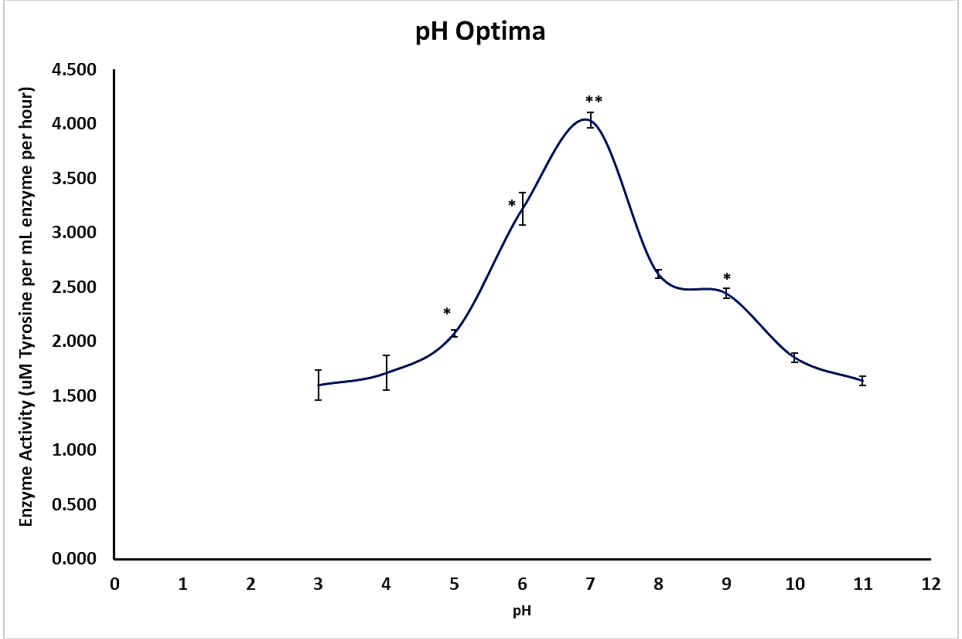


Figure 5.41: Graph depicting optimum pH of Mir2*Pro* clone. '*' represent p<0.05 and '' represent p<0.01**

5.18.5. *Effect of metal ions on protease enzyme activity*

Metal ions work as cofactors for enzymatic activities. The effect of different divalent cations on the protease enzyme (Mir2*Pro*) activity was evaluated and results obtained at 2 mM and 5 mM metal ion concentrations were compared. The most effective ions were determined to be Mg^{2+} at 5 mM followed by Ca^{2+}, Fe^{2+}, Cu^{2+}, Mn^{2+} at 2 mM concentration compared to the metal ion control. 5mM Mg^{2+} increased the enzyme activity to 247% and 2mM Ca^{2+} increased the activity to 193%. However, Cu^{2+}, Co^{2+} and Zn^{2+} showed the reduction in enzyme activity at 5mM concentration to 12.5, 16.5 and 3.08% respectively (Table 5.3 and Figure 5.42). These results were found to corroborate with the result obtained by Sharma *et al.,* (2021), in which they found the positive impact of Mg^{2+} and Ca^{2+} ions on the protease enzyme activity obtained from myxobacteria (Sharma *et al.,* 2021). Furthermore, numerous proteases obtained from *Bacillus spp.,* have also been reported to stimulate alkaline protease activity on the addition of Mg^{2+} and Ca^{2+} ions (Deng *et al.,* 2010; Annamalai *et al.,* 2014; Yang *et al.,* 2020).

Table 5.3: Effect of different divalent metal ions on protease enzyme activity

2 mM	Mean Activity (U/mL)	Percentage Relative Activity (%)	5 mM	Mean Activity (U/mL)	Percentage Relative Activity (%)
Control	4.03 $\pm$ 0.12		Control	4.03 $\pm$ 0.12	

Ca^{2+}	7.77 ± 0.61	193	Ca^{2+}	7.60 ± 0.11	189
Co^{2+}	1.42 ± 0.15	35.1	Co^{2+}	0.67 ± 0.14	16.5
Cu^{2+}	6.59 ± 0.79	164	Cu^{2+}	0.50 ± 0.11	12.5
Fe^{2+}	7.09 ± 0.35	176	Fe^{2+}	6.22 ± 0.07	154
Mg^{2+}	9.84 ± 0.09	244	Mg^{2+}	9.95 ± 0.03	247
Mn^{2+}	6.31 ± 0.12	157	Mn^{2+}	3.72 ± 0.13	92.2
Zn^{2+}	0.87 ± 0.05	21.6	Zn^{2+}	0.12 ± 0.01	3.08

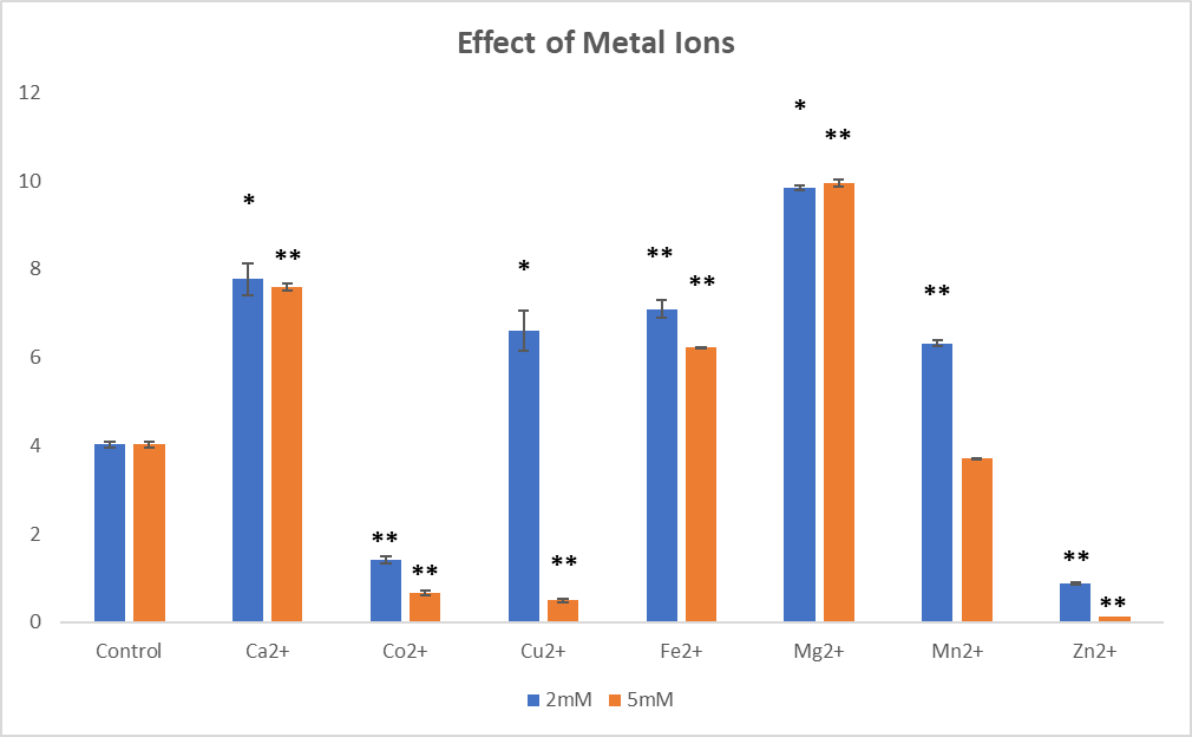


Figure 5.42: Bar graph depicting effect of metal ions on Mir2*Pro* clone. '*' represent p<0.05 and '' represent p<0.01**

5.18.6. *Effect of detergents on protease enzyme activity*

The effect of different detergents on protease enzyme (Mir2*Pro*) activity was also assessed compared to the detergent-free control. The presence of non-ionic detergent i.e., Tween-20 (2% and 5%) and Triton X-100 (2% and 5%) showed an increase in the activity of the enzyme. 5% Triton X 100 increased the activity to 537.9% and 5% Tween 20 increased the activity to 234%. Whereas 2% Triton X 100 increased the activity to 201% and 2% Tween 20 increased the activity to 146% as compared to the control. However, the reduced enzyme activity was accorded for 2% and 5% SDS i.e., 13% and 25% respectively (Table 5.4 and Figure 5.43). These results were found to align with those reported by Sharma *et al.,* (2021). The same study

also reported about the stability of protease enzyme obtained from myxobacteria isolate in the presence of SDS, Triton X-100 and Tween 20 (Sharma *et al.,* 2021).

Table 5.4: Effect of different detergents on protease enzyme activity

Detergents 2%	Mean Activity (U/mL)	Percentage Relative Activity (%)	Detergents 5%	Mean Activity (U/mL)	Percentage Relative Activity (%)
Control	4.031 ± 0.12		Control	4.031 ± 0.12	
SDS	0.524 ± 0.11	13	SDS	1.011 ± 0.21	25
Tween 20	5.906 ± 0.08	146.5	Tween 20	9.453 ± 0.57	234.5
Triton X 100	8.12 ± 0.18	201.4	Triton X 100	21.684 ± 0.18	537.9

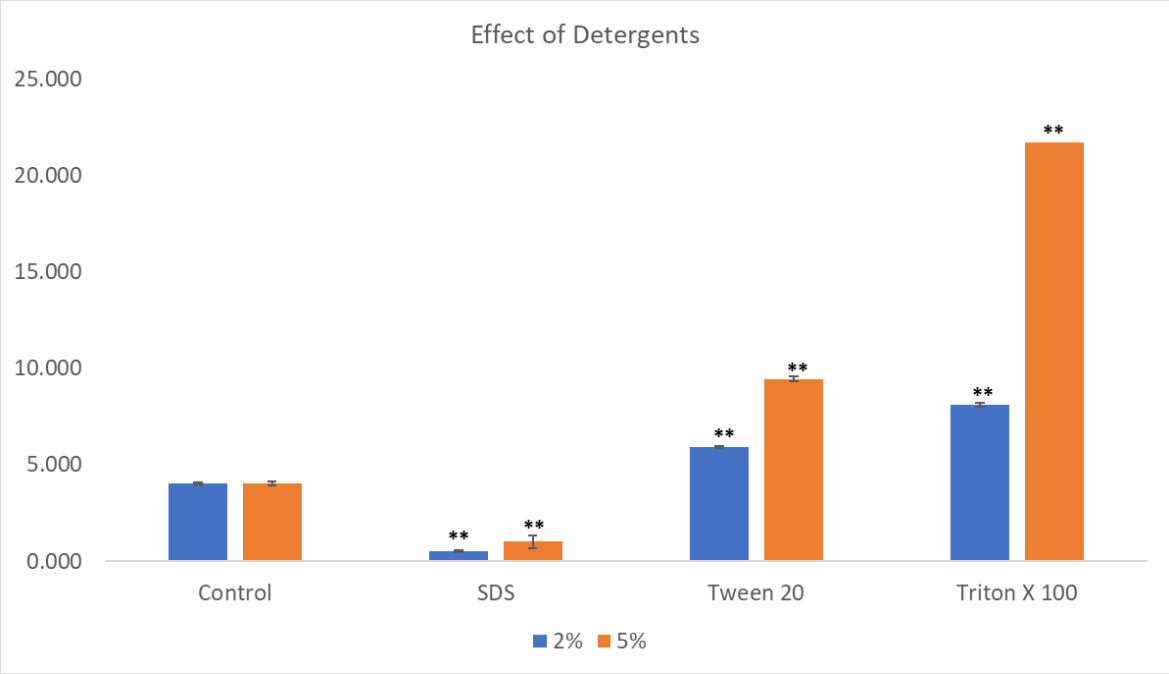


Figure 5.43: Bar graph depicting effect of detergent on Mir2*Pro* clone. '*' represent p<0.05 and '' represent p<0.01**

5.19. Analysis of antibacterial activity of clone containing the PKS gene

To assess the antibacterial activity of the clone Mir2*PKS*, the cells were harvested and subjected to lysis as mentioned in section 4.9.2. Supernatant and cell-free extract were used as extracellular and intracellular source for the zone of inhibition study. The antibacterial activity was performed against the *Bacillus cereus* MTCC 8714, *Escherichia coli* MTCC 1679 and

Pseudomonas aeruginosa MTCC 2582 through the agar diffusion method. As per the observation, both CFE and supernatant could not generate any zone of inhibition for the above-mentioned bacterial strains (Figure 5.44). This is the first report about the assessment of the cloned PKS gene for antibacterial activity via agar diffusion method.

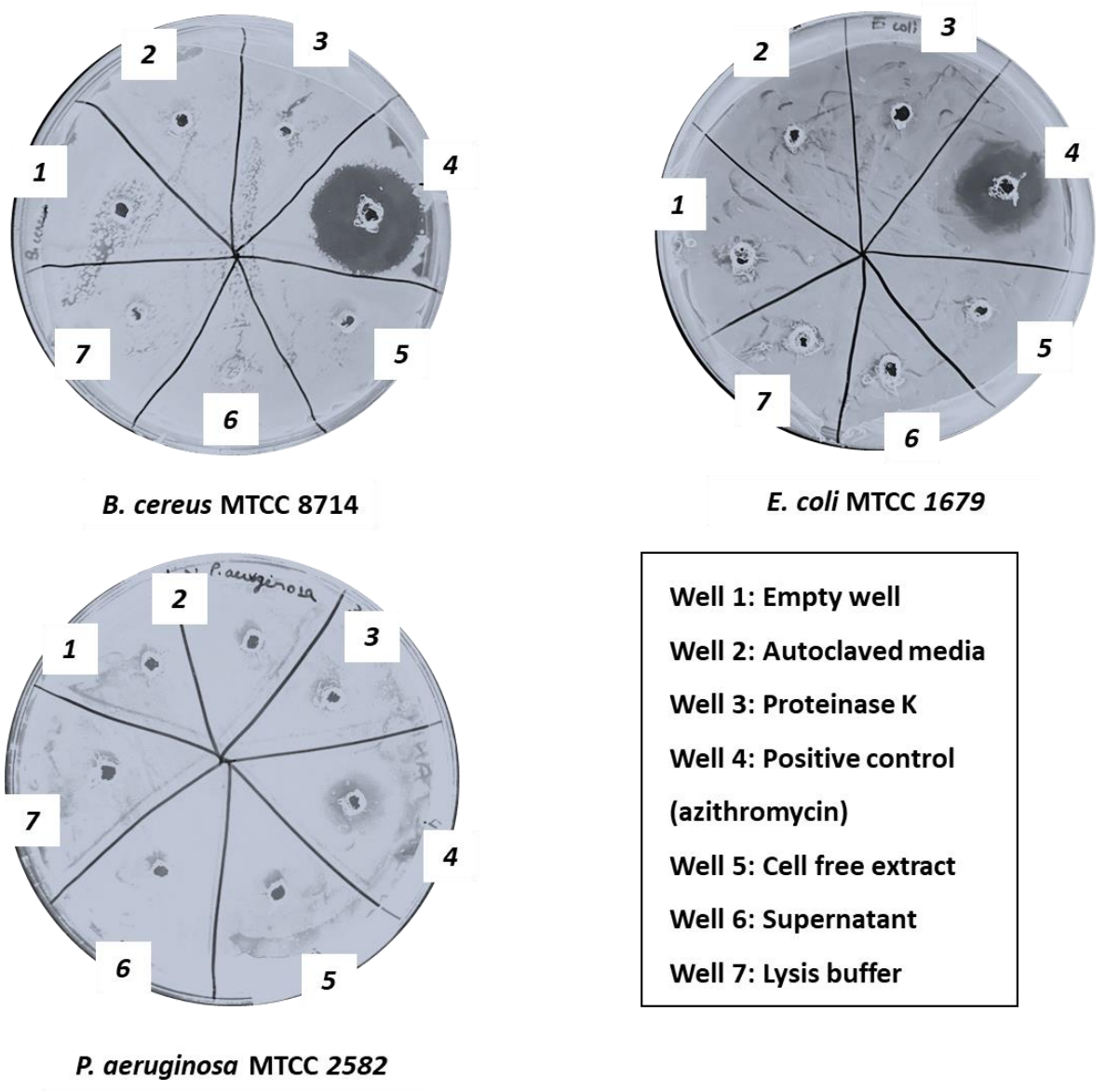


Figure 5.44: Antibacterial assessment of Mir2*PKS clone* against *Bacillus cereus* MTCC 8714, *Escherichia coli* MTCC 1679 and *Pseudomonas aeruginosa* MTCC 2582

5.20. Induction and molecular weight determination of the PKS

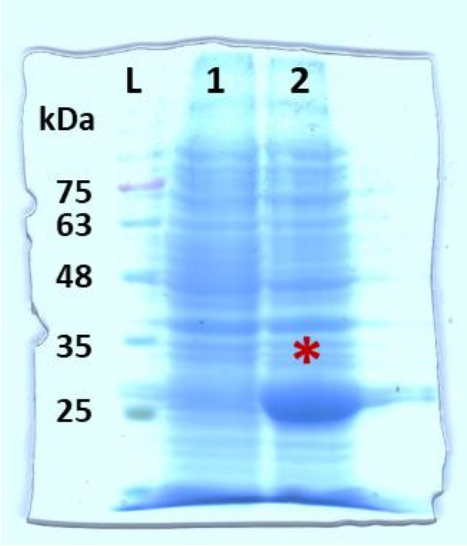


Figure 5.45: SDS PAGE (10%) showing IPTG induced and uninduced protein expression of the PKS enzyme. Lane L: Protein marker, Lane 1: Uninduced protein and Lane 2: IPTG-induced protein expression (50 µg/mL)

Polyketides represent an extremely diverse and significant group of natural products. Moreover, polyketides have diverse applications and functions like antibiotics, antioxidants, antitumor agents, immunosuppressants and pigments (Paterson and Lam, 2018). Biosynthesis of polyketides needs the systematic operation of specific enzymes named as polyketide synthases (PKS). In general, PKSs are group of large enzymes that are classified into three categories i.e., type I, II and III. In which, PKS Type I are multi-domain large enzymes that function in modular or iterative manner. Typically, the PKS Type I minimally encompasses three domains i.e., an acyltransferase (AT), an acyl carrier protein (ACP) and keto synthase (KS). These three domains selectively stimulate and catalyzes the decarboxylative claisen condensation reaction between the extender unit and the growing polyketide chain (Staunton and Weissman 2001). This objective is focused on generating the recombinant PKS enzyme in bulk that can be utilized in multiple industries.

To express the PKS gene, gene was cloned in pEZY19 vector followed by transformation in the BL21 (DE3) expression strain of *E. coli*. A colony of the clone was inoculated and primary culture was prepared, followed by secondary culture and IPTG induction of the His-tagged PKS gene as mentioned in the section 4.20. Induced and uninduced bacterial cells were harvested and subjected to lysis followed by SDS-PAGE. Induced cells showed the expression of PKS gene of molecular weight (~25 kDa) as shown in the Figure 5.45. Uninduced cells did not show the expression of the gene.

5.21. Partial purification of antimicrobial compounds from Mir2 and clone (Mir2*PKS*)

Mir2 was grown in casitone broth containing cycloheximide (25 mg/mL) for 5-7 days. Whereas, clone Mir2*PKS* was grown in LB broth containing ampicillin (100 µg/mL) for 24 hours at 37°C. These cultures were further subjected to organic extraction method. Different solvent systems were used for the extraction of the compound but the best results were obtained by chloroform: methanol (9:1) ratio. The extract of both Mir2, clone Mir2*PKS* and antimicrobial compounds were subjected for partial purification on TLC plates and visualized for discrete band (Figure 5.46). From the results, it was inferred that solvent system chloroform: methanol (9:1) ratio showed the presence of band of compound extracted from Mir2 close to R1 i.e. Azithromycin (reference macrocyclic drug), which indicate the presence of macrocyclic drug. Whereas, no compound was observed for the clone Mir2*PKS*.

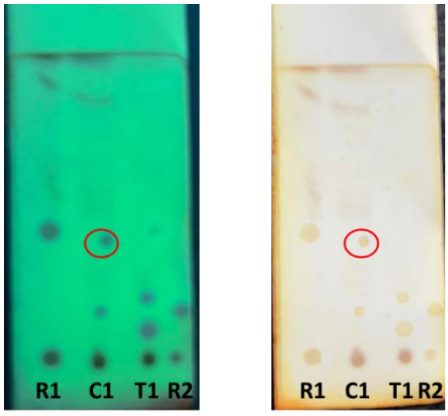


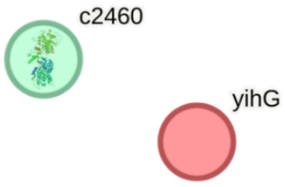
Figure 5.46: TLC plate of antimicrobial compound visualized at short wavelength (254 nm) and iodine exposure, where R1: Reference drug azithromycin; C1: Crude extract of Mir2 (control), T1: Extract of clone Mir2*PKS* and R2: Reference drug vancomycin.

5.22. Computational analysis of the cloned PKS gene sequence for the lack of antimicrobial activity.

To identify the probable cause for no such activity, the sequence study of clone Mir2*PKS* was performed. As mentioned in the section 3.20. PKS work in a protein complex in which acyltransferase and acyl carrier protein are key players for the synthesis of antimicrobial compounds. In silico protein-protein interaction was performed for the PKST1 from *Myxococcus* sp. and acyltransferase from *E. coli* through STRING software. The amino acid sequence for both proteins was submitted in the STRING database, and interaction was searched in the *E. coli* since the recombinant protein was thrived in the *E. coli* expression strain. STRING showed no possible interaction among the submitted proteins as shown in Figure

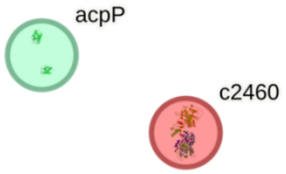
5.47, suggesting the possible reason for no synthesis of antimicrobial compounds in *E. coli* harbouring the recombinant *PKS*.

a)



S.No.	Source of Input Sequence	Protein ID	Protein Name	Sequence Match with Input Sequence
1.	*Myxococcus xanthus*	*c2460*	Putative Polyketide Synthase	E. coli sequence match 33.3%
2.	*E. coli*	*yihG*	Inner Membrane Acyl transferase	E. coli sequence match 98.4%

b)



S.No.	Source of Input Sequence	Protein ID	Protein Name	Sequence Match with Input Sequence
1.	*Myxococcus xanthus*	*c2460*	Putative Polyketide Synthase	E. coli sequence match 33.3%
2.	*E. coli*	*acpP*	Inner Membrane Acyl transferase	E. coli sequence match 100%

Figure 5.47: Protein-protein interaction of *Myxococcus* PKS with *E. coli* acyl carrier protein (yihG and acpP)

Though synthesis of polyketide molecules is a conserved mechanism in the microbes, but the presence of sequence variation might be the reason for incomplete complex formation. To test this hypothesis, the amino acid sequences for all the 3 proteins (PKS, ACP and AT) from *Myxococccus* sp. and *E. coli* were subjected to an alignment study by Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/). As shown in Figure 5.48-5.50 and Annexure 2. All these three proteins from *Myxococcus* sp. are completely different from *E. coli*. This clearly suggests that cloning a single gene of PKS for recombinant protein synthesis is not enough for the generation of antimicrobial compounds in the *E. coli* expression strain.

```
ABF89709.1_polyketide_synthase_type_I_Myxococcus_xanthus_DK_1622]  VVQPVLFAMQVALAALWRSWGIAPDAVVGHSMGEVAAAHVAGALSLEDAARIICLRSQLL  1231
AZH85684.1_polyketide_synthase_Escherichia_coli                   ----------------------------------------DGD--------YRCTDGRYV  995
                                                                                                          *          *  .: :

ABF89709.1_polyketide_synthase_type_I_Myxococcus_xanthus_DK_1622]  RRKSGQGAMAVVELGLAQAREALAGYESRLSVAVS-----NSARSTVLSGDIDALESLLP  1286
AZH85684.1_polyketide_synthase_Escherichia_coli                   RARPIEH------QQRESLLTELAGYCEGFQAIPDTIARAGDRLYEMMSGAEEPVAIIFP  1049
                                                                  * :  :           .    **** . :..  .     ..    ::**  : :  ::*

ABF89709.1_polyketide_synthase_type_I_Myxococcus_xanthus_DK_1622]  KLESQGVFCRRVKVNVASHSPQMDSLKDDLLRVLDGVAPQGAPVPIYSTVTGQTSDGADF  1346
AZH85684.1_polyketide_synthase_Escherichia_coli                   QSASDGVEVLYQEFSF---GRYFNQIAAGVLRGIVQTRQPRQPLRILEVGGGT--GG---  1101
                                                                  :  *:**     :...   .  ::.:  .:** :  .     *: * ..  *   .*

ABF89709.1_polyketide_synthase_type_I_Myxococcus_xanthus_DK_1622]  HPAYWVSNLREPVLFHGAVEQLLADGFTVLVEVSPHPVLLAPIEETLRESKQGAIALASA  1406
AZH85684.1_polyketide_synthase_Escherichia_coli                   -TTAWLLPE-----------------------L---------------------------  1110
                                                                    : *:                          :

ABF89709.1_polyketide_synthase_type_I_Myxococcus_xanthus_DK_1622]  RRQMPERRCLLESLAALYAWGCAVDWKALHPVKGRVVALPRYPWQRERFWLPDEAEVDAQ  1466
AZH85684.1_polyketide_synthase_Escherichia_coli                   -NGVPALEYHFTDISALFTRRAQQKFA------------DYDFVKY---SELDLEKEAQ  1153
                                                                   . :*  .  : .::**::  .  .:               * : :       : * :**

ABF89709.1_polyketide_synthase_type_I_Myxococcus_xanthus_DK_1622]  PEGV-------VLADRK--GHPLIGG---SLSSSVQPGTHFWERTVSTAAFPYLADHCVW  1514
AZH85684.1_polyketide_synthase_Escherichia_coli                   SQGFQAQSYDLIVAANVIHATRHIGRTLDNLRPLLKPGGRLLMREITQ---PMRLFDFVF  1210
                                                                   :*.       ::* .   .   **    .*   ::** ::  * ::    *    . *:

ABF89709.1_polyketide_synthase_type_I_Myxococcus_xanthus_DK_1622]  GDVVFPGAGYV--------------EMALSAGAE---VLGETGLVLEDVSFSEMLALEPG  1557
AZH85684.1_polyketide_synthase_Escherichia_coli                   GPLVLPLQDLDAREGELFLTTAQWQQQCRHAGFSKVAWLPQDGSPTAGMSEHIILATLPG  1270
                                                                  * :*:*  .                : .  ** .    * : *    .:*   :**  **

ABF89709.1_polyketide_synthase_type_I_Myxococcus_xanthus_DK_1622]  QSRRVQVVLTEEEPGRATFQIASRAEGEMSWRKHAAGTVRRDARAAAVVESPEALRARVT  1617
AZH85684.1_polyketide_synthase_Escherichia_coli                   QAVSAVTFTAPSEPVLG-QALTDNGDYLADWSDCAGQ-------------PERFNA---  1312
                                                                  *:  . .. : .**  .   ::...:   .* . *.               ** :.*

ABF89709.1_polyketide_synthase_type_I_Myxococcus_xanthus_DK_1622]  VAVSAEAHYQRRQAQGLMYGPTFQGLRGIWRSEQEALGRLEVSDTVALEAGAYRLHPALL  1677
AZH85684.1_polyketide_synthase_Escherichia_coli                   ---------------------R---WQEAWRLLSQRHGD----------------------  1327
                                                                                        :  **  .:  *

ABF89709.1_polyketide_synthase_type_I_Myxococcus_xanthus_DK_1622]  DAGLQVAVELLAPLTATSAPATHVPVGIGRIRFFQRPGRAAWARVKVRGEGAASERERTF  1737
AZH85684.1_polyketide_synthase_Escherichia_coli                   --AL-----------PVEPPPVAAPEWLGKVRL-------SWQNEA--------------  1353
                                                                    .*           .. * . .*  :*::*:       :* .

ABF89709.1_polyketide_synthase_type_I_Myxococcus_xanthus_DK_1622]  DFWLLDEQGQVLLELEALRLFRLDAGASARKELGAWLYQVDWEERPLPAE-LAWPEQTPG  1796
AZH85684.1_polyketide_synthase_Escherichia_coli                   -------------------FSRGQMRVEARHPAGEWLPLSPAAPLPAPQTHYQW------  1388
                                                                                    : * :  ..**:  * **        * *     *

ABF89709.1_polyketide_synthase_type_I_Myxococcus_xanthus_DK_1622]  SWLILQDG---GGVGQRLSAQLLTRGETCVLVAPREAYRLTGPRSAEVDPRNPEHWRRLL  1853
AZH85684.1_polyketide_synthase_Escherichia_coli                   RWTPLNVASIDHPLTFSFSAGTLARSDEL------AQYGII------HDPH---ASSRL-  1432
                                                                   *  *: .     :   :**  *:*.:          * :        **:      **

ABF89709.1_polyketide_synthase_type_I_Myxococcus_xanthus_DK_1622]  TDLLGAGVPPCRGVVHLWSLDLASTEALTPQALEDSRRLGTTSVLHL------VQALSGA  1907
AZH85684.1_polyketide_synthase_Escherichia_coli                   ---------------------------MIVEESEDTLALAEKVIAALTASAAGLIVVTRR  1465
                                                                                            :  :  **:  *. . :  *      : .::

ABF89709.1_polyketide_synthase_type_I_Myxococcus_xanthus_DK_1622]  GWRDAPRLWLATRGARSAGKVAERVAVAQAPLLGLGQVLAVEQPELRCTRVDLEGGADVA  1967
AZH85684.1_polyketide_synthase_Escherichia_coli                   AWRVEEN---------------EALSASHHALWALLRVAANEQPERLLAAIDLAENT--P  1508
                                                                  .**   .              * ::.::  * .* :* * ****   : :**  .:

ABF89709.1_polyketide_synthase_type_I_Myxococcus_xanthus_DK_1622]  ADALLRELSSTSFEDQTAWR---GGTRRVARLARAADALSAREPATLLRE-DGTYLITGG  2023
AZH85684.1_polyketide_synthase_Escherichia_coli                   WETLHQGLSAVSLS--QRWLAARGDTLWLPSLTPN-TGCAAELPANVFTGDSRWHLVTGA  1565
                                                                   ::* : **:.*:.    *    *.*  :  *:    . :*. **.::   .  :*:**.

ABF89709.1_polyketide_synthase_type_I_Myxococcus_xanthus_DK_1622]  LGGLGLELARWLVSQGARHLLLMGRRAPSAEAEQALAALREAGARVG-SFQGDVARLEDV  2082
AZH85684.1_polyketide_synthase_Escherichia_coli                   FGGLGRLAVNWLREKGARRIALLAPRVDESWLRD----VEGGQTRVCRCDVGDAGQLATV  1621
                                                                  :****   ..** .:***:: *:. *. .:  .:    :. . :**  .  **..:*  *

ABF89709.1_polyketide_synthase_type_I_Myxococcus_xanthus_DK_1622]  TGALARVEDAMPPLRGVFHAAGLLEDGLLLNLTEARFASVSAPKVLGSWNLHAQTRHLPL  2142
AZH85684.1_polyketide_synthase_Escherichia_coli                   LDDL----AANGGIAGAIHAAGVLADAPLQELDDHQLAAVFAVKAQAASQLLQTLRNHDG  1677
                                                                   . *     *   : *.:****:* *. * :* : ::*:* * *. .: :*    *:

ABF89709.1_polyketide_synthase_type_I_Myxococcus_xanthus_DK_1622]  EHFVLFSSVAASLGTPGQSNYASANAFMDALAQARRA-EGLPALSINWGTWTQVGLAAAQ  2201
AZH85684.1_polyketide_synthase_Escherichia_coli                   RYLILYSSAAATLGAPGQSAHALACGYLDGLAQQFSTLDAPKTLSVAWGAWGESGRAATP  1737
                                                                  .:::*:**.**:**:**** :* * .::*.***   : :.  :**: **:* : * **:

ABF89709.1_polyketide_synthase_type_I_Myxococcus_xanthus_DK_1622]  SIRGERLEARGLGGMAPDKALAVLGMLLGQERPQ-LSVMAFEPRQWLGFYLAAAQSPYFT  2260
AZH85684.1_polyketide_synthase_Escherichia_coli                   EMLA-TLASRGMGALSDAEGCWHLEQAVMRGAPWRLAMRVFTD------KMPPLQQALFN  1790
                                                                  .: .  * :**:*.::  :.   *   : :  *  *:: .*        :   *.  *.

ABF89709.1_polyketide_synthase_type_I_Myxococcus_xanthus_DK_1622]  KLAQEPSSRPSVSAGKSRIREQLEAARASERRGLLDAHLRELIG-GVLRMAPARIEPRTP  2319
AZH85684.1_polyketide_synthase_Escherichia_coli                   ISATEKAATPVIPPADDNA---FN-GSLSDETAV-MAWLKKRIAVQLRLSDPASLHPNQD  1845
                                                                    * * :: * :  ....    :: .  *:. .:  * *:: *.  :    ** :.*.

ABF89709.1_polyketide_synthase_type_I_Myxococcus_xanthus_DK_1622]  LVTFGLDSLMSMEIRNRLEAALGLKLSATVVWTYPTVAALAPFLAEKLALPLEDSRPEPA  2379
AZH85684.1_polyketide_synthase_Escherichia_coli                   LLQLGMDSLLFLELSSDIQHYLGVRINAERAWQDLSPHGLTQLICSKPEATPAASQPEVL  1905
```

```
ABF89709.1_polyketide_synthase_type_I_Myxococcus_xanthus_DK_1622]  VVQPVLFAMQVALAALWRSWGIAPDAVVGHSMGEVAAAHVAGALSLEDAARIICLRSQLL  1231
AZH85684.1_polyketide_synthase_Escherichia_coli                   ----------------------------------------DGD--------YRCTDGRYV  995
                                                                                                          *          *  .: :

ABF89709.1_polyketide_synthase_type_I_Myxococcus_xanthus_DK_1622]  RRKSGQGAMAVVELGLAQAREALAGYESRLSVAVS-----NSARSTVLSGDIDALESLLP  1286
AZH85684.1_polyketide_synthase_Escherichia_coli                   RARPIEH------QQRESLLTELAGYCEGFQAIPDTIARAGDRLYEMMSGAEEPVAIIFP  1049
                                                                  * :  :           .    **** . :.. .     ..    ::**  : :  ::*

ABF89709.1_polyketide_synthase_type_I_Myxococcus_xanthus_DK_1622]  KLESQGVFCRRVKVNVASHSPQMDSLKDDLLRVLDGVAPQGAPVPIYSTVTGQTSDGADF  1346
AZH85684.1_polyketide_synthase_Escherichia_coli                   QSASDGVEVLYQEFSF---GRYFNQIAAGVLRGIVQTRQPRQPLRILEVGGGT--GG---  1101
                                                                  :  *:**     :...   .  ::.:  .:** :  .     *: * ..  *   .*

ABF89709.1_polyketide_synthase_type_I_Myxococcus_xanthus_DK_1622]  HPAYWVSNLREPVLFHGAVEQLLADGFTVLVEVSPHPVLLAPIEETLRESKQGAIALASA  1406
AZH85684.1_polyketide_synthase_Escherichia_coli                   -TTAWLLPE-----------------------L----------------------------  1110
                                                                    : *:                         :

ABF89709.1_polyketide_synthase_type_I_Myxococcus_xanthus_DK_1622]  RRQMPERRCLLESLAALYAWGCAVDWKALHPVKGRVVALPRYPWQRERFWLPDEAEVDAQ  1466
AZH85684.1_polyketide_synthase_Escherichia_coli                   -NGVPALEYHFTDISALFTRRAQQKFA------------DYDFVKY---SELDLEKEAQ  1153
                                                                   . :*  .  : .::**::  .  .:               * : :       : * :**

ABF89709.1_polyketide_synthase_type_I_Myxococcus_xanthus_DK_1622]  PEGV-------VLADRK--GHPLIGG---SLSSSVQPGTHFWERTVSTAAFPYLADHCVW  1514
AZH85684.1_polyketide_synthase_Escherichia_coli                   SQGFQAQSYDLIVAANVIHATRHIGRTLDNLRPLLKPGGRLLMREITQ---PMRLFDFVF  1210
                                                                   :*.       ::* .   .   **    .*   ::** ::  * ::    *    . *:

ABF89709.1_polyketide_synthase_type_I_Myxococcus_xanthus_DK_1622]  GDVVFPGAGYV--------------EMALSAGAE---VLGETGLVLEDVSFSEMLALEPG  1557
AZH85684.1_polyketide_synthase_Escherichia_coli                   GPLVLPLQDLDAREGELFLTTAQWQQQCRHAGFSKVAWLPQDGSPTAGMSEHIILATLPG  1270
                                                                  * :*:*  .                : .  ** .    * : *    .:*   :**  **

ABF89709.1_polyketide_synthase_type_I_Myxococcus_xanthus_DK_1622]  QSRRVQVVLTEEEPGRATFQIASRAEGEMSWRKHAAGTVRRDARAAAVVESPEALRARVT  1617
AZH85684.1_polyketide_synthase_Escherichia_coli                   QAVSAVTFTAPSEPVLG-QALTDNGDYLADWSDCAGQ--------------PERFNA---  1312
                                                                  *:  . .. : .**  .   ::...:   .* . *.               ** :.*

ABF89709.1_polyketide_synthase_type_I_Myxococcus_xanthus_DK_1622]  VAVSAEAHYQRRQAQGLMYGPTFQGLRGIWRSEQEALGRLEVSDTVALEAGAYRLHPALL  1677
AZH85684.1_polyketide_synthase_Escherichia_coli                   ----------------------R---WQEAWRLLSQRHGD---------------------  1327
                                                                                        :  **  .:  *

ABF89709.1_polyketide_synthase_type_I_Myxococcus_xanthus_DK_1622]  DAGLQVAVELLAPLTATSAPATHVPVGIGRIRFFQRPGRAAWARVKVRGEGAASERERTF  1737
AZH85684.1_polyketide_synthase_Escherichia_coli                   --AL-----------PVEPPPVAAPEWLGKVRL-------SWQNEA--------------  1353
                                                                    .*           .. * . .*  :*::*:       :* .

ABF89709.1_polyketide_synthase_type_I_Myxococcus_xanthus_DK_1622]  DFWLLDEQGQVLLELEALRLFRLDAGASARKELGAWLYQVDWEERPLPAE-LAWPEQTPG  1796
AZH85684.1_polyketide_synthase_Escherichia_coli                   -------------------FSRGQMRVEARHPAGEWLPLSPAAPLPAPQTHYQW------  1388
                                                                                  : * :  ..**:  * **        * *     *

ABF89709.1_polyketide_synthase_type_I_Myxococcus_xanthus_DK_1622]  SWLILQDG---GGVGQRLSAQLLTRGETCVLVAPREAYRLTGPRSAEVDPRNPEHWRRLL  1853
AZH85684.1_polyketide_synthase_Escherichia_coli                   RWTPLNVASIDHPLTFSFSAGTLARSDEL------AQYGII------HDPH---ASSRL-  1432
                                                                   *  *: .     :  :**  *:*.:          * :        **:      **

ABF89709.1_polyketide_synthase_type_I_Myxococcus_xanthus_DK_1622]  TDLLGAGVPPCRGVVHLWSLDLASTEALTPQALEDSRRLGTTSVLHL------VQALSGA  1907
AZH85684.1_polyketide_synthase_Escherichia_coli                   ---------------------------MIVEESEDTLALAEKVIAALTASAAGLIVVTRR  1465
                                                                                           :  :  **:  *. . :  *      : .::

ABF89709.1_polyketide_synthase_type_I_Myxococcus_xanthus_DK_1622]  GWRDAPRLWLATRGARSAGKVAERVAVAQAPLLGLGQVLAVEQPELRCTRVDLEGGADVA  1967
AZH85684.1_polyketide_synthase_Escherichia_coli                   AWRVEEN---------------EALSASHHALWALLRVAANEQPERLLAAIDLAENT--P  1508
                                                                  .**   .               * ::.::  * .* :* * ****   : :**  .:

ABF89709.1_polyketide_synthase_type_I_Myxococcus_xanthus_DK_1622]  ADALLRELSSTSFEDQTAWR---GGTRRVARLARAADALSAREPATLLRE-DGTYLITGG  2023
AZH85684.1_polyketide_synthase_Escherichia_coli                   WETLHQGLSAVSLS--QRWLAARGDTLWLPSLTPN-TGCAAELPANVFTGDSRWHLVTGA  1565
                                                                   ::* : **:.*:.    *    *.*  :  *:    . :*. **.::   .  :*:**.

ABF89709.1_polyketide_synthase_type_I_Myxococcus_xanthus_DK_1622]  LGGLGLELARWLVSQGARHLLLMGRRAPSAEAEQALAALREAGARVG-SFQGDVARLEDV  2082
AZH85684.1_polyketide_synthase_Escherichia_coli                   FGGLGRLAVNWLREKGARRIALLAPRVDESWLRD----VEGGQTRVCRCDVGDAGQLATV  1621
                                                                  :****   ..** .:***:: *:. *. .:  .:    :. . :**  .  **..:*  *

ABF89709.1_polyketide_synthase_type_I_Myxococcus_xanthus_DK_1622]  TGALARVEDAMPPLRGVFHAAGLLEDGLLLNLTEARFASVSAPKVLGSWNLHAQTRHLPL  2142
AZH85684.1_polyketide_synthase_Escherichia_coli                   LDDL----AANGGIAGAIHAAGVLADAPLQELDDHQLAAVFAVKAQAASQLLQTLRNHDG  1677
                                                                   . *     *   : *.:****:* *. * :* : ::*:* * *. .: :*    *:

ABF89709.1_polyketide_synthase_type_I_Myxococcus_xanthus_DK_1622]  EHFVLFSSVAASLGTPGQSNYASANAFMDALAQARRA-EGLPALSINWGTWTQVGLAAAQ  2201
AZH85684.1_polyketide_synthase_Escherichia_coli                   RYLILYSSAAATLGAPGQSAHALACGYLDGLAQQFSTLDAPKTLSVAWGAWGESGRAATP  1737
                                                                  .:::*:**.**:**:**** :* * .::*.***   : :.  :**: **:* : * **:

ABF89709.1_polyketide_synthase_type_I_Myxococcus_xanthus_DK_1622]  SIRGERLEARGLGGMAPDKALAVLGMLLGQERPQ-LSVMAFEPRQWLGFYLAAAQSPYFT  2260
AZH85684.1_polyketide_synthase_Escherichia_coli                   EMLA-TLASRGMGALSDAEGCWHLEQAVMRGAPWRLAMRVFTD------KMPPLQQALFN  1790
                                                                  .: .  * :**:*.::  :.   *   : :  *  *:: .*         :   *.  *.

ABF89709.1_polyketide_synthase_type_I_Myxococcus_xanthus_DK_1622]  KLAQEPSSRPSVSAGKSRIREQLEAARASERRGLLDAHLRELIG-GVLRMAPARIEPRTP  2319
AZH85684.1_polyketide_synthase_Escherichia_coli                   ISATEKAATPVIPPADDNA---FN-GSLSDETAV-MAWLKKRIAVQLRLSDPASLHPNQD  1845
                                                                    * * :: * :  ....    :: .  *:. .:  * *:: *.  :    ** :.*.

ABF89709.1_polyketide_synthase_type_I_Myxococcus_xanthus_DK_1622]  LVTFGLDSLMSMEIRNRLEAALGLKLSATVVWTYPTVAALAPFLAEKLALPLEDSRPEPA  2379
AZH85684.1_polyketide_synthase_Escherichia_coli                   LLQLGMDSLLFLELSSDIQHYLGVRINAERAWQDLSPHGLTQLICSKPEATPAASQPEVL  1905
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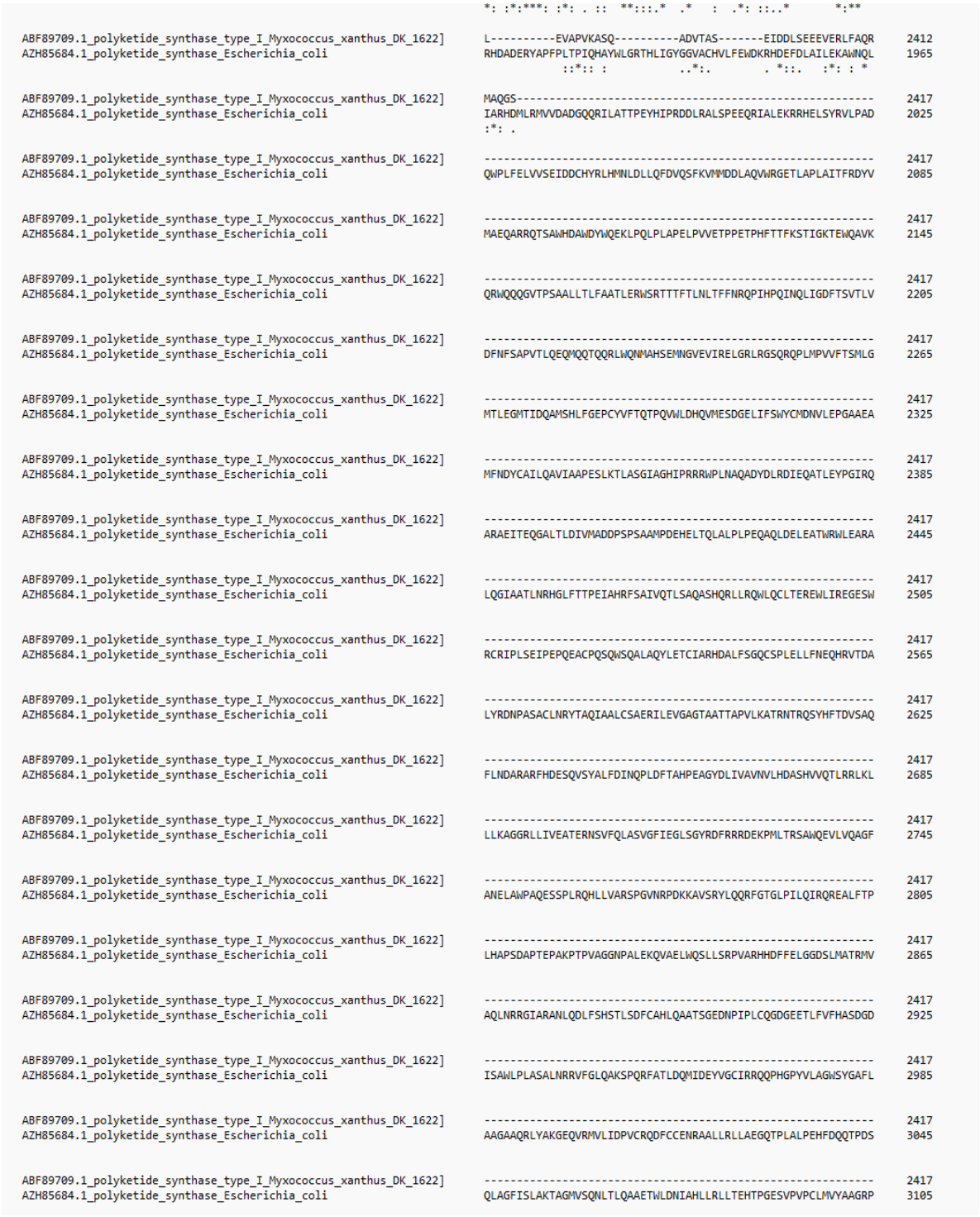

Figure 5.48: Amino acid alignment of polyketide synthase type I in *Myxococcus* and *E. coli* using Clustal Omega

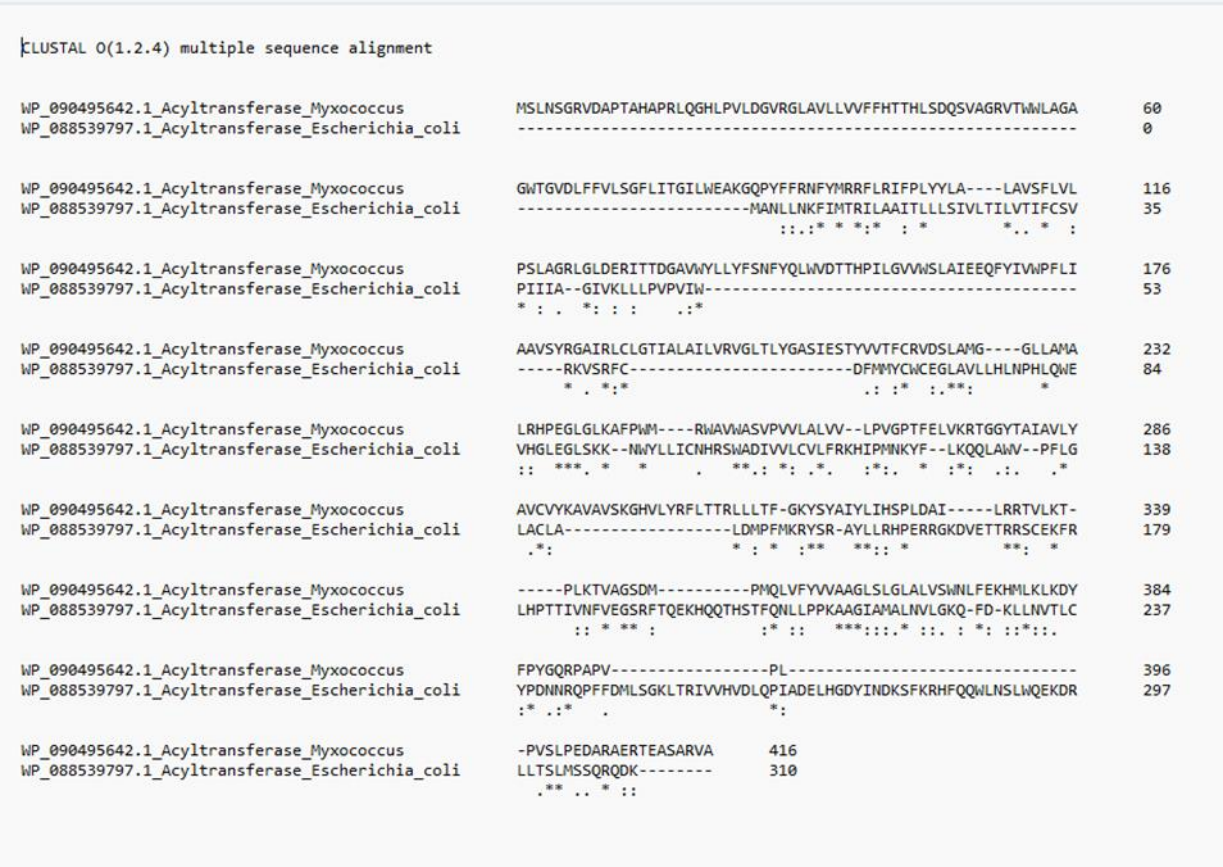

Figure 5.49: Amino acid alignment of acyltransferase in *Myxococcus* and *E. coli* using Clustal Omega

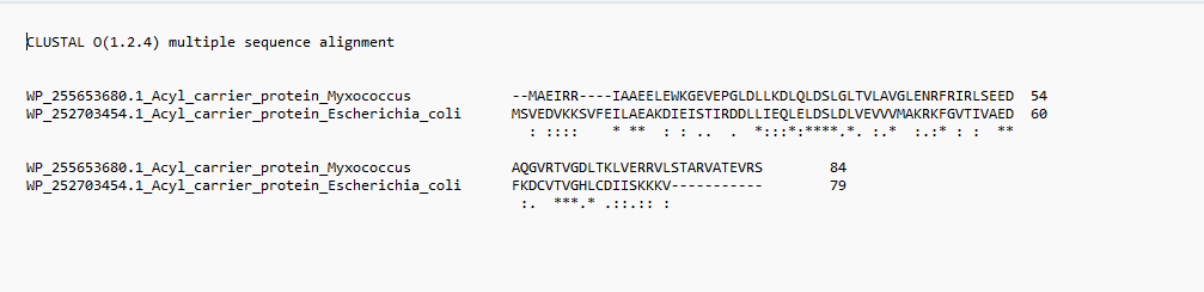

Figure 5.50: Amino acid alignment of acyl carrier protein in *Myxococcus* and *E. coli* using Clustal Omega

Myxococcus species have been well-comprehended to produce a distinct bioactive secondary metabolites, that effectively inhibits the growth of competing bacterial species (Kaur *et al.*, 2017). These secondary metabolites are generally polyketides, which are structurally complex compounds and their different forms has other clinical applications. Typically, polyketides can be sourced from biological sources like actinomycetes or chemically synthesised via semi- or total chemical synthesis. While isolating compounds from biological material is relatively easy in contrast to chemical synthesis, which poses challenges, often constrained by specific reactions types and resulting in low quantities of desired products (Paterson and Lam, 2018). After the development of genetic engineering, heterologous expression systems are used to

synthesize and produce large amounts of proteins/metabolites. Members of the polyketide synthase gene family are ones which are involved in the synthesis of such polyketides posing antibacterial activities (Musiol-Kroll and Wohlleben, 2018). In the present study, we tried the to replicate the PKS system in *E. coli* for the expression of the PKS gene to get the active polyketide in large amounts at low cost.

Functional polyketide synthases are present in the form of large protein complexes. As per structural studies, the PKS domains are organised into units termed as modules. Each module harbours a set of domains which are required for one elongation and modification cycle (Shen, 2003). Thus, a minimal requirement for a functional PKS consists of AT, ACP and KS domain. The AT is typically encoded as an independent gene. ATs within polyketide assembly lines are responsible for loading simple units, that are derived from precursors (thioester-activated) on the ACPs of a PKS. Subsequently, the PKS initiates the decarboxylative claisen-like condensation of the recently incorporated unit into the pre-existing polyketide chain (Staunton and Weissman, 2001). Ultimately, the synthesized polyketide chain is liberated from the final module of the assembly line, generally facilitated by thioesterase (TE). Tailoring enzymes (if existing) further alter the intermediate to yield the end product (Musiol-Kroll and Wohlleben, 2018).

The polyketide engineering has complex modularity and functionality. Thus, these systems require a deep understanding of PKS modification. PKSs serve as the chief enzymes in the polyketide biosynthetic machinery and are organized into modular structures. Within each module, a complete set of necessary domains exists for a single elongation as well as possible modification cycle (Gulder *et al.,* 2011). Disruptions of the PKS modular architecture, breakdown of the polypeptide integrity of PKS and interference during the protein-protein interactions are few recurrent reasons for the inadequate expression of modified PKSs. These findings prompt us for further investigate and perform the inclusive mechanistic as well as structural analysis of the PKS assembly lines (Khosla *et al.,* 2014). In the current study, we focus on PKS engineering and overlook the interactions among AT-ACP-KS, and the current study highlights the crucial role of interdomain communication in polyketide biosynthesis.

CHAPTER 6

SUMMARY AND CONCLUSION

Myxobacteria are unicellular, gram-negative, rod-shaped and obligate aerobic bacteria that belong to the δ-proteobacteria family and form multicellular structures. These microbes are known to inhabit diverse environments like soil, tree bark, herbivore dung, marine environment and decaying plant material. They are usually mesophiles and grow within a pH range of 5-9 and temperature 6-38°C.

Myxobacteria have became an attractive microbial species as it has been reported to produce diverse bioactive compounds and suitable candidate to meet the increasing demand for distinct bioactive molecules. As per published literature, they have been stated to produce more than 100 fundamental structures and 500 variations of bioactive compounds to date. These bioactive compounds are known to exhibit anti-bacterial, anti-fungal, antiviral, antiparasitic, and cytotoxic potential activity.

Owing to the habitable environment for myxobacteria, the Kashmir Valley was selected as it is an undisturbed and unexplored area with rich wetland biodiversity. This also increases the chances of discovering novel strains with unique traits. These features aligned with the objective of improving the limited knowledge about myxobacteria in the Indian habitat. Therefore, the current study aimed to explore the diverse habitat of Kashmir Valley, specifically its wetland, to unveil novel myxobacterial strains.

In the current study, myxobacteria were isolated from the soil of two wetlands of Kashmir Valley (named Hokersar and Mirgund) and identified using morphological characteristics and 16s rRNA-based sequencing. Further, the isolates were screened for the production of enzymes and bioactive compounds. Furthermore, the genes responsible for the production of protease enzyme and bioactive compound (polyketide synthase) were identified and amplified for *in-vitro* expression in *E. coli*. Purified protease enzyme was investigated for optimal temperature, pH, effect of metal ions and detergent. Whereas, polyketide synthase encoding clone was screened for antibacterial activity. The outcomes of the study have been concluded in following bullet points.

- The isolates demonstrated the typical morphological characteristics of myxobacteria.
- Moreover, 16s rRNA-based sequencing revealed these three isolates, i.e., Hok1, Mir1 and Mir2 to be Uncultured *Anaeromyxobacter* sp. (86.5%), *Pyxidicoccus fallax* (99.93%) and *Myxococcus* sp. (100%) respectively.

- These isolated strains were evaluated for their potential to synthesise various enzymes. The preliminary results revealed promising results for the isolate Mir2, which showed the presence of extracellular protease in high amounts.
- Furthermore, the myxobacteria isolate demonstrated antibacterial potential against *Bacillus cereus* MTCC 8714*, Escherichia coli* MTCC 1679 and *Pseudomonas aeruginosa* MTCC 2582.

Considering these promising findings, the study delved into identifying the genes responsible for encoding the industrial enzyme and bioactive compounds.

- The PCR-based screening targeting the specific gene encoding for enzymes led to successful identification and sequencing of the gene, which showed similarity with serine protease. This amplified gene of interest encoding for protease was cloned using gateway cloning, primarily in pENTR-D-Topo (entry vector) and secondary into pEZY19 (destination/expression vector).
- The pEZY19 plasmid was transformed into the BL21 (DE3) expression strain of *E. coli* for protein expression incorporated with His-Tag. Subsequently, the His-tagged protease was purified and evaluated on SDS-PAGE.
- The purified protein from the recombinant clone was further subjected to investigate the optimal conditions for enzyme activity, involving temperature, pH, effect of metal ions and detergents. The result for the protease enzyme obtained from the recombinant clone (Mir2*Pro*) revealed it to be extracellular in nature, with optimal activity at 40°C and a pH of 7.0. Additionally, the finding regarding the effect of metal ions like Mg^{2+}, Ca^{2+} and Fe^{2+} and detergents like Tween-20 and Triton X showed a positive impact on the enzyme activity of the protease.
- Additionally, the study delved into determining the presence of polyketide synthase (PKS), which is important for polyketide synthesis, a known class of bioactive molecules. The PCR-based screening targeting the specific gene encoding for PKS led to successful identification and sequencing of the gene, which showed similarity with polyketide synthase. This amplified gene of interest encoding for protease was cloned using gateway cloning, primarily in pENTR-D-Topo (entry vector) and secondary into pEZY19 (expression vector). The pEZY19 plasmid was transformed into the BL21 (DE3) expression strain of *E. coli* for protein expression incorporated with His-Tag. Subsequently, the His-tagged PKS was evaluated on SDS-PAGE.

- The recombinant clone (Mir2*PKS*) containing the PKS gene cloned in pEZY19 vector was used as the seed culture and evaluated for antibacterial activity against tested strains like *Bacillus cereus* MTCC 8714*, Escherichia coli* MTCC 1679 and *Pseudomonas aeruginosa* MTCC 2582.
- The result of recombinant clone (Mir2*PKS*) supernatant as well as cell-free extract did not show any significant difference in activity against the test strain. Further, to elucidate the underlying cause for the absence of antibacterial activity, a sequence analysis of the recombinant clone was conducted.
- PKS is well-known to work as complex in which acyltransferase and acyl carrier protein plays pivotal role for the synthesis of antimicrobial compound. For this, In-silico protein-protein interaction was studied using STRING software by providing the amino acid sequence as the input to the STRING database.
- The output obtained from STRING showed no discernible interaction among the submitted protein, suggesting a possible reason for no synthesis of antimicrobial compound in *E. coli* harbouring the recombinant PKS. Moreover, polyketide molecules synthesis is a conserved mechanism in microbes, the presence of sequence variation may contribute to incomplete complex formation. To test this hypothesis, amino acid sequences of the three proteins (PKS, ACP, and AT) from both *Myxococcus* and *E. coli* were subjected to alignment studies via Clustal Omega. The result obtained revealed that all three proteins from *Myxococcus* showed substantial difference from *E. coli*. This observation highlights the insufficiency of cloning a single gene of PKS for recombinant protein synthesis is not enough for the generation of antimicrobial compounds in the *E. coli* expression strain. Thus, present study highlights the significance of interdomain communication for polyketide synthesis and provide the valuable insights about challenges and complexities involved for obtaining bioactive compounds via genetic manipulation of myxobacterial strain.

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APPENDIX

Annexure 1

Contig Sequence of Different Myxobacterial Strains are as followed:

>Contig Hok1

GGTACCCACAAATAAAACGTGCTGGAGCCACAGGATGAGATACCCTGGTAGTCCA
CGCCGTATACGATGAGTGCTAGGTGTTGGGGGTTTCAATACCCTCAGTGCCGCACC
TAACGCAATAAGCACTCCGCCGGGGGAGTACGCTCGCAAGAGTGAAACTCAAAG
GAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACG
CGAAGAACCTTACCAGGTCTTGACATCCCGCTGACCGTCCTAGAGATAGGGCTTC
CCTTCGGGGCAGCGGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAG
ATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCTTTAGTTGCCAGCATTCA
GTTGGGCACTCTAGAGAGACTGCCGTCGACAAGACGGAGGAAGGCGGGGATGAC
GTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGCTGGTA
CAACGGGGAGCTAGCTCGCGAGAGTATGCCAATCTCTTAAAACCAGTCTCAGTTC
GGATTGCAGGCTGCAACTCGCCTGCATGAAGTCGGAATCGCTAGTACTCGCGGAT
CAGCATGCCGCGGTTGAATACGTTCCCGGGGCGTTGTACACACCGCCCAGTCACA
CCACGTGGAGTTTGCAACACCCGAAATCTGTGAAGTAACCGCCCGGGAGCCCGCC
CCCCGAAGTTGGGGTCAGTGACTGGGTGTGAAGACCATAACCCCGGTGCTTGGGG
TACCCACAAATAAAACGTGCTGGAGCCACAGGATGAGATACCCTGGTAGTCCACG
CCGTATACGATGAGTGCTAGGTGTTGGGGGTTTCAATACCCTCAGTGCCGCACCTA
ACGCAATAAGCACTCCGCCGGGGGAGTACGCTCGCAAGAGTGAAACTCAAAGGA
ATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCG
AAGAACCTTACCAGGTCTTGACATCCCGCTGACCGTCCTAGAGATAGGGCTTCCCT
TCGGGGCAGCGGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATG
TTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCTTTAGTTGCCAGCATTCAGTT
GGGCACTCTAGAGAGACTGCCGTCGACAAGACGGAGGAAGGCGGGGATGACGTC
AAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGCTGGTACAA
CGGGGAGCTAGCTCGCGAGAGTATGCCAATCTCTTAAAACCAGTCTCAGTTCGGA
TTGCAGGCTGCAACTCGCCTGCATGAAGTCGGAATCGCTAGTACTCGCGGATCAG
CATGCCGCGGTTGAATACGTTCCCGGGGCGTTGTACACACCGCCCAGTCACACCA
CGTGGAGTTTGCAACACCCGAAATCTGTGAAGTAACCGCCCGGGAGCCCGCCCCC
CGAAGTTGGGGTCAGTGACTGGGTGTGAAGACCATAACCCCGGTGCTTGG

>Contig Hok2

GAGGGAGCTTGCTCCCAAAGATTAGCGGCGGACGGGTGAGTAACACGTGGGCAA
CCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAATATC
TATTTATACATATAATTAGATTGAAAGATGGTTCTGCTATCACTTACAGATGGGCCCG
CGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCC
GACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTAC
GGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACG
CCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGGGAAGAACA
AGTATCGGAGTAACTGCCGGTACCTTGACGGTACCTAACCAGAAAGCCACGGCTA
ACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATT
GGGCGTAAAGCGCGCGCAGGCGGTTCCTTAAGTCTGATGTGAAAGCCCACGGCTC
AACCGTGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAAAGTGG
AATTCCAAGTGTAGCGGTGAAATGCGTAGAGATTTGGAGGAACACCAGTGGCGAA
GGCGACTTTCTGGTCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAAC
AGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAG
GGTTTCCGCCCTTTAGTGCTGCAGCAAACGCATTAAGCACTCCGCCTGGGGAGTA
CGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGA
GCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCT
GACAATCCTAGAGATAGGACTTTCCCCTTCGGGGGACAGAGTGACAGGTGGTGCA
TGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCA
ACCCTTGATCTTAGTTGCCAGCATTTAGTTGGGCACTCTAAGGTGACTGCCGGTGA
CAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGG
CTACACACGTGCTACAATGGATGGTACAAAGGGCTGCAAGACCGCGAGGTTTAGC
CAATCCCATAAAACCATTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAA
GCCGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCC
TTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGGGG
TAA

>Conting Mir1

GGGGCAACCCTTAGTAGAGCGGCGCACGGGTGCGTAACACGTGGATAATCTGCCT
GGATGCCCGGGATAACCAGTCGAAAGATTGGCTAATACCGGATAAGCCCACGGCC
TCTTCGGAGACTGAGGGAAAAGGTGGCCTCTGTATACAAGCTATCACAACCAGAT
GAGTCCGCGGCCCATCAGCTAGTTGGCGGGGTAATGGCCCACCAAGGCAACGAC
GGGTAGCTGGTCTGAGAGGACGATCAGCCACACTGGAACTGAGACACGGTCCAG
ACTCCTACGGGAGGCAGCAGTGGGGAATTTTGCGCAATGGGCGAAAGCCTGACG
CAGCAACGCCGCGTGTGTGATGAAGGTCTTCGGATTGTAAAGCACTTTCGACCGG

GACGAAAACCCGTAGCCCAACACGCTACGGCTTGACGGTACCGGGAGAAGAAGC
ACCGGCTAACTCTGTGCCAGCAGCCGCCGGTAATACAGAGGGTGCAAGCGTTGTT
CGGAATTATTGGGCGTAAAGCGCGTGTAGGCGGCGTGACAAGTCGGGTGTGAAAG
CCCTCAGCTCAACTGAGGAAGTGCGCCCGAAACTGTCGTGCTTGAGTGCCGGAG
AGGGTGGCGGAATTCCCGAAGTAGAGGTGAAATTCGTAGATATCGGGAGGAACAC
CGGTGGCGAAGGCGGCCACCTGGACGGTAACTGACGCTGAGACGCGAAAGCGTG
GGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGAACTA
GGTGTCGTGGGAGTTGACCCCCGCGGTGCCGAAGCTAACGCATTAAGTTCTCCGC
CTGGGAAGTACGGTCGCAAGACTAAAACTCAAAGGAATTGACGGGGGCCCGCAC
AAGCGGTGGAGCATGTGGTTTAATTCGACGCAACGCGCAGAACCTTACCTGGTCT
TGACATCCTTGGAATCCCTCAGAGATGAGGGAGTGCCCGCAAGGGAACCAAGAG
ACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCC
GCAACGAGCGCAACCCTCGCCTTTAGTTGCCACGCAAGTGGATCTCTAGAGGGAC
TGCCGGTGTTAAACCGGAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCTTTA
TGACCAGGGCTACACACGTGCTACAATGGCCGGTACAGAGCGTTGCCAACCCGCG
AGGGGGAGCTAATCGCATAAAACCGGTCTCAGTTCAGATTGGAGTCTGCAACTCG
ACTCCATGAAGGCGGAATCGCTAGTAATCGCAGATCAGCACGCTGCGGTGAATAC
GTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTCGATTGCTCCAG

>Contig Mir2

GGGCAACCCTTAGTAGAGCGGCGCACGGGTGCGTAACACGTGGATAATCTGCCTG
GATGCTCGGGATAACCAGTCGAAAGATTGGCTAATACCGGATAAGCCCACGGTTTC
TTCGGAGACTGAGGGAAAAGGTGGCCTCTGTATACAAGCTATCACAACCAGATGA
GTCCGCGGCCCATCAGCTAGTTGGCGGGGTAATGGCCCACCAAGGCGACGACGG
GTAGCTGGTCTGAGAGGACGATCAGCCACACTGGAACTGAGACACGGTCCAGAC
TCCTACGGGAGGCAGCAGTGGGGAATTTTGCGCAATGGGCGAAAGCCTGACGCA
GCAACGCCGCGTGTGTGATGAAGGTCTTCGGATTGTAAAGCACTTTCGACCGGGA
CGAAAACCCGTAGCCCAACACGCTACGGCTTGACGGTACCGGGAGAAGAAGCAC
CGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCAAGCGTTGTTCGG
AATTATTGGGCGTAAAGCGCGTGTAGGCGGCGTGACAAGTCGGGTGTGAAAGCCC
TCAGCTCAACTGAGGAAGTGCGCCCGAAACTGTCGTGCTTGAGTGCCGGAGAGG
GTGGCGGAATTCCCCAAGTAGAGGTGAAATTCGTAGATATGGGGAGGAACACCGG
TGGCGAAGGCGGCCACCTGGACGGTAACTGACGCTGAGACGCGAAAGCGTGGGG
AGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGAACTAGGT
GTCGTGGGAGTTGACCCCCGCGGTGCCGTAGCTAACGCATTAAGTTCTCCGCCTG

GGAAGTACGGTCGCAAGACTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAG
CGGTGGAGCATGTGGTTTAATTCGACGCAACGCGCAGAACCTTACCTGGTCTTGA
CATCCTCGGAATCTCTCAGAGATGAGGGAGTGCCCGCAAGGGAACCGAGAGACA
GGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCA
ACGAGCGCAACCCTCGCCTTTAGTTGCCACGCAAGTGGATCTCTAGAGGGACTGC
CGGTGTTAAACCGGAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCTTTATGA
CCAGGGCTACACACGTGCTACAATGGCCGGTACAGAGCGTTGCCAACCCGCGAGG
GGGAGCTAATCGCATAAAACCGGTCTCAGTTCAGATTGGAGTCTGCAACTCGACT
CCATGAAGGCGGAATCGCTAGTAATCGCAGATCAGCACGCTGCGGTGAATACGTT
CCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTCGATTGCTCCAGAAGT
CACCTCACCA

Annexure 2

Sequences used in Clustal Omega Analysis

>ABF89709.1_polyketide_synthase_type_I_Myxococcus_xanthus_DK_1622]
MSGSRESARNGFVLTGLRQRFGFGIDPFFLVSGLSRGRRTASVVISFAEEKPGFRACQ
LEVRVSPQNGLSKAGLAEVRARLTAVLAQRLGIEAHTLDVRERFSLYGLDSLKATGF
IAEVGAMLGRSLSPTLAWEYPTLDGLARYLAGERDGASSHSLARIARAHEPIAIIGLA
CRYPQAADPEAFWRLLVGGTDAITEVPPDRWDVNRLYDRDPAAPGKVISRWGGYL
DRVDGFDPLFFGISPKEALHMDPQQRLMLELSWEALEDAGIAADRLQGSPTAVCFGA
AWMDYEMTLQRFGMKRISSYTSTGYHHSVLANRVSYVLGLRGPSFSIDSACSSSLTA
AHLACESLRRGESTLALVGGVNLTIAPESTVSLSKLGALSPDGHCYTFDARANGFVR
GEGAGVAVLKPLSLALADGDAIYCVIRGSAINNNGGSNGLTAPNPKAQAEVIRQACA
NAGVEPAEVQYVEAHGTGTQLGDPLEAQALGEVLGAGRPGGKPLLIGSCKTNIGHL
EAAAGITGLIKTALCIKHRVLPASLHYEKPNPLIPFEALGLAVHHTLGDWPEPDRKLI
AGVSSFGFGGANCHVVMEEARLPEARLLHLSGETTEALRGAAQGLLDRVTAREHLP
LAELLRAAEADAGTHAHRLTVTVRTRKDLRAGLENFLAGVPRPGVSVGTVAEDAV
RKPVFVYSGHGCQWPRMGLPLLDTEPVFRATLLRCDALIREYEGWSLLEVLAADDA
AARLSRLDIGLPAIVSVEMALTDLWRSWGIEPGAVVGHSIGEVSAAYAAGVLDIEDTI
RVVCAESRLMHTQAGKGSLAVVGVPWAEAAELLVGYEGRLFRAIDSGAGSTVLSG
DVDALAEVLASLQQRGVFCRQVDIDVPVHSPRMDVLADALTEELRDIRPRPARVPLI
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>WP_090495642.1 MULTISPECIES: acyltransferase [Myxococcus]

MSLNSGRVDAPTAHAPRLQGHLPVLDGVRGLAVLLVVFFHTTHLSDQSVAGRVTW
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FLVLPSLAGRLGLDERITTDGAVWYLLYFSNFYQLWVDTTHPILGVVWSLAIEEQFYI
VWPFLIAAVSYRGAIRLCLGTIALAILVRVGLTLYGASIESTYVVTFCRVDSLAMGGL
LAMALRHPEGLGLKAFPWMRWAVWASVPVVLALVVLPVGPTFELVKRTGGYTAIA
VLYAVCVYKAVAVSKGHVLYRFLTTRLLLTFGKYSYAIYLIHSPLDAILRRTVLKTPL
KTVAGSDMPMQLVFYVVAAGLSLGLALVSWNLFEKHMLKLKDYFPYGQRPAPVPL
PVSLPEDARAERTEASARVA

>WP_088539797.1 acyltransferase [Escherichia coli]

MANLLNKFIMTRILAAITLLLSIVLTILVTIFCSVPIIIAGIVKLLLPVPVIWRKVSRFCDF
MMYCWCEGLAVLLHLNPHLQWEVHGLEGLSKKNWYLLICNHRSWADIVVLCVLFR
KHIPMNKYFLKQQLAWVPFLGLACLALDMPFMKRYSRAYLLRHPERRGKDVETTR

RSCEKFRLHPTTIVNFVEGSRFTQEKHQQTHSTFQNLLPPKAAGIAMALNVLGKQFD
KLLNVTLCYPDNNRQPFFDMLSGKLTRIVVHVDLQPIADELHGDYINDKSFKRHFQQ
WLNSLWQEKDRLLTSLMSSQRQDK

>WP_255653680.1 MULTISPECIES: acyl carrier protein [Myxococcus]

MAEIRRIAAEELEWKGEVEPGLDLLKDLQLDSLGLTVLAVGLENRFRIRLSEEDAQG
VRTVGDLTKLVERRVLSTARVATEVRS

>WP_252703454.1_Acyl_carrier_protein_Escherichia_coli

MSVEDVKKSVFEILAEAKDIEISTIRDDLLIEQLELDSLDLVEVVVMAKRKFGVTIVAE
DFKDCVTVGHLCDIISKKKV

LIST OF PUBLICATIONS

1. **Dhanjal, D. S.,** Singh, S., Kumar, V., Ramamurthy, P. C., Chopra, C., Wani, A. K., Singh, R. & Singh, J. (2023). Isolation and Characterization of Cellulase-Producing Myxobacterial Strain from the Unique Niche of Mirgund Wetland from the North-Western Himalayas. *Journal of Applied Biology and Biotechnology*, *11*(5), 119-125.

OTHER RELEVANT PUBLICATIONS

1. **Dhanjal, D. S.,** Singh, R., & Chopra, C. (2020). Recent Advances in the Discovery Antimicrobials through Metagenomics. *Frontiers in Anti-Infective Drug Discovery: Volume 8*, *8*, 159.
2. **Dhanjal, D. S.,** Chopra, R. S., & Chopra, C. (2020). Metagenomics and enzymes: the novelty perspective. *Metagenomics: Techniques, Applications, Challenges and Opportunities*, 109-131.
3. Mohan, A., **Dhanjal, D.S.,** Chopra, C., & Singh, R. (2021). Myxobacterial Metabolites: A Promising Resource for Big Pharma, Global Emerging Innovation Summit (GEIS-2021), 360.
4. Bhardwaj, S., Mehra, P., **Dhanjal, D.S.,** Sharma, P., Sharma, V., Singh, R., Nepovimova, E., Chopra, C. & Kuča, K. (2022). Antibiotics and Antibiotic Resistance-Flipsides of the Same Coin. *Current Pharmaceutical Design, 28(28), 2312-2329.*
5. Thakur, P., Chopra, C., Anand, P., **Dhanjal, D. S.,** & Chopra, R. S. (2018). Myxobacteria: unraveling the potential of a unique microbiome niche. *Microbial Bioprospecting for Sustainable Development*, 137-163.

LIST OF CONFERENCES

- Participated and gave Oral Presentation at the "Virtual International Conference on Emerging Trends in Applied Sciences" jointly organized by Veer Narmad South Gujarat University, Surat, India and Vyatka State University, Kirov, Russia (2021)
- Participated and gave Oral Presentation and stood '**Third**' at "International Conference on Sustainability: Life on Earth 2021 (ICS-LOE 2021)" at LPU, Phagwara, Punjab